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OPTIMIZING EMBRYONIC DEVELOPMENT, EGG HATCHABILITY, AND LARVAL SURVIVAL OF ASIAN SEABASS, *Lates calcarifer* USING PAPAYA LEAF EXTRACT (*Carica papaya*) TREATMENTS

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ABSTRACT

Embryonic development of Asian seabass, *Lates calcarifer* is a critical phase in the success of larval rearing production in a hatchery. Low production of larvae has been the drawback of the Asian seabass aquaculture owing to diseases and microbial infection to the eggs, causing low egg viability. This study aims to evaluate the effect of different doses of papaya leaf extracts during embryonic development to improve egg hatchability and larval survival. Six different doses of papaya leaf extracts of 2, 4, 6, 10, 20, and 25 mL were used as treatments arranged triplicates in five liter aquaria. The newly fertilized eggs (99 eggs) were immersed in each treatment for five minutes and fifteen seconds. Subsequently, the eggs were moved into 10 L incubation tanks, and samples were collected for embryogenesis observation. The hatchability of eggs was significantly different among treatments. The hatchability of larvae in group with dose of 4 mL was the best treatment (93.94 ± 0.1%) followed by 2 mL (93.88 ± 3%), and 6 mL (90.91 ± 3.0%). The different doses of papaya leaf extract significantly affected larval survival. The highest survival rate of larvae was 2 mL (93%) followed by 4 mL (90%) and 6 mL (70%). There was no significant difference in the hatching time of larvae. The fastest hatching time occurred at the dose of 6 mL about 13 hours and 30 minutes, followed by 2 mL and 4 mL treatment for about 14 hours. This study recommends that the doses of 2 mL or 4 mL of papaya leaf extracts have potential impacts on the improvement of larval rearing production for Asian seabass hatchery.

KEYWORDS: Asian seabass; hatchability; survival rate; larval production; embryogenesis

INTRODUCTION

Asian seabass or barramundi (*Lates calcarifer*) is an economically valuable aquaculture species in Asia Pacific regions (Jaya *et al*., 2013; Ezraneti & Windarti, 2015; Yaqin *et al*., 2018; Windarto *et al*., 2019). This species contains high nutritional values and is a fast-growing fish for aquaculture development (Kuznetsova *et al*., 2014; Siddik *et al*., 2016; Heriyati *et al*., 2016). The fish has been widely cultured by small and large-scale aquaculture industries due to relatively simple rearing techniques and more tolerance to environmental fluctuations (Rayes *et al*., 2013; Putri *et al*., 2018).

High demand for fish fingerlings required hatcheries to increase fry production annually for the development of Asian seabass aquaculture (Migaud *et al*., 2013; Matthew *et al*., 2015; Bista *et al*., 2019). However, increasing the production of larvae has been a more challenging aspect in hatcheries due to low larval production indicated by low egg hatchability

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(Shadrin & Pavlov, 2015; Ochokwu *et al*., 2015). At the post-spawning stage, the low hatching rate of eggs was commonly caused by microbe infestations or disease infections which leads the eggs to be unable to hatch (Petrescu-Mag *et al*., 2011; Caipang *et al*., 2011; Caipang *et al*., 2014).

The use of commercial drug treatments to deal with disease outbreaks can increase additional input that reduces the profit of the business in Indonesian small-scale hatcheries. Thus, it needs alternative raw materials that are abundantly available, efficient, and effective for improving eggs' hatchability and larval survival. Papaya leaf extract is one of the raw materials known as the source of papain enzyme (Zusfahair *et al*., 2014) containing proteolytic and antimicrobial activity (Saeed *et al*., 2014; Lismawati *et al*., 2017), and carpain which is commonly used for its antimicrobial properties (Sari *et al*., 2016) and immunostimulant (Monica *et al*., 2017).

Previous studies showed that the papain enzyme provides a positive response for improving fish survival (Arief *et al*., 2016; Nuraeni *et al*., 2018) and it has been applied for improving eggs hatchability and decreasing abnormalities of larvae in milkfish (*Chanos chanos*) hatcheries (Haser *et al*., 2018). This study aims to evaluate the effect of different doses of papaya leaf extracts on embryonic development and improvement of egg hatchability and larval survival.

MATERIALS AND METHODS

The research was carried out at the Aceh Besar Brackishwater Aquaculture Center (BPBAP) Ujung Batee, Aceh, Indonesia from May to June 2018. The leaves of papaya were collected from the papaya farmer in Aceh. The papaya leaves were packed in a plastic bag and kept fresh for further sample preparation. The eggs of Asian seabass were collected from the BPBAP hatchery facility. The eggs were produced from the semi-artificial reproduction of Asian seabass broodstock with a female and male ratio of 2:1.

