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**Research Article**

# Hatchability improvement in ephippia *Moina macrocopa* through substitution of rice bran suspension with fishmeal suspension

**Mubarak A.S.[1*]; Jusadi D.[2]; Muhammad Zairin J.R.[2]; Suprayudi M.A.[2]**

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**Abstract**

Ephippia *Moina macrocopa* hatching relates to the quality of nutrients received by the parent. Increasing the quality of the parent feed can improve the quality of the nutrient in eggs (ephippium) and can increase hatchability. This study was conducted to examine the addition of fatty acids in the feed through replacing rice bran suspension with fishmeal suspension on the quality and quantity of ephippia *M. macrocopa*. The treatment in this research was a substitution of rice bran suspension with fishmeal suspension by 0, 15, 30, and 45%. This study indicated that substitution of rice bran suspension with fishmeal suspension of 30% and 45% in Moina culture produced the same ephippia production but with a higher degree of hatching. Moreover, the substitution of rice bran suspension with fishmeal suspension by 30% increased the concentration of n-3 fatty acids (1.15%) in the egg of ephippia *M. macrocopa*.

**Keywords:** Mating, Hatching rate, Fatty acid, Egg, Ephippia

1-Department of marine science, Faculty of Fisheries, Airlangga University, Kampus C Mulyorejo Surabaya 60113, East Java, Indonesia
2-Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Kampus IPB Dramaga. Bogor 16680, West Java, Indonesia
Corresponding author's Email: shofy.ua@gmail.com

## Introduction

The use of Moina as a substitute for artemia as a natural feed of shrimp and fish larvae is optimal if it could produce ephippia Moina which has high hatchability (Mubarak *et al*., 2020a). Our previous research has succeeded in producing *M. macrocopa* sexual females, more than 90% of the total female offspring production from the culture using the mother density of 660 ind/L, 37-80 mg/L rice bran, and 3.86 mg/L gold fish faeces (Mubarak *et al*., 2020b). The ephippia *M. macrocopa* from this culture, using a combination of several induction factors, produced a large number of ephippia (6069 grains/L) but with a low hatchability value (<20%). Based on preliminary research, the mating *M. macrocopa* with the male-female sex ratio of 9:30 produced ephippia containing two eggs as much as 100%. The success of producing ephippia *M. macrocopa* should be supported by high ephippia hatching rates. The degree of hatching of ephippia is related to the quality of nutrients received by the mother (Putman *et al*., 2015), so improving the quality of the parent feed will improve the nutrient quality in the egg (ephippium) and the degree of hatching ephippia.In copepod species, egg production and hatching are associated with the availability of eicosapentaenoic acids (EPA) in a feed. The production of Cladoceran ephippia is limited by the availability of polyunsaturated fatty acids, especially EPA, which influences the hatching rate (Choi *et al.,* 2016). The fat and fatty acid content of rice bran, especially linoleic acid (6.35–6.85%), are high, but α-linolenic acid (0.20–0.27%) is low (Faria *et al.,* 2012). So *M. macrocopa* has produced ephippia with a low degree of hatching when feeding with rice bran suspension. Fishmeal has EPA and DHA fatty acids of 0.01% and 0.21% of the dry weight (Stevanato *et al*., 2008), so substituting rice bran suspension feed with fishmeal suspension will increase the concentration of EPA and DHA received by the parent and the eggs produced. Fishmeal has a high protein content and high concentration of arginine (7% of dry weight) (Haghbayan and Mehrgan, 2015). High protein and amino acid contents in the feed may decrease ephippia production in *Daphnia. pulex* (Koch *et al*., 2009; Koch *et al*., 2011), although proteins are needed to increase the growth of embryo *M. macrocopa* (Li *et al.,* 2008).

Based on the above analysis, the substitution of rice bran suspension with fishmeal suspension becomes limited to produce ephippia *M. macrocopa* with high quality and quantity. This study was conducted to examine the addition of n-3 fatty acids in the feed by replacing rice bran suspension with fishmeal suspension on the quality and quantity of ephippia *M. macrocopa.*

## Materials and methods

A Completely Randomized Design with four treatments, and each treatment was repeated three times. The treatments are, including 1) control with 100% rice bran suspension feed, 2) rice bran suspension 85% plus fishmeal 15% suspension, 3)

70% rice bran suspension plus 30% fishmeal suspension, and 4) rice bran suspension 55% plus fishmeal suspension 45% from total daily rice bran suspension feed as in Table 1.

