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Reproductive Aspect and Embryonic Development of Wader pari Fish (*Rasbora lateristriata* Bleeker 1854) from Malang East Java

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ABSTRACT
Indonesia is well known for its high freshwater fish diversity. Wader pari (*Rasbora lateristriata*) is one of the endemic fish, which is very popular, but experienced massive exploitation in wild, due to high market demand. High demand and high economic value of this fish in the market induced a massive exploitation in the wild. This situation is leading to a possible population decline and resulted in extinction. however, so far the cultivation of wader pari fish has not been carried out The fish also has potential as an animal model in research. However, the embryonic development of the fish has not been studied, yet. This study analyzed the reproductive performance and described the stages of embryonic development of the wader pari fish originated from Malang Province, East Java, Indonesia. The number of eggs and normal embryonic development were observed visually and documented using stereomicroscope and time-lapse imaging method. Data on egg quality and hatching percentage were analyzed by analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at 5% level. The results showed the number of good quality eggs was higher than that of poor quality at the percentage of 76%. Moreover, the hatching rate was 44%, which mostly embryos that hatched were at the period of 24 to 30 hours after fertilization. The stages of embryonic development observed respectively are the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching stage.

Keywords: *Embryo development, Morphogenesis, Rasbora lateristriata*

1. INTRODUCTION

Indonesia is well known for its high freshwater fish diversity. Wader pari (*Rasbora lateristriata*) is one of the endemic fish, which spread from Sumatra, Kalimantan, Java to Papua. Recently, the Rasboran is one of the community culinary favorites, leading to increasing market demand thus threaten its already vulnerable existence in nature. It is unfortunate that until now, cultivation of wader parifish has not been carried out as an effort to maintain its population and to fulfill the market demand. If this situation persists, it will lead to a possible population decline and resulted in extinction. This is also in line with the Department of Marine Affairs and Fisheries of East Java, which states that Wader pari is one of the local fish species that is starting to be threatened because it has not been widely cultivated.

Some important informations for cultivation effort are fish embryo development (embryogenesis) and larval development [1]. Informations on the early development and life of fish provide important parameters in fish larvae production [2]. Good quality eggs are eggs that have a low mortality rate at the time of fertilization, the eggs hatch into larvae with good growth [2]. Many factors affect egg quality, one of which is the quality of the broodstock.

Availability of good quality male and female gametes in aquaculture is necessary to obtain subsequent generations. In addition, In addition, the eggs produced contain maternal factors and other components origin

from maternal, such as the environment and broodstock management techniques that affect egg quality [3]. The embryonic development of the wader pari fish especially those originated from Malang has not been studied, yet. This study analyzed the reproductive performance and described the stages of embryonic development of the wader pari fish originated from Malang East Java Province, Indonesia.

2. MATERIAL AND METHODS

2.1. Test Organisms

The research was carried in the Laboratory of Animal Structure and Development, Faculty of Biology, Universitas Gadjah Mada. This research used wader pari fish (*R. lateristriata*) from Malang, East Java. Wild-type fish were maintained, reared and staged, under laboratory conditions. Prospective wader fish brood from Malang maintained in a closed system with circulating filtered water at a temperature of 28-29 °C and pH 7.0-7.5. Fish are fed with commercial feed three times a day in the morning, afternoon, and evening.

2.2. Gonad maturity selection and fish mating

The male dan female broods was checked for gonad maturity by massaging the fish's abdomen. The embryos were obtained by natural mating, which was conducted by ratio of 2 male and 1 female at mating chamber. The fish were set at 16:00 - 17:00 one day before the mating. The mating was conducted at 01:00 - 05:00. The embryos were collected, then washed with egg water and used for further experiment or kept at 28.5 °C.

2.3. Embryo care and development analysis

Spawning eggs were transferred to the egg medium. Time-lapse imaging was conducted to obtain live image of embryos development process during the early stage of fish embryo development. Picture series were recorded using light microscope (DM750, Leica Microsystems) equipped with a microscope camera (ICC50E, Leica Microsystems) which positioned at the center of a glass-bottom dish. Egg qualities were analyzed by randomly taking 300 eggs and placing them in 3 glass-bottom dish to observe their morphology from the cleavage to blastula period using the microscope camera with various magnification. The eggs that have been observed then stored at room temperature to observe their hatchability on the next day. The data were analyzed by analysis of variance (ANOVA) and further analyzed with Duncan's Multiple Range Test (DMRT) at a 5% level.

3. RESULT AND DISCUSSION

The embryonic development of *R. lateristriata* eggs are described in Table 1. The elapsed time from fertilization was calculated assuming that the eggs were spawned at 05:00. The embryonic development was divided into six period: zygote, cleavage, blastula, gastrula, segmentation, pharyngeal, and hatching period [4]. Details of development features are presented in Table 1 and Fig 1 (A-T).

