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The Effect of Incubation Temperature on Hatching Period, Hatching Rate and Embryonic Development of Barramundi (*Lates calcarifer,* Bloch 1790)

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Abstract

Despite the widespread development of *Lates calcarifer* (Barramundi) cultivation since the 1970s, scientific documentation on the effect of incubation temperature on the hatching period, hatching rate, and embryo development remained limited. Temperature is a crucial physicochemical factor that significantly affects the early stages of embryo development. A total of 100 eggs were placed in incubation containers with a volume of 4 liters, and subjected to temperature treatments of 26, 28, 30, and 32°C. This study aims to examine the effects of incubation temperatures on the hatching period, hatching rate, embryo development, and to determine the optimal incubation temperature to improve hatchery techniques. The results show that incubation temperature plays an important role in the hatching period, hatching rate and embryo development. The 32°C treatment resulted in the highest values for all study parameters: a hatching period of 633.3±11.5 minutes post-fertilization and a hatching rate of 86.25±4.45%. Conversely, the 26°C treatment had the lowest values, with a hatching period of 1050±10.0 minutes post-fertilization and a hatching rate of 72.92±6.24%. Embryo development indicated that the Australian strain developed faster than the Asian strain. Additionally, embryos incubated at 26°C were able to develop and survive until hatching, although this was the lowest result in this study. The findings of this study are essential for supporting efforts to optimize larval rearing practices and improve hatchery-level operations in the future.

Introduction

Lates calcarifer (Barramundi) is a species of great commercial importance for aquaculture in Southeast Asia and Australia (Katersky and Carter, 2005). The cultivation of barramundi began in Asia and Australia in the 1970s and 1980s and has since undergone rapid expansion (MacKinnon, 1989; Thépot and Jerry, 2015). In Indonesia, the Fisheries Research and Development Center, in collaboration with the Japan International Cooperation Agency (JICA), successfully developed barramundi hatchery techniques in 1991. Key factors contributing to the rapid cultivation of barramundi include their rapid growth rate, high fecundity, and high tolerance to various cultivation conditions (Partridge et al., 2008; Simanjuntak et al., 2021). Despite the widespread and long-standing development of barramundi cultivation, scientific documentation on the influence of incubation temperature on hatching period, hatching rate, and embryonic development remains limited (Thépot and Jerry, 2015).

High-quality eggs produced from quality broodstock gametes are crucial for producing quality juveniles, which is a critical step in enhancing production

(Mylonas et al., 2010; Pangreksa et al., 2016). Therefore, controlling environmental temperature in aquaculture is crucial to maintaining species-specific breeding conditions and preventing developmental abnormalities and low hatching rates (Arenzon et al., 2002; Tucker et al., 2002). Temperature is an important physicochemical factor that significantly affects embryonic development and the success of aquaculture production (Viader-Guerrero et al., 2021), particularly in producing quality fish larvae (Boucher et al., 2014). Eggs incubated at higher temperatures experience faster embryonic development, causing larvae to hatch earlier (Imsland et al., 2019), and the yolk sac is absorbed more quickly (Brown et al., 2011). Conversely, lower temperatures caused delays in embryo development and reduced viability (Tucker, 2002; Georgakopoulou et al., 2010). Thépot and Jerry (2015) have reported the optimal incubation temperature for the Australian strain of *L. calcarifer* providing critical insights into species-specific requirements for early developmental stages. However, differences due to strain variation could be interesting to explore, particularly whether there are disparities in embryonic development between the Asian and Australian strains. In intensive aquaculture efforts, maximizing all aspects, including early embryo development, ensures optimal results in both quality and quantity (Imsland et al., 2019; Przybyla et al., 2023).

This study aims to examine the effect of incubation temperature on the hatching period, hatching rate, embryo development, and to provide a reference to improve hatchery techniques for barramundi cultivation. To identify the optimal temperature range for hatching Lates calcarifer, we used the thermal regime employed by Thépot and Jerry (2015) for the Australian strain of *L. calcarifer*. The results may potentially enhance existing hatchery protocols, providing valuable insights particularly applicable to domesticated strains of *L. calcarifer*. Such advancements could support efforts to optimize larval-rearing practices and improve the overall efficiency of aquaculture operations for this economically significant species.