Sample Preparation

Papaya leaf extraction followed Haser *et al*. (2018) protocol. The papaya leaves (excluding the main stem) were selected and properly cleaned with water and kept in the freeze dryer at a temperature of -108°C until dried. The dried leaves were extracted and made into powder. The leaf powder samples (10 g) were diluted with ethanol (200 mL) and the dilution was filtered using a Whatman paper screen (Whatman No.1) to remove unwanted materials. Then, the filtrated samples were condensed in a rotary evaporator at 45°C for 1.5 hours. The papaya leaf extract was stored in a refrigerator for further use in this experiment.

Experimental Set-Up

Six different doses of papaya leaf extracts of 2, 4, 6, 10, 20, and 25 mL were used as treatments arranged triplicates in 5 liter aquaria filled with seawater. The control group was omitted in this experiment because previous study which was conducted with similar environment showed that eggs hatchability in *Lates calcarifer* without treatment under optimum temperature were low i.e. 50% (Khalfianur, 2019). The newly fertilized eggs (n = 99) were selected and immersed in each solution for five minutes and fifteen seconds. Subsequently, the eggs were moved into 10 L incubation tanks filled with seawater. A total of 10 egg samples were collected from each aquarium and placed in the petri dish. Egg samples were observed under the binocular microscope (Olympus Bx14) with 4x magnification for embryogenesis observation. The process was also recorded using a digital camera. The embryogenesis phases including morula, blastula, gastrula, and organogenesis were monitored until they were hatched. The abnormality of the larvae was also investigated in this study. About 10 healthy larvae collected from each tank were reared for 30 days to observe the treatment effects of papaya leaf extracts on the survival rate of larvae.

During the rearing period, the larvae were placed in 30 cm x 30 cm x 40 cm aerated aquaria filled withh 35 L of water. The larvae were fed with egg yolk at satiation; 50% of the water was replaced every two days to avoid elevated ammonia concentration in the water. Observations for abnormalities and survival were done on daily basis.

Data Analysis

The hatching rate and survival performance of Asian seabass eggs treated with different doses of papaya leaf extracts were calculated using the following methods. The newly hatched larvae and unhatched eggs were counted from each tank. The hatching rate (HR, %) was calculated using a formula explained by Andriyanto *et al*. (2013):

$$HR = \frac{(a+b)}{(a+b+c)} \times 100$$

where: a is the number of normally hatching eggs, b is the number of abnormal hatching eggs, and c is the number of unhatched eggs

Survival rate (SR, %) were calculated using the formula:

$$SR = \frac{Nt}{No} \times 100\%$$

where: Nt is the final number of larvae and N_0 is the initial number of larvae

The effect of treatments was analyzed using one way ANOVA W-Tuckey post-hoc test at a 95% level of confidence.

RESULT AND DISCUSSION

Result

Embryonic Development

The morula division stage was indicated by the division of single-cell mitosis to create two smaller and same-sized cells that occurred 30 minutes after fertilization. The blastula stage was characterized by the invasion of the yolk forming germinal ring. At this phase, some parts of the yolk were still not covered by blastoderm. The blastula phase occurred one hour after fertilization. The next stage of embryogenesis was the gastrula. During gastrula, the yolk was expanded and was fully covered by the blastoderm. This phase was started 6-7 hours after fertilization. After that, organogenesis can be observed as the formation of the head and tail which occurred 9-13 hours and 30 minutes after fertilization. The larvae hatched after three hours and 30 minutes to 15 hours after fertilization. The stages of embryogenesis during this study are described in (Figure 1).

Embryonic development of morula and blastula commonly occurred at a similar time in all treatments. Variability started to observe at the gastrula stage. The complete time of the development stage of Asian seabass embryogenesis in this study can be seen in Table 1.

The Table 1, shows that the range of hatching time observed was from 13 hours and 30 minutes to 15 hours. The most effective dose of papaya leaves for the hatching time of Asian seabass eggs occurred at the dose of 6 mL. The larvae hatched after 810 minutes or 13 hours and 30 minutes, followed by treatment of 2 mL and 4 mL doses for about 14 hours. The longer hatching time occurred in treatments with doses of 10 mL/L, 20 mL/L, and 25 mL/L; extracts on the hatching time of Asian seabass embryonic development.