Table 1. Period and time of embryo development wader pari fish

Development period	Time (hpf*)
Fertilized egg	00:00
Zygote	00:45
Cleavage	57:00 to 02:01
Blastula	02:36 to 05:19
Gastrula	06:12 to 7:12
Segmentation	05:19 to 09:58
Pharyngeal	15:10 to 24:00
Hatching	24:00

*hours post fertilization

3.1. Zygote period

Fertilized eggs until the early cleavage stage are included in the zygote period. After fertilization, activation of cytoplasmic movement occurs. The non-yolk cytoplasm will move towards the animal poles and the cytoplasm will separate from the yolk at the vegetal pole [4]. This area of the cytoplasm is called the blastodisc. The cytoplasmic movement will form a convex on the animal pole of eggs (Fig 1-A).

3.2. Cleavage period

The cleavage pattern of wader pari fish is meroblastic cleavage. This cleavage occurs only in the blastodisc region, while the yolk area does not divide. Cleavage occurs vertically from the animal poles to the vegetal poles and ends at the outer edge of the yolk. The first cleavage occurred in the blastodisc from the animal pole toward the vegetal pole region (Fig 1.B). This division produces two equal blastomeres at 57 minutes after fertilization. The second cleavage occurs meridionally and perpendicular to the groove of the first cleavage (Fig 1.C) producing in four equal blastomere about 1 hour 9 min after fertilization. The third cleavage occurs similarly and parallel to the first division which results in eight cells arranged in two rows of four cells and an arrangement of two parallel rectangular rows (Fig 1.D) about 1 hour 21 minutes after fertilization. The fourth cleveage was produces 16 cells that occur perpendicular to the direction of the first division (Fig 1.E). This cleavage divides 8 blastomeres into 16 blastomeres in a 4 x 4 arrangement. In step 16 the cell division occurs "completely" where there are 4 blastomeres in the center and surrounded by 12 other blastomeres. These 12 blastomeres are called marginal blastomeres [5] The 16 cell stage took place at 1 hour 33 minutes. The fifth cleavage is parallel with the first and third cleavage