Material and Methods

Broodstock Maintenance

The barramundi broodstock were maintained at the Mariculture Development Centre, Ambon in accordance with the Indonesian National Standard (SNI: 01-6145-1999) for aquaculture set by the Ministry of Marine Affairs and Fisheries of the Republic of Indonesia. Broodstock were kept in fiberglass tanks with a volume 3 of M^3 and fed fish and squid amounting to 3–5% of body weight twice a day. Entering the spawning period, the feed was given once a day, supplemented with vitamins (2 g/broodstock) to enhance nutrition. When the broodstock met the criteria, they were transferred to a concrete tank for spawning. Spawning was conducted using a 1:1 ratio of male to female broodstock, with broodstock ages ranging from 2 to 3 years. The average weight of male broodstock was 2.5–3 kg, while female broodstock averaged 3.5–5 kg. The salinity in the spawning tank ranged from 29-30 ppt, and dissolved oxygen levels ranged from 4.53-5.11

Egg Collection, Selection, and Incubation

Spawning typically occurred between 4 and 5 am, after which eggs were collected using PVC pipes (3-inch diameter) split lengthwise into two parts and placed on the surface of the water to collect floating eggs. Good-quality barramundi eggs are characterized by their buoyancy, transparency, and round shape (Ulfani et al., 2018). Selected eggs were placed in containers filled with seawater for further selection, with floating eggs being used for incubation and hatching. The total egg production from the spawning was 2.7 million eggs. The eggs were transferred to plastic containers with a volume of 4 L of water, which had been prepared in advance with a GH50 Aquarium Submersible Glass Heater (Aquatop, California, USA) to maintain stable temperatures of 26, 28, 30, and 32°C, with three replicates for each temperature. A total of 100 eggs were placed in each treatment container. To prevent thermal shock, eggs were gradually introduced into the containers along with 500 mL of water. The experimental containers were placed in a semi-outdoor environment where the light intensity was consistent with natural conditions ensuring that the photoperiod and light levels corresponded to those typically experienced in the species' natural habitat. Salinity in the incubation containers was measured to range from 30 to 32 ppt, and dissolved oxygen levels ranged from 6.17 to 6.47 mg/L.

Egg Observation

Prior to incubation, eggs were observed to determine their initial phase. A total of 10 eggs were randomly selected for observation, and this process was repeated throughout the duration of the observation. At 30-minute intervals, damaged eggs were removed during incubation to prevent water quality degradation (Pangreksa et al., 2016). The developmental stages of the *L. calcarifer* Australian strain, as previously documented by Thépot and Jerry (2015), were used as a reference to determine the embryonic developmental stages in this study. These stages were employed to compare the development between the Asian and Australian strains. The microscope used during the observation was the Zeiss Binocular Stereo microscope (Carl Zeiss AG, Oberkochen, Germany). The determination of hatching period and hatching rate was calculated using the following formula (Thomas et al., 2020):

$$\text{Hatching period (h)} = t_{50} - t_1$$

$$\text{Hatching time (\%)} = \frac{N_h}{N_f} X\ 100$$

Where t_{50} and t_1 were the time at 50% of larvae hatching and the time at the beginning of fertilization, respectively. N_h and N_f were the number of eggs hatched and the total eggs stocked, respectively.

To evaluate the volume consumption of the yolk sac, the following was used to calculate;

$$\text{Yolk sac volume } (mm^3) = 0.1667\ \pi\ L\ A^2$$

Where L is the length and A is the height of the yolk sac (Blaxter and Hempel, 1966).

Statistical Analysis

The effect of incubation temperatures on hatching period and rate were analyzed using one-way ANOVA, followed by Tukey's test, with the statistical software R version 3.4.3 and the "stats" package (R Core Team, 2020). The difference between each treatment was considered significant at P<0.05 and all data were presented as an average±standard deviation.

Result

Hatching Period

The ANOVA test of the effect of incubation temperature on hatching period yielded significant values across all treatments (P<0.05). The incubation temperature treatment at 32°C had the highest hatching period value of 633.3±9.43 minutes. In contrast, the incubation temperature of 26°C had the lowest hatching period value in this study, at 1050.0±8.16 minutes. The hatching period values for the 28°C and 30°C treatments were 916.7±4.71 minutes and 754.3±12.28 minutes, respectively (Figure 1a).