**Table 1: Rice bran suspension concentrations for induction of production of males, sexual females and the culture of *M. macrocopa* offspring.**

| Day | Rice bran Suspension (mg/L) | | |
|---|---|---|---|
| | Induction of males | Induction of ephippia | Rearing offspring |
| 1 | 29.60 | 37.00 | 37.00 |
| 2 | 32.64 | 44.88 | 44.88 |
| 3 | 36.86 | 53.85 | 53.85 |
| 4 | 40.39 | 53.85 | 64.32 |

*M. macrocopa* mating culture used 48 and 70-hours-old females, respectively. Each culture used 30 and nine females and males in a vessel containing one *Moina*/mL (Koch *et al*., 2009; Koch *et al*., 2011). Containers and freshwater were replaced 100% every day during mating culture, and additional feeds were simultaneously added to each treatment. *M. macrocopa* sexual females were produced from the culture with a parent density of 660 ind/L using aeration (28 mL/min), rice bran 56-80 µg/parent suspension, and 3.86 mg/L goldfish faeces. *M. macrocopa* male offspring was produced from the culture with a parent density of 660 ind/L and using a rice bran-45-54 µg/parent suspension. The males and females are obtained on the third day and separated from the mother by screening using a 1 mm nylon filter. The offsprings were cultured with a density of 1000 ind/L with rice bran suspension feed that supports the production of ephippia (Zadereev and Lopatina, 2007). During the rearing period, *M. macrocopa* was fed with the rice bran suspension concentrations, as shown in Table 1. The cultured was conducted in a confined space with 700-900 lux and 50-100 lux night daytime lighting. On the second day (24-hour age), males were identified and separated, especially in the culture, for the provision of sexual females. Males were kept until three days (72 hours) before use in mating culture.

Suspension of rice bran and the fish meal was prepared by homogenizing as much as one hundred grams of each rice bran, and the fishmeal were suspended in 500 mL of water using a blender at a speed of 2000 rpm for 5 minutes twice. The second suspension was done 30 minutes after the first suspension. Then the suspensions were filtered using 2 mm, 0.1 mm, and 40 µm filters. The suspension that passed the filtration then had more water added to reach a volume of 500 mL. The proximate analysis results showed that the rice bran suspension contained 7.4% organic materials, 0.83% protein, and 0.79% fat, whereas the fishmeal suspension contained 8.13% organic materials, 6.53% protein, and 0.34% fat.

The collection and counting of ephippia Moina was done from five to six days post-hatch. The ephippia were identified based on containing eggs using a binocular microscope (100x magnification). The percentage of ephippia from the total population and the percentage of ephippia containing two, one, and no eggs were calculated using the following equation:

$$ephipia\ from\ the\ total\ population\ (\%) = \frac{\text{Ephippia production every liter}}{\text{Population Moina every liter}}\ x\ 100$$

$$ephipia\ containing\ (n)\ eggs\ (\%) = \frac{\text{Number of ephippia containing (n)}\ eggs}{\text{Total ephippia productioan}}\ x\ 100$$

Note: n is the number of eggs in the ephippia**.**

The water quality was assessed for dissolved oxygen, pH, temperature, total ammonia, and hardness during the culturing period.

Ephippia, with hatching rate determined using ephippia *M. macrocopa* containing two eggs, was stored wet in a microtube containing distilled water at a 200 eggs/mL density. After being stored for two months, ephippia from each treatment was incubated in a glass container containing 300 mL of water with a lighting intensity of 1800 lux. The hatching *Moina* was removed and counted on the second and third days. The degree of hatching ephippia was calculated based on the equation (Haghparast *et al.,* 2012). Where Ii is the hatching index and Ni is the number of larvae hatched.

$$Hatching\ rate = \overset{15}{\underset{i-3}{?}} \frac{N\ i}{N\ e}\ x\ I\ i$$

Fatty acid analysis was performed on ephippia in 100% rice bran suspension feed treatment and 70% rice bran suspension plus 30% fishmeal suspension. *M. macrocopa* sexual females were produced from cultured with a parent density of 660 ind/L using aeration (28 mL/min), rice bran 56-80 µg/parent suspension, and 3.86 mg/L goldfish faeces. *M. macrocopa* male offspring were produced from a culture with 660 ind/L parent density and a rice bran 45-54 µg/parent suspension. Then offsprings were cultured with 10 liters of water using rice bran suspension feed (5 mL/L first day up to 10 mL/L on the fourth day). Ephippia is harvested by turning water, so ephippia and dirt collect in the center of the tub. Ephippia was separated from dirt and washed three times before it was stored. Ephippia *M. macrocopa* fatty acids was analyzed using 10 grams each (wet weight) based on the Fardiaz*'s* method (1989) using Shimadzu GC-2010 Gas ChromatographyThe observational data were tested with Wallace Kruskal (non-parametric) to reveal a significantly different result and identify the treatment with the best response at a 95% confidence level.