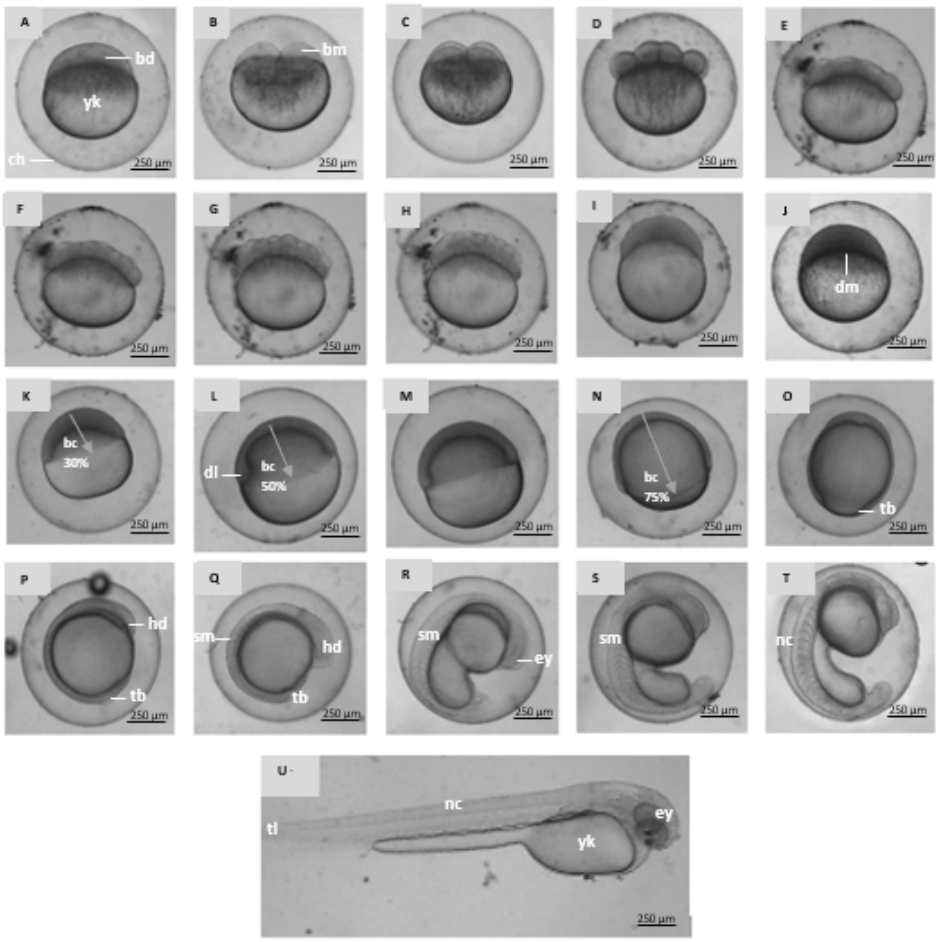

Figure 1. (1-21) Embryonic development of *R. lateristriata:* (1) zygote period; (2) 2 cell stage; (3) 4 cell stage; (4) 8 cell stage; (5) 16 cell stage; (6) 32 cell stage; (7) 64 cell stage; (8) 256 cell stage; (9) hight stage; (10) dome stage; (11) 30% epiboly; (12) 50% epiboly; (13) germ ring; (14) 70% epiboly; (15) 90% epiboly; (16) bud stage; (17) 1-10 somite; (18) 14 somites; (19) 17 somites; (20) 20-25 somites; (21) hatching period; blastomere closure 30% (bc 30%); blastomere closure 50% (bc 50%); blastomere closure 75% (bc 75%); blastodisc (bd); blastomere (bm); dorsal lip (dl); epiboly (ep); eye (ey); head (hd); notochord (nt); somites (sm); tailbud (tb); tail (tl); and yolk (yk). Scale bar: 250 µm; magnification: 10x.

furrow (Fig 1.F). This cleavage produces blastomere with a 4 x 8 arrangement blastomere. The cell stage took place at 1 hour 49 minutes after fertilization. At 2 hours 1 minute, the blastomere cells in the deep will be covered by the marginal blastomere. This covering cell is called the Enveloping Layer (EVL) [4](Fig 1.G).

3.3. Blastula period

The cleveage that occurs continuously causes an increase in the number of cells and a decrease in cell size. In the early blastula stage, the blastomeres are arranged to form a solid semi-circular mound of cells attached to the yolk (Fig 1.H) occurs at 2 hours 36 minutes after fertilization. At 2 hours 57 minutes after fertilization, blastomere was distinguished from the previous stage based on the number and shape. When viewed from the side (Fig 1.I), more than 11 pile EVL of the animal pole to the margin of the yolk will be seen. At the high stage, a rotational movement begins to occur in the yolk. Then at 4 hours 23 minutes, the yolk rises toward the animal pole to form a dome (Fig 1.J) A striking and rapid change between the yolk and blastodisc cells is a sign that epiboly begins [4].

In this study, egg quality was observed from the cleavage phase to the blastula. Good quality eggs are eggs that can be fertilized and developed into normal embryos [6]. The observation results of egg quality in this study showed that there was a difference in the percentage of eggs with normal and abnormal structures in the cleavage and blastula phases which can be seen in Table 2. The analyzed data ANOVA and DMRT showed a significant difference between replicates ($p<0.05$).

Table 2. Percentage of egg quality

Development period	Percentage (%)	
	Normal	Abnormal
Cleavage	76 ± 3	24 ± 3
Blastula	63 ± 2	37 ± 2

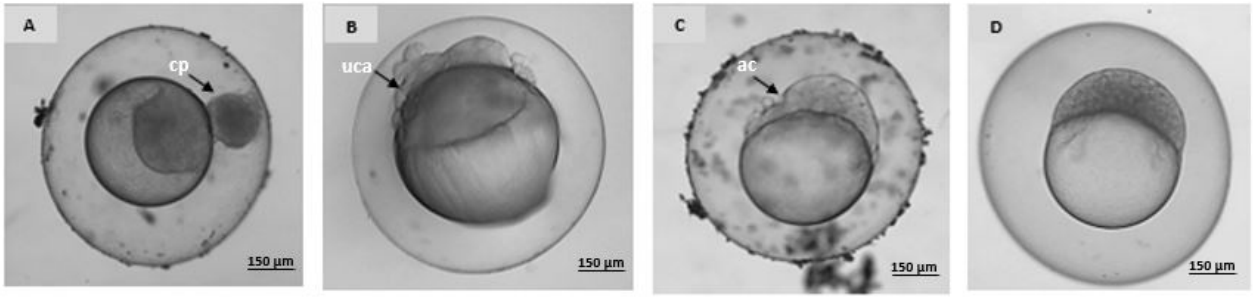

Figure 2. Comparison between abnormal and normal cells 10x. (A, B, C) abnormal cells and (4) normal cells. Cells protrude (cp); unclear cell arrangement (uca); and asymmetric cell (ac). Magnification: 10x.

The data showed that 76% of eggs were developed normally from one cell to blastula stage (Fig 1. A-O). Meanwhile, abnormal egg cells (24%), fail to develop normally and usually reach to 2-cell stage only. The abnormal egg cell morphology was consist of one or two asymmetrical blastomeres at the early stage, and develop into an irregular shape of cells that protrude from the cell group, which were unequal in size. Moreover, the abnormal category also includes any type of blastomere deformity, asymmetric cell position, unequal cell size, incomplete cell adhesion, and unclear cell arrangement.