Hatching Rate

Based on the results of the ANOVA test for the effect of incubation temperature on hatching rate, it yielded an insignificant value (P>0.05). The 32°C treatment had the highest hatching rate of 86.25±4.45%, while the 26°C treatment had the lowest hatching rate at 79.92±6.24%. The hatching rates for the 28°C and 30°C treatments were 78.75±7.36% and 82.50±1.77%, respectively (Figure 1b). Generally, in all temperature treatment groups, eggs that ceased developing were identified by their opaque white color. These eggs were classified as damaged and considered to have failed to develop to the hatching stage.

Effect of Temperature on the Development Stage

Initial Development

The diameter of barramundi eggs was 0.54±0.01 mm (n=20). At 20 minutes post-fertilization, the cytoplasmic movement triggered the cortical cytoplasm to move toward the animal pole and formed a structure called the blastodisc (Figure 2a). It was difficult to distinguish successfully fertilized eggs from unfertilized ones; generally, fertilized eggs appeared clear with a visible perivitelline space. Eggs that failed to fertilize and develop tended to be white and opaque. The zygote stage started from fertilization to the beginning of cell division, with a transition period of approximately 30±0.0 minutes post-fertilization (Table 1).

Cleavage Stage

The cleavage pattern in barramundi embryos is classified as meroblastic. This specific cleavage is restricted to the blastodisc region of the embryo, while the yolk remains unsegmented. The process is characterized by vertical divisions extending from the animal pole to the vegetal pole, concluding at the

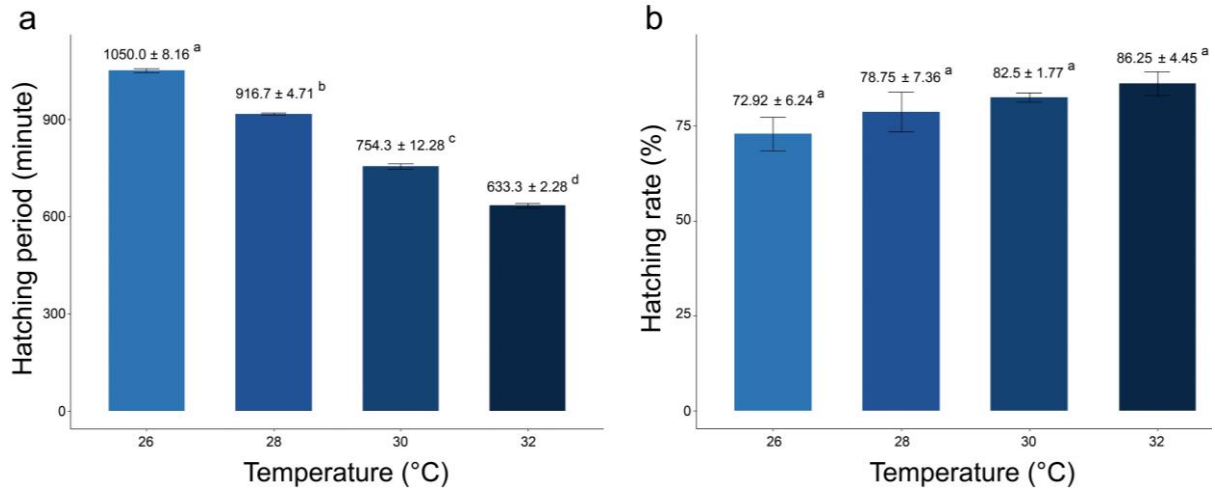

Figure 1. Hatching period and hatching rate of *Lates Calcarifer* egg incubation at different temperature (mean±SD). (a) Hatching period and (b) hatching rate. Different superscripts denote significant different (P<0.05).