## Results

*M. macrocopa* male offsprings were identified from smaller body sizes, abaout 1.2-1.5 mm, and longer antennas than females (Fig. 1A). *M. macrocopa* sexual females have the same morphology as the female parthenogenesis, and sexual females can be identified after synthesizing ovarian eggs that have large sizes and are darker after 78-85 hours (Fig. 1B).

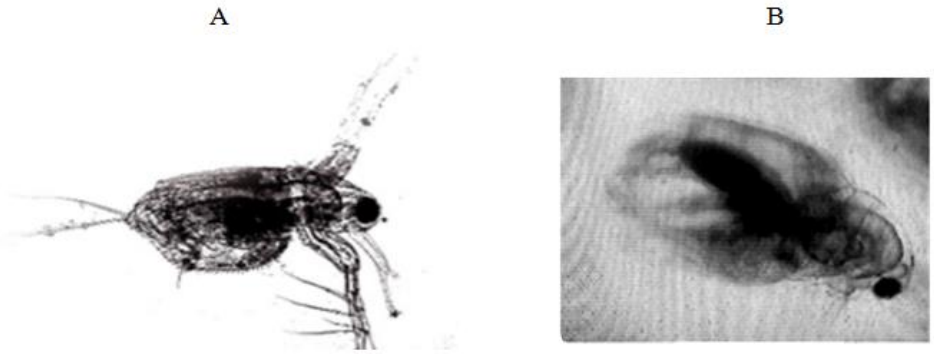



**Figure 1: The morphology of *M. macrocopa* male (A) and sexual females (B).**

*Moina's* survival rate was not affected by the substitution treatment of rice bran suspension with fishmeal suspension. The survival rate of *M. macrocopa* after one day of cultured was 95-99% and decreased after generating a third-day ephippium of 65-70% (Fig 2A and 2B).

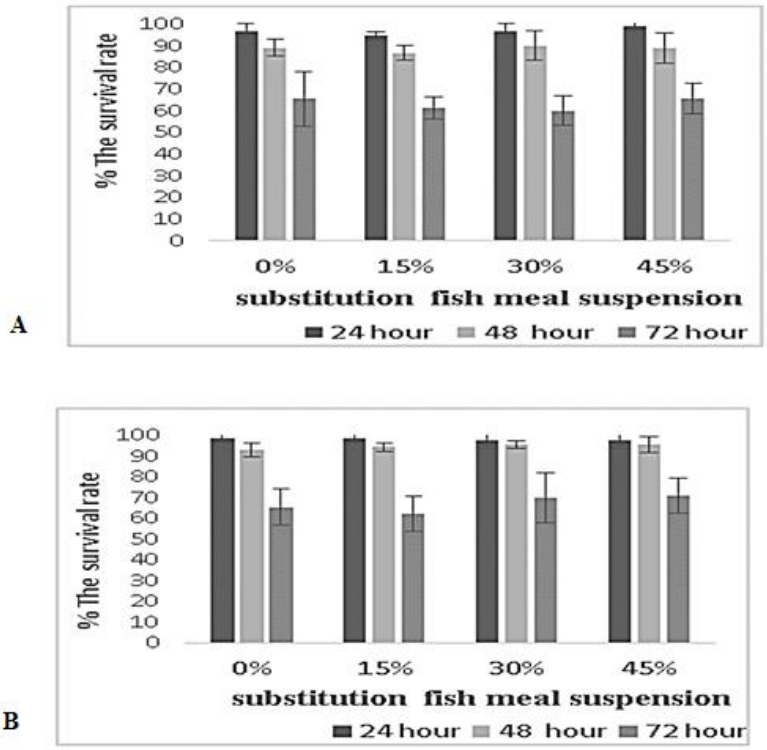



**Figure 2: The survival rate of the 70-hour-old (A) and 48-hour old *M. macrocopa* cultured using 0-45% rice bran suspension substitution with fishmeal suspension after rearing for 24, 48, 72 hours. Captions, without superscripts do not differ significantly ($p$>0.05).**

The female age in the mating affects the percentage of ephippia production in the total female. *M. macrocopa* mating using a 70-hour-old female child resulted in a percentage of ephippia production of 84-100% of females. *M. macrocopa* mating using a 48-hour-old female child resulted in a percentage of ephippia production of a lower female number of 29-33% of the total female (Fig. 3A and Table 2).