ANOVA that was run to the time required for eggs to hatch as larvae showed that doses of the extract significantly affect the duration of embryogenesis (Table 1). The shortest duration occurred to group-3 or eggs treated with 6 mL/L of papaya leaf extract, followed by group-1 and 2 with extract concentration of 2 mL/L and 4 mL/L, respectively.

The above results suggested, to shorten embryogenesis duration, application of 6 mL/L of papaya extract will give the best result.

This study also showed that, the embryogenesis of Asian seabass treated with papaya leaf extract has a shorter period compared to untreated samples.

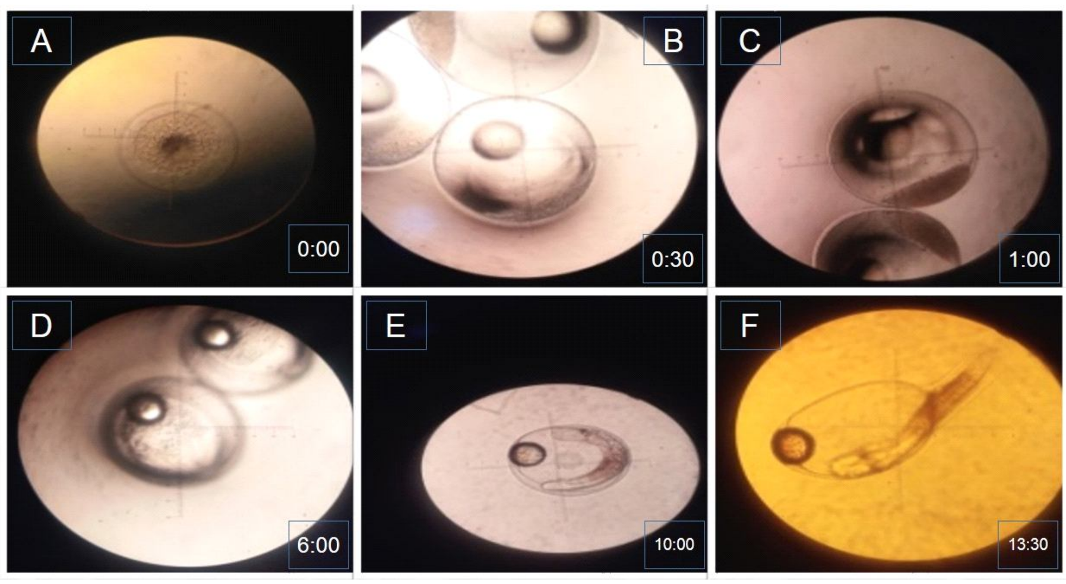

Figure 1. The embryonic stage of the Asian seabass embryo: (a) a single celled embryo; (b) morula division; (c) blastula phase; (d) gastrula occur after six hours; (e) organogenesis starts at 10 hours after fertilization; (f) final stage of organogenesis after 13 hours.

Table 1. The development stage of the Asian seabass embryo treated with six different doses of papaya leaf extracts

Development stage (Minutes)	Treatments (mL)					
	2	4	6	10	20	25
Morula	30 ± 0.474	30 ± 0.668	30 ± 0.792	30 ± 0.542	30 ± 0.667	30 ± 0.047
Blastula	60 ± 0.24	60 ± 0.953	60 ± 0.259	60 ± 0.141	60 ± 0.471	60 ± 0.527
Gastrula	420 ± 0.311	420 ± 0.082	360 ± 0.189	420 ± 0.151	360 ± 0.382	420 ± 0.275
Organogenesis	540 ± 0.33	810 ± 0.212	600 ± 0.943	540 ± 0.481	780 ± 0.471	780 ± 0.236
Larva	840 ± 0.047^{a}	840 ± 0.822^{a}	810 ± 0.189^{b}	870 ± 0.141^{c}	900 ± 0.236^{d}	900 ± 0.527^{d}

Description:* values represent hours and minutes after fertilization

Previous studies showed that the duration of normal embryo development from fertilization to hatching time ranged from 24 hours to less than 34 hours (Hasibuan *et al*., 2018). The application of the papaya leaf extract can shorten embryogenesis and organogenesis durations by up to 20 hours. The acceleration of embryogenesis may be occurred due to papain effects, a proteolytic enzyme, contained in papaya leaves (Enferadi *et al*., 2018). The enzyme reduces the adhesivity of the egg's outer membrane by breaking down the eggs' mucus layer's glycoprotein. This leads to increased oxygenation of eggs which is beneficial for embryo development and it can accelerate embryogenesis duration (Habibah, 2017). However, at high doses of papaya leaf extract administration can have a negative effect on the embryos by the virtue of its teratogenic (delayed development) effect. This effect is assumed to be the result of its constituents' compound activities such as saponin (De Castro *et al*., 2015). Studies showed that ethanolic extract can pass through membrane layer systems and inhibit blastomeres' mitosis (Wabo *et al*., 2011). Hence, eggs immersed in higher extract concentration experience slightly delayed growth and hatching time, although the delays are not statistically significant.