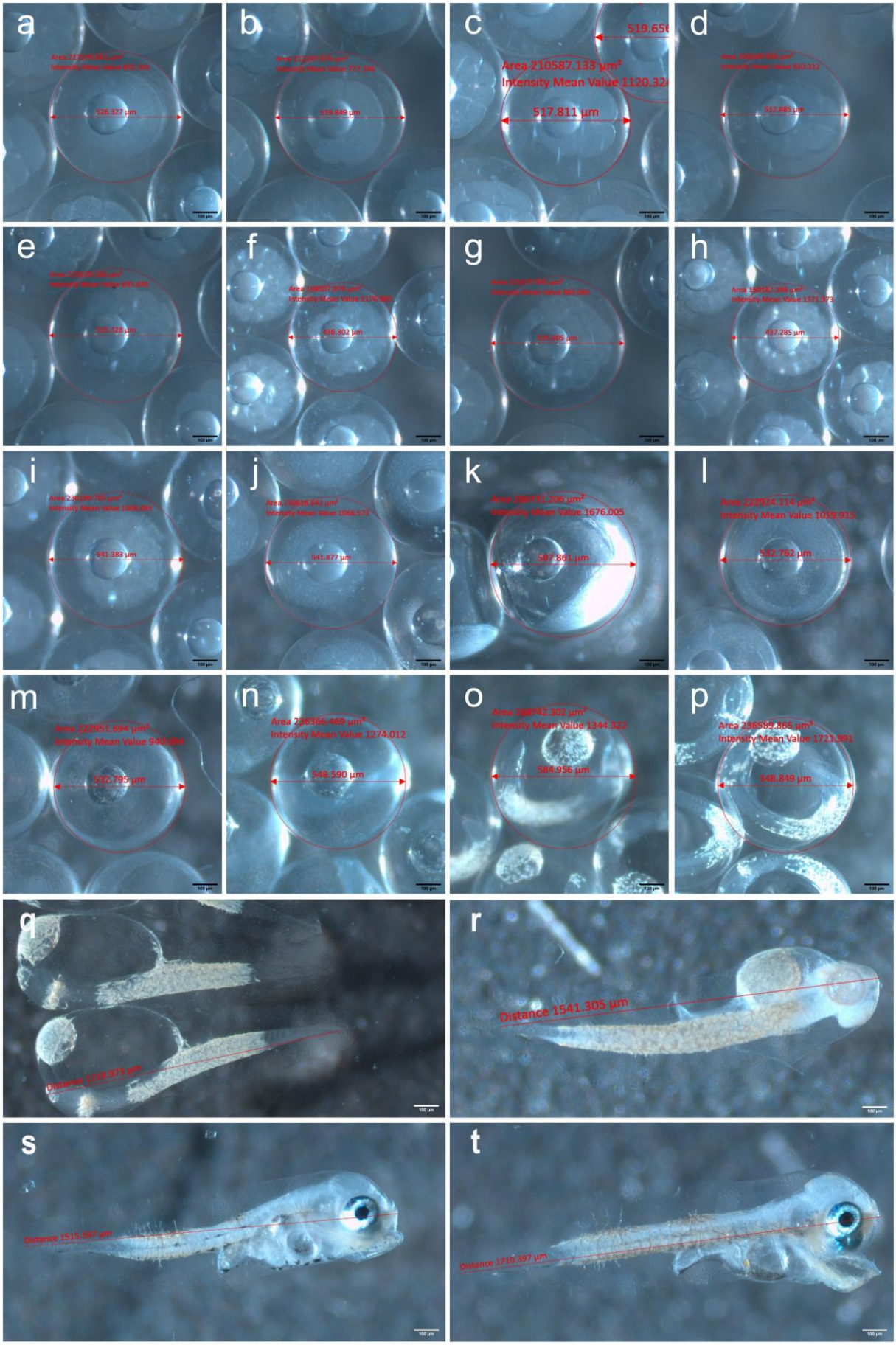

Figure 2. Developmental stages of *Lates calcarifer* embryo and larvae: (a) 1-cell; (b) 2-cell; (c) 4-cell; (d) 8-cell; (e) 16-cell; (f) 32-cell; (g) 64-cell; (h) 128-cell; (i) 256-cell; (j) high stage to oblong transition; (k) dome; (l) 50% epiboly; (m) 90% epiboly; (n) tail bud stage; (o) 2-somite; (p) 20-somite; (q) newly hatched larvae; (r) 24 hours post-hatching; (s) 48 hours post-hatching; (t) 72 hours post-hatching. Scale bar 100 µm.

Table 1. Effects of temperature on the rate of development of *Lates calcarifer* embryos from zygote stage until hatching (mean ± S.D).