**Table 2: Production and degree of hatching ephippia M. macrocopa from mating culture using rice bran suspension feed and fish meal suspension substitution equal to 0-45%.**

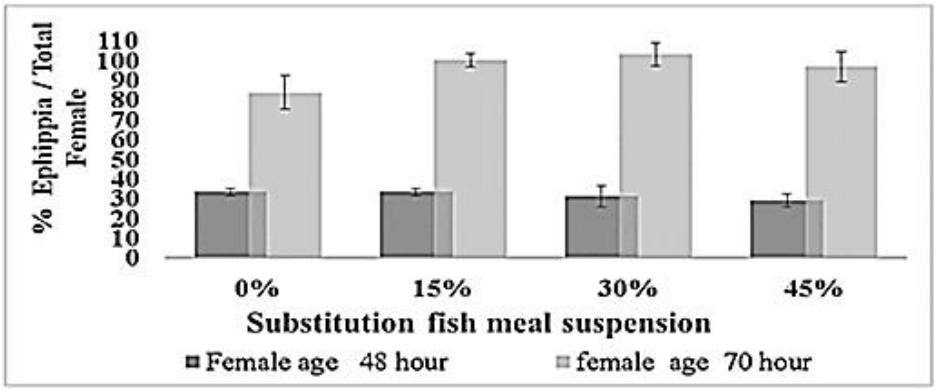
| Parameter | Substitution of fishmeal suspension | | | |
|---|---|---|---|---|
| | 0% | 15% | 30% | 45% |
| **Female aged 48 hours** | | | | |
| Ephippia production (grain/L) | 256±25[a] | 256±25[a] | 239±82[a] | 221±51[a] |
| Ephippia cont 2 eggs (%) | 93.33±5,8[a] | 100±0.0[a] | 100±0.0[a] | 100±0.0[a] |
| Degree of hatching (%) | 34.45±8.3[a] | 35,15±6.05[a] | 56.85±7.1[b] | 56.85±7.1[b] |
| **Female aged 70 hours** | | | | |
| Ephippia production (grain/L) | 615±135[a] | 692±51[a] | 759± 128[a] | 769±115[a] |
| Ephippia cont 2 eggs (%) | 86.0±3.0[a] | 94±0.91[a] | 92.4±1.75[a] | 96.7±1.5[a] |
| Degree of hatching (%) | 34.26±5.6[a] | 41.05±1.37[a] | 46.76±6.2[b] | 45.64±6.6[b] |

Different superscripts of each parameter showed significant differences ($p$<0.05).

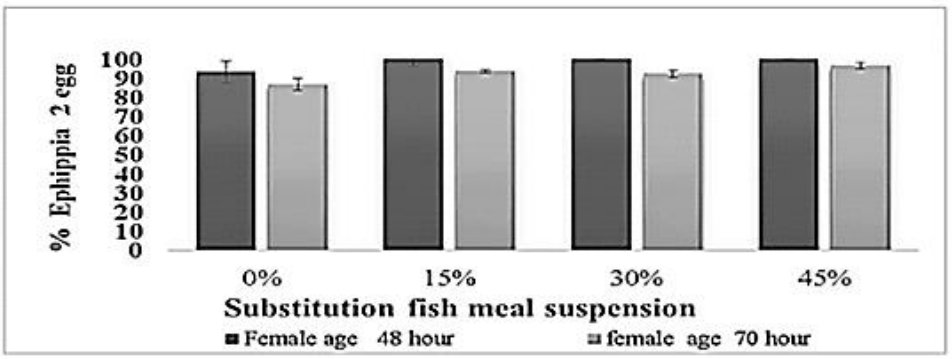
Substitution of rice bran suspension with fishmeal suspension in *M. macrocopa* mating culture did not affect the number of ephippia containing two eggs produced. The male-female sex ratio of 9:30 in the mating of *M. macrocopa* produces ephippia containing two high eggs using either a 48-hour-old or a 70-hour-old female, which is 93.9-100% of the ephippia produced (Fig. 3B).

Substitution of rice bran suspension with fishmeal suspension in *M. macrocopa* mating culture affects the degree of hatching of ephippia produced. The substitution of rice bran suspension with fishmeal suspension of 30% and 45% produced ephippia with the highest degree of hatching, either in mating using 48-hour-old females (55.7-55.9%) and using 70-hour-old females (46.7-46.8%). *M. macrocopa* mating culture using rice bran suspension feed produced ephippia with the lowest hatching rate of 30-31% (Fig. 4).