Egg Hatchability and Survival Rate

Hatchability of the eggs administered with papaya leaf extract was significantly different among treatments. The dose of 2, 4, and 6 mL provided the greater results ranging from 90.91 ± 3.0 to 93.94 ± 0.1%. The hatchability of the eggs treated with the dose of 10 and 20 mL of papaya leaf extract ranged from 65.81 ± 1.73 to 69.70 ± 3.0%, while the last treatment (25 mL) performed the lowest hatching rate (38.38%). The results indicate that the higher the concentration of the papaya leaf extracts, the lower the hatching rate of eggs.

Analysis of variance (Figure 2) shows that the different doses of papaya leaf extract significantly affected larvae survival. Similar to egg hatchability, survival rates tend to decrease as the concentration of the extract increase (Figure 2).

Abnormalities

Larvae with longer hatching times tend to suffer from deformities more than those that hatch earlier even though they were not statistically significant (Table 2). Most deformities are related to the shape of the bodies and bones of the larvae as shown in Figure 3. Both analysis results suggest that a high

Table 2. The effects of different doses of papaya leaf extract on hatchability and abnormality of Asian seabass larvae

Treatments (mL)	Hatchability (%)	Abnormality (%)
2	93.88 ± 3^{a}	1.67 ± 0.58
4	93.94 ± 0.1^{a}	1.30 ± 0.58
6	90.91 ± 3^{a}	1.00 ± 1.0
10	69.70 ± 3^{b}	2.67 ± 2.08
20	65.81 ± 1.73^{b}	3.67 ± 2.08
25	38.38 ± 1.73^{c}	2.67 ± 1.15

Note: Means within the same row with different superscripts are significantly different ($P < 0.05$)

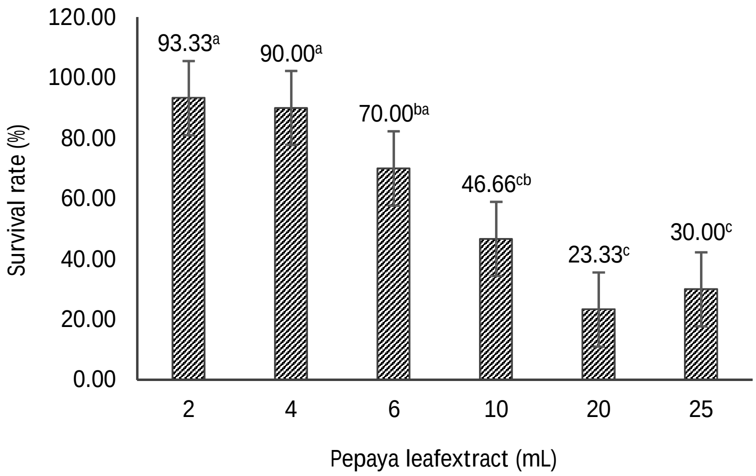

Figure 2. The survival rate of Asian seabass larvae treated with six different doses of papaya leaf extracts.

concentration of papaya leaf extract can be detrimental for both seabass eggs and larvae.

This study demonstrates that immersing Asian seabass eggs in the ethanolic extract at doses of 2, 4, and 6 mL is effective to increase hatchability up to two folds. A previous study demonstrated that the normal hatching rate of Asian seabass eggs ranges from 44.67% to 80% (Hasibuan *et al*., 2018; Ulfani *et al*., 2018). Fungi and microbes are known to have negative effects on eggs hatching rate success. The thinner mucous layer of fish eggs causes them to be less adhered to other objects and hence, eggs become less susceptible to bacterial, sporal, and fungal infection (Hartri *et al*., 2018). This less adhesivity effect is coupled with the antifungal and antibacterial activity of papaya leaves (Baskaran *et al*., 2012; Saputra *et al*., 2019; Saptiani *et al*., 2016). Therefore, immersing eggs at a low extract concentration overcomes problems in egg hatching rate caused by fungal and bacterial infections.