Stage	Temperature (°C)			
	26	28	30	32
1-cell	30.7±0.5^{a}	30.7±1.1^{a}	30.3±0.5^{a}	30.0±0.0^{a}
2-cell	42.3±1.5^{b}	40.3±0.5$^{a.b}$	39.3±0.5^{a}	40.0±1.0$^{a.b}$
4-cell	48.3±0.5^{b}	46.7±1.5^{b}	42.7±0.5^{a}	41.7±0.5^{a}
8-cell	59.3± 0.5^{b}	58.0±1.0^{b}	51.3±0.5^{a}	50.3±0.5^{a}
16-cell	77.0±1.0^{d}	72.7±0.5^{c}	62.7±0.5^{b}	60.0±1.0^{a}
32-cell	88.7±1.5^{d}	84.7±0.5^{c}	72.7±0.5^{b}	70.0±0.0^{a}
64-cell	109.3±0.5^{d}	104.0±1.0^{c}	82.3±0.5^{b}	79.0±1.0^{a}
128-cell	124.7±0.5^{d}	117.7±1.1^{c}	91.7±1.1^{b}	89.0±1.0^{a}
Oblong	252.0±2.0^{d}	192.0±2.0^{c}	160.0±2.0^{b}	142.7±3.7^{a}
Dome	359.3±1.5^{d}	291.0±2.6^{c}	224.0±5.2^{b}	198.7±1.5^{a}
50% epiboly	418.0±2.0^{d}	368.0±2.0^{c}	323.3±3.0^{b}	289.7±0.5^{a}
90% epiboly	530.7±2.0^{d}	445.3±3.0^{c}	367.0±3.6^{b}	326.0±1.7^{a}
Tail bud	614.0±4.0^{d}	557.7±2.5^{c}	435.0±4.3^{b}	395.0±2.0^{a}
20 somites	854.3±4.0^{d}	728.0±7.2^{c}	595.7±5.1^{b}	492.0±2.6^{a}
Hatching	1050.0±10.0^{d}	916.7±5.7^{c}	754.3±15.0^{b}	633.3±11.5^{a}

* within the same row, the with different superscript letters were significantly different ($p<0.05$) determined by Tukey's test.

periphery of the yolk. This results in an increased number of progressively smaller cells (Figure 2b–g). The time taken to reach the 64-cell stage at an incubation temperature of 32°C was 79±1.0 minutes post-fertilization (Table. 1).

Blastula Stage

The blastula stage is characterized by the spherical shape of the blastodisc when observing the animal poles. The blastula period can be separated into early (synchronous) and late (asynchronous) stages, occurring respectively before and after the mid-blastula transition (Soman et al., 2021). The synchronous blastula period when cell division reached 128 cells at 90 minutes post-fertilization with an incubation temperature of 32°C (Figure 2h) (Table.1). At this stage, the blastodisc has an ellipsoidal shape when viewed on the animal pole and the yolk syncytial layer (YSL) can be clearly distinguished. The asynchronous period starts from the oblong, sphere, and dome stages (Retnoaji *et al.*, 2023). At the dome stage occurs at 198±1.5 minutes post-fertilization with an incubation temperature of 32°C (Table. 1), where the yolk moves towards the animal pole, forming a dome (Figure 2k).

Gastrula Stage

The gastrula stage is marked by the beginning of epiboly. During the epiboly stage, the germ ring covered both the animal and vegetal poles. The dorsal-ventral axis is characterized by the formation of a shield, where the blastoderm partially closes over the yolk at 50% epiboly (Sule et al., 2004) (Figure 2l). The 50% epiboly stage is achieved 289±0.5 minutes post-fertilization at an incubation temperature of 32°C (Table. 1). The embryo shield extends towards the animal pole and appears to cover almost the entire yolk at the 90% epiboly stage at 326.0±1.7 minutes post-fertilization (Figure 2m–n). Development at this stage determined the anterior-posterior axis, with the anterior eventually developing into the head and the posterior into the tail (Thépot and Jerry, 2015).

Segmentation Stage

The segmentation stage is characterized by the formation of segments called somites (Figure 2o–p). Initially, somite development is rapid but slows as their number increases. In vertebrate development, the primary body axis underwent a developmental process that led to the formation of the posterior part (Güralp et al., 2017). This process is crucial for forming various tissues, including vertebrae, skeletal muscle, and dermis (Retnoaji et al., 2023). During the segmentation stage, the embryo's length expands in response to the increasing number of somites. At an incubation temperature of 32°C, the time taken to reach 20 somites was 492±2.6 minutes post-fertilization (Table. 1).