Fig 2 (A-C) shows that there is a protrusion with cells from the blastomere group. The unusual arrangement of blastomeres has abnormal cleavage. Abnormalities in these blastomeres have a greater potential for greater development [7]. Several factors cause poor egg quality, including lack of fertilization, egg activation problems, developmental delays, embryonic death, and embryo abnormalities. [6;8-9] In addition, it can also be influenced by conditions that come from the parent and environmental factors that support the occurrence of a malformation [9].

3.4. Gastrula period

One of the movements that occur during the period of gastrulation is epiboly. Embryonic development at the epibolic stage is described by the percentage of blastoderm covering the yolk area. The 30% epiboly stage is defined as the amount of blastoderm that closes the yolk is 30% (Fig 1.K) about 5 hours 19 minutes after fertilization. At 6 hours 12 minutes, 50% of the area of the animal pole and vegetal pole covered by the blastomere (Fig 1.L). Together with epiboly, other movements of the blastoderm such as involution produce a germ ring at the edge of the blastoderm at 6 hours 15 minutes (Fig 1.M). The blastoderm consists of two layers of cells at the germ-ring stage, namely the epiblast and the hypoblast [4]. At 7 hours 12 minutes, the blastoderm covers the ¾ yolk area (Fig 1.N) and at 9 hours 18 minutes, a yolk plug is visible (Fig 1.O). The yolk plug is a yolk area that is not covered by the blastoderm (Fig 1.A-O). The end of the gastrula period is marked by the closure of 100% yolk closure by the blastoderm and the formation of a tailbud. Buds are formed anteriorly and posteriorly (Fig 1.P). The anterior buds will develop into the head, while the posterior buds will develop into the tail.

3.5. Segmentation Period

Segmentation period is the process of somite formation. Over time there was an increase in the number of somites which increased the length of the embryo linearly. The formation of the first pair of 1-10 somites occurs in the anterior region. The formation of a new pair of somites continues from the anterior to the posterior region in 11 hours 54 minutes after fertilization (Fig 1.Q). At 14 somites, Kuppfer's vesicle could be observed, this Kupffer's vesicles functions to determine the right and left orientation of organ formation (Fig 1.R). At 13 hours 53 minutes after fertilization, 17 somites have been formed (Fig 1.S). Entering 14 hours 39 minutes after fertilization, the somites already numbered 20 pairs. There is a clear elongation of the tail of the embryo.

3.6. Pharyngula period

As a result of the segmentation period, the embryo has a set of somites that extend to post-anal tail, these characteristics indicate that the embryo is ready to enter the pharyngeal period, besides that the embryo's notochord has developed well and the hollow nervous system has begun to extend anteriorly.

The prim or pharyngeal period is a period where there is a migration from the germ layer so that it will form skin or other organ linings such as pigments. At the primordial stage, it will be characterized by the formation of aortic arches, pigmentation in the eyes and epithelium, dorsal, ventral, and pectoral fins, vessels, pericardial cavity, eyes with lenses that surround the retina, and brain development which is divided into the cerebellum, midbrain, and hindbrain (Fig 1.T)

3.7. Hatching period

The egg hatching time observation result showed different hatching periods for each egg. First egg

hatching was occured at 24 hours after fertilization, and continue to occur up to 30 hours after fertilization.

The hatching rate was measured up to 30 hpf, which was 44% of eggs that hatch at a certain time. Hatching is the last stage in the incubation period as a result of several processes, one of which is the larvae coming out of their shells. The analyzed data ANOVA and DMRT showed a significant difference between replicates ($p<0.05$). Table 3 shows that there is an increase in the percentage of hatching. This hatching time can vary for each individual, depending on the environmental conditions that affect it.

Table 3. Hatching rate

Hatching time (hpf*)	Percentage (%)
24	40.67
30	44.00

*Hours post fertlization

Hatching can occur due to two things, 1) mechanical factor, when the embryo often changes its position due to lack of space in its shell, or because the embryo is bigger than its shell environment, 2) enzymatic factor, when enzymes and other chemical elements were secreted by the Endoderm glands in the pharyngeal region of the embryo. Factors that can cause low hatching rates are eggs that do not develop after fertilization and changes in the physiological abilities of eggs during embryogenesis [9].

AUTHORS' CONTRIBUTIONS

BR designed the project/main conceptual ideas and research outline. AAR and SF fish care and part of research setting. HZ, KA developed theories and verified analytical methods. HZ and KA conducted research. The results and manuscript final are carried out based on the discussions and contributions of all authors

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