However, a higher concentration of papaya leaf extract can be fatal for the egg hatching rate. Wabo *et al*. (2011) examined unsuccessfully hatched eggs of *Heligmosomoides bakeri* which possess blastomeres destroyed when eggs immersed in a high dose of aqueous and ethanolic extract of papaya seeds. The low hatching rate is believed to be influenced by the carotene alkaloid content in papaya leaf extract, which is toxic and could interfere mechanistically with the hatching enzyme function and it inhibits egg hatchability (Supriati *et al*., 2010; Andriyanto *et al*., 2013; Ardhardiansyah *et al*., 2017; Firdaus *et al*., 2017; Haser *et al*., 2018). We argued that the absence of this effect on eggs treated in low doses of papaya leaf

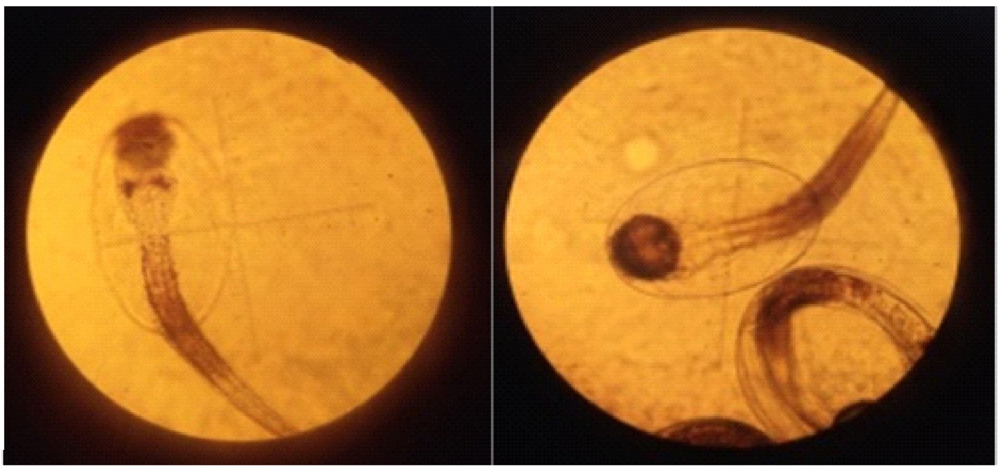

Figure 3. Type of deformity: scoliosis to the fish larvae.

extracts may be due to inadequate concentration to pass through the eggs membrane system, therefore it becomes less potent to the embryo.

The low hatchability is accompanied by lower survival rates at Asian seabass eggs treated in extract concentrations of 10, 20, and 25 mL. The reasons behind the larvae's low survival in the three treatments cannot be explained within this study since they are selected from similarly healthy larvae from each treatment. Further physiological performance observation must be done on the larvae to get a conclusive explanation. Interestingly, although the dose of 6 mL has a similar effect on hatchability and deformity to treatment with doses of 2 mL and 4 mL, it has lower effects on larval survival than the other two treatments.

From the above explanation, doses of 2 mL and 4 mL, statistically provided similar good results in terms of embryogenesis duration, hatchability, larval survival, and abnormalities. However, the descriptive figures tends to show that the dose of 2 mL gave the better results compared to the dose of 4 mL. Only in abnormalities that the dose of 4 mL can outweigh the results given by the dose of 2 mL.

The dose of 6 mL/L provided better result for the duration of embryogenesis, however the survival of larvae in this group, eventhough it does not significantly different from the group of 2 and 4 mL/L, it also not significantly different from the group of 10 mL/L which makes the survival of the larvae within this group being in the grey area of the two post-hoc groups.

Therefore, practitioners will face two options depend on the parameters that needed to optimize. If the hatchery wants a shorter duration of embryogenesis with lower abnormalities then the dose suggested is 6 mL/L with the risk that the larvae survival is somewhat lower. If the hatchery wants a higher survival by accepting 30 minutes delays in hatching time, then it can chose to use the dose of 2 mL/L.

CONCLUSION

Embryonic development, egg hatchability, and larval survival of Asian seabass can be improved using the papaya leaf extract. Low doses of papaya extracts are demonstrated to be able to improve egg hatching rates and larval survival. Higher concentration of papaya leaf extracts resulted in detrimental effects to the eggs and larvae. The doses of either 2 mL and 6 mL of papaya leaf extracts is the two recommended doses to apply for better hatchery production of Asian seabass larvae in the first step of larvae rearing. This study also suggests that future research must address the effect of papaya leaf extract administered during embryonal development on the later larvae physiological performance.

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