Hatching Period

The intensity of twitching movements increases, and the chorionase hatching enzyme is secreted, allowing the embryo to penetrate the chorion (Figure 2q) (Blaxter, 1969; Thépot and Jerry, 2015). Upon hatching, the larva's head is still attached to the yolk sac, which has a volume of 0.08±0.01 mm^3. The oil globule, auditory vesicles, and developing eyes are clearly visible (Figure 3a). The newly hatched larvae were transparent, measuring of 1.15±0.03 mm (n=20), with several melanophore pigments seen on the larval body (Figure 3b). Just post-hatching, the larvae floated upside down because of the yolk sac and oil globule, despite fin folds surrounding the posterior part, which are not yet sturdy enough for swimming (Figure 3b). The mouth or jaw remained closed for up to 24 hours post-hatching, with nutrients supplied by the yolk sac and oil globule, which contained proteins, lipids, and glyceride fats crucial for the larvae and are absorbed immediately

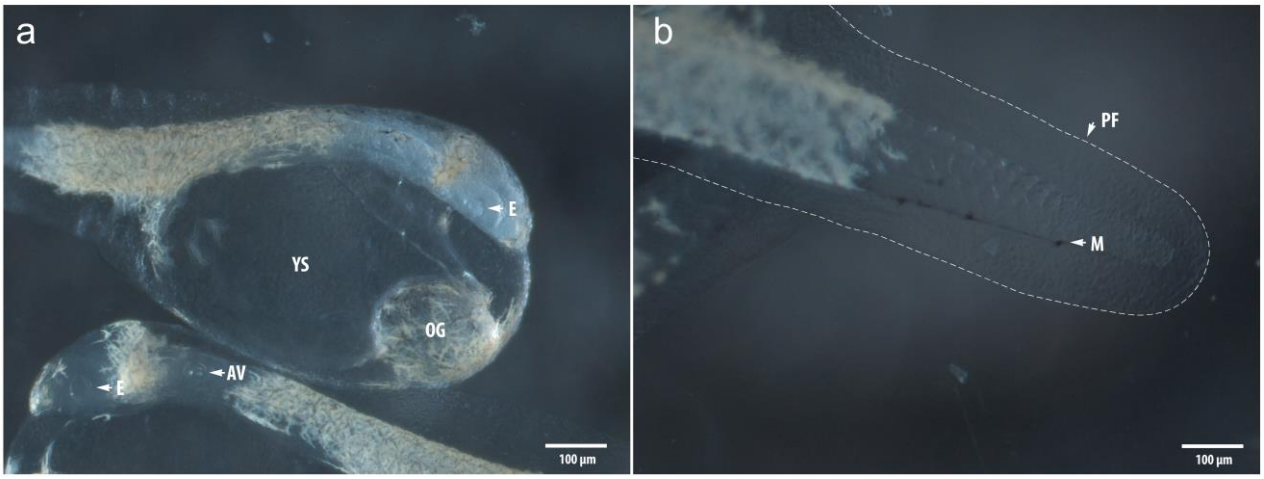

Figure 3. Detailed photomicrographs of the head and tail of *Lates calcarifer* embryos. (a) An embryo at the early segmentation stage showing key structures: E (Eye), AV (Auditory Vesicle), OG (Oil Globule), and YS (Yolk Sac). (b) The dotted line indicated the posterior finfold (PF) of an embryo and highlighting the M (Myomeres). Scale bar 100 µm.

(Gajbhiye et al., 2022). At 32°C, the time taken until hatching was 633.3±11.5 minutes post-fertilization (Table. 1).

Effect of Temperature on Developmental Rate

The incubation temperature caused a significant difference in the hatching period ($P<0.05$), with hatching durations for the 26°C, 28°C, 30°C, and 32°C treatments being 1050.0±10.0 minutes, 916.7±5.7 minutes, 754.3±15.0 minutes, and 633.3±11.5 minutes post-fertilization, respectively. At the beginning of development, there were no significant differences between all treatments observed until reaching the 16-cell stage, where the 26°C treatment had the slowest progression at 88.7±1.5 minutes post-fertilization, and the 32°C treatment had the fastest progression at 70.0±0.0 minutes post-fertilization ($P<0.05$). During the later stages of barramundi embryo development, namely from blastula and gastrula to hatching, significant differences were observed across all incubation temperature treatments (Table. 1). At 120 minutes post-fertilization, a difference was observed in the incubation temperature 32°C treatment, with some eggs already developing to the oblong and dome stages. After 720 minutes post-fertilization, eggs in the 32°C incubation treatment began hatching, while eggs in other treatments did not show the same progress. The effect of temperature on the development rate is clearly seen in the documentation presented in Figure 4.

Discussion

The study confirmed that incubation temperature significantly influences the embryogenesis of barramundi, with Australian strains generally developing faster than Asian strains at the same temperatures (Thépot and Jerry, 2015). This aligns with the results of this study, where the time taken for hatching at an incubation temperature of 30°C was 754.3±15.0 minutes post-fertilization, slower compared to Australian strains, which hatched at 717.0 minutes

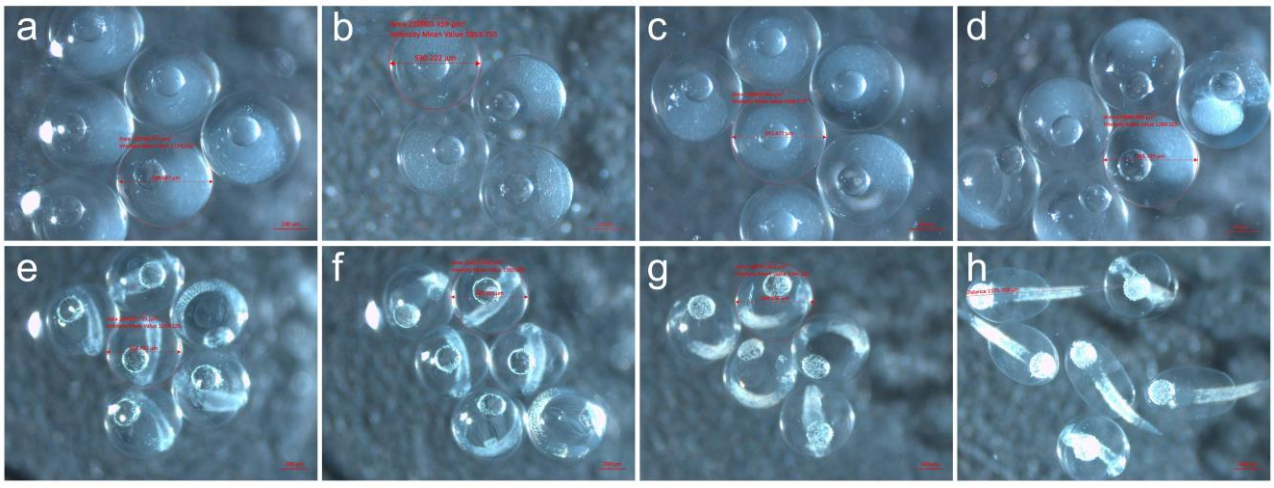

Figure 4. Differences in the embryo development of *Lates calcarifer* at 120 minutes and 720 minutes post-fertilization. (a) at 26°C, (b) at 28°C, (c) at 30°C, and (d) at 32°C at 120 minutes post-fertilization. (e) at 26°C, (f) at 28°C, (g) at 30°C, and (h) at 32°C at 720 minutes post-fertilization. Scale bar 200 µm.

post-fertilization at the same temperature (Thépot and Jerry, 2015). However, a difference was observed at 26°C. In this study, eggs managed to survive until hatching, taking 1050.0±10.0 minutes post-fertilization, with a hatching rate of 72.92%. These findings contrasted markedly with those reported by Thépot and Jerry (2015) for Australian strains, where embryogenesis failed, and the eggs did not survive hatching, resulting in a hatching rate of 0%. The reasons for this discrepancy are not fully understood but might include factors such as poorly maintained fluctuations in incubation temperature or the quality of gametes from the broodstock used (Castranova and Woods, 2005; Lim et al., 2017).

Maneewongsa and Tattanon (1982) explained that for *L. calcarifer* Asian strain, at an incubation temperature of 27°C, 1-cell development occurred at 35 minutes post-fertilization, and hatching occurred at 1020 minutes post-fertilization. At temperatures of 30-32°C, this was achieved within 720-840 minutes post-fertilization. The results in this study also showed similar findings; in all incubation temperature treatments, the average development of the 1-cell stage was achieved within 30±0.5 minutes post-fertilization, and hatching was achieved within 1,050±10.0 minutes post-fertilization. The 1-cell stage development showed no significant difference across all temperature treatments ($P<0.05$), likely because the eggs were incubated at temperatures appropriate to the treatment (Thépot and Jerry, 2015). At 30°C and 32°C, the time taken was 754.3±15.0 minutes post-fertilization and 633.3±11.5 minutes post-fertilization, respectively (Table. 1), similar to those observed in the study by Maneewongsa and Tattanon (1982).

The observed data in this study indicated that the incubation temperature of 32°C resulted in the highest hatching rate at 86.25%, while the 26°C treatment had the lowest hatching rate at 79.92%. The hatching rates at 28°C and 30°C were 78.75% and 82.50%, respectively. However, the hatching rates across different incubation temperature treatments did not differ markedly ($P>0.05$). These results contrast with those reported by Thépot and Jerry (2015), who found that the incubation temperature significantly affected the hatching rate of the Australian strain of *L. calcarifer*. In their study, the highest hatching rate was at 30°C (86.6%) and the lowest at 34°C (42.6%), with temperatures of 26°C and 36°C failing to support development, resulting in a hatching rate of 0%. The reasons for these discrepancies are unclear, but it should be noted that each species has a different tolerance range (Soman et al., 2021), and the quality of gametes from the broodstock is crucial for the success of fertilization, hatching, and low mortality (Mylonas et al., 2010).

Several prior studies indicated that the egg and larval stages were highly sensitive to environmental conditions, particularly temperature, which greatly influenced development and survival of black seabass (*Centropristis striata*), white sturgeon (*Acipenser transmontanus*), lake whitefish (*Coregonus clupeaformis*), lumpfish (*Cyclopterus lumpus*), ocellaris clownfish (*Amphiprion ocellaris*), longfin yellowtail (*Seriola rivoliana*) (Berlinsky et al., 2004; Boucher et al., 2014; Lim et al., 2017; Imsland et al., 2019; Soman et al., 2021; Viader-Guerrero et al., 2021). Generally, physiologically, fish survived within a specific temperature range known as the species' tolerance range. If fish lived in environments outside this tolerance range (extreme), it resulted in stress and developmental failure (Soman et al., 2021, Naz et al., 2023). In ectothermic animals, temperature was the primary factor influencing embryonic development on a commercial scale and could potentially cause hatchery problems by speeding up or slowing down hatching rates provided that the species tolerance range was considered (Thépot and Jerry, 2015; Ashaf-Ud-Doulah et al., 2021).

This study demonstrated that temperature affects the hatching period and embryonic development. However, it should be noted that there was no significant difference in the hatching rate for the *L. calcarifer* Asian strain. This lack of significant difference may be attributed to the relatively small number of eggs used or the high quality of the eggs, which may have made them more tolerant to temperature variations. This study did not assess the long-term effects of temperature variation on yolk sac utilization, larval growth, and development. Therefore, further comprehensive research is necessary to understand these aspects better. Such information would be valuable and could contribute significantly to aquaculture practices aimed at optimizing larval survival and growth rates, which are crucial for improving larval-rearing protocols and enhancing the overall efficiency of aquaculture operations.

Conclusion

This comprehensive research thoroughly explained the effects of incubation temperature on the hatching period, hatching rate, and embryo development of Barramundi, which is not widely discussed. Incubation temperature played an important role in the hatching period and hatching rate, with the 32°C treatment showing the highest values across all study parameters, with a hatching period of 633.3±11.5 minutes post-fertilization and a hatching rate of 86.25%. The temperature of 26°C had the lowest values in the hatching period and hatching rate parameters, which were 1050±10.0 minutes post-fertilization and 72.92%, respectively. Embryo development produced similar results to those explained by Maneewongsa and Tattanon (1982) as well as Thépot and Jerry (2015), indicating that the Australian strain developed faster compared to the Asian strain. Embryos at an incubation temperature of 26°C could still develop and survive until hatching, although this was the lowest result in this study.

Ethical Statement

This study utilized eggs obtained from broodstock barramundi at the Marineculture Development Center, Ambon, Indonesia, and broodstock management as well as breeding procedures were conducted in accordance with the Indonesian National Standard (SNI: 01-6145-1999). All procedures were performed meticulously to ensure the welfare of the embryos and complied with relevant guidelines. Ethical approval was not required for this study as it involved non-invasive observation of fish embryos, which did not cause suffering or stress to the animals. According to the Indonesian Animal Welfare Act and related regulations, non-invasive research on fish embryos is exempt from ethical approval requirements because it does not involve the use of chemicals, anesthesia, or other harmful procedures.

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Author Contribution

JR: Conceptualization, writing-original draft, data curation, formal analysis, investigation, methodology, and visualization. JMFS: Supervision, writing-review, and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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