The results of the rice bran suspension fatty acid analysis had higher n-6 fatty acids (linoleic, arachidonic, gamma linolenic) (28.48%). In contrast, fishmeal suspension had higher n-3 fatty acids EPA and DHA (14.84%). Ephippia *M. macrocopa* mating, resulting from using rice bran suspension substitution feed with fishmeal suspension 30%, had a higher concentration of n-3 fatty acid (1.15%) and a lower concentration of n-6 fatty acid (22.54%) than ephippia production from culture using rice bran suspension feed (Table 3).




A




B

**Figure 3: The production of ephippia to the total female (A) and ephippia percentage contained two eggs (B) of *M. macrocopa* marinated with substitution feed of rice bran suspension with fishmeal suspension of 0-45%. Captions, without superscripts do not differ significantly ($p$>0.05).**

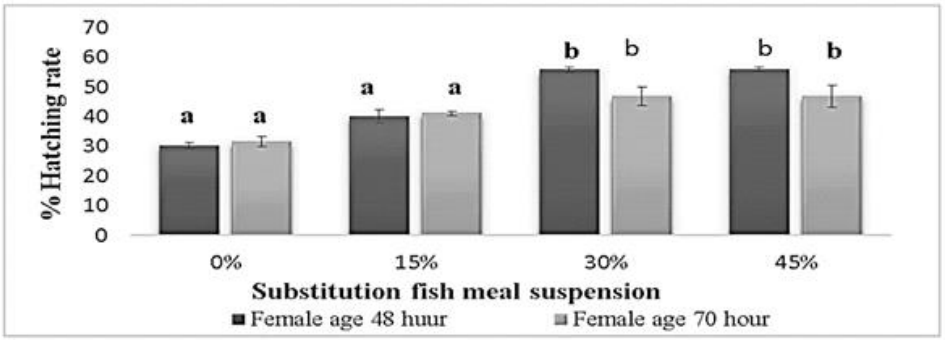



**Figure 4: The degree of hatching ephippia, the result of *M. macrocopa* mating culture using substitution of rice bran suspension feed with fishmeal suspension of 0-45%. Captions, different superscripts at the same *Moin*a age, show significant differences ($P$ < 0.05).**

Water quality during mating culture was temperature 28-30º C, pH 7.5-8, and hardness 55 mg/L CaCO3. Dissolved oxygen at the start of the study was 4 mg/L and decreased at the end of the study to 2.5 mg/L. Substitution of rice bran suspension with fishmeal suspension leads to an increase in total ammonia at a 45% substitution rate causing the total ammonia to increase to 1.5 mg/L (Table 4).

**Table 3: The concentration of fatty acids ephippia *M. macrocopa* (% w / w fat) from culture using rice bran suspension and fishmeal suspension substitution by 30%.**

| NO | Fatty acid | Ephippia *M. macrocopa* | | Rice bran | Fish Flour |
|---|---|---|---|---|---|
| | | 100% SD | 70% SD +30% STI | | |
| 1 | 12:0 | 0.17±0.00 | 0.17±0.00 | | 0.06 |
| 2 | 13:0 | 0.04±000 | 0.04±0.00 | | 0.03 |
| 3 | 14:0 | 1.25±0.06 | 1.51±0.12 | 0.3 | 2.67 |
| 4 | 15:0 | 0.29±0.01 | 0.32±0.01 | 0.02 | 0.06 |
| 5 | 16:0 | 18.78±1.31 | 21.9±0.31 | 14.60 | 17.27 |
| 6 | 16:1n-7 | 5.89±0.44 | 4.52±0.54 | 0.01 | 3.06 |
| 7 | 17:0 | 0.12±0.00 | 0.17±0.01 | 0.03 | 1.08 |
| 8 | 18:0 | 2.37±0.10 | 3.00±0.33 | 1.21 | 6.25 |
| 9 | 18:1n-7 | 0.05±0.00 | 0.04±0.01 | | 0.07 |
| 10 | 18: 1n-9 | 29.37±1.73 | 26.22± 3.02 | 31.83 | 8.02 |
| 11 | 18:2n-6 | 26.65±1.23 | 22.30±2.11 | 28.48 | 1.57 |
| 12 | 20:0 | 0.11±0.00 | 0.11±0.01 | 0.04 | 0.30 |
| 13 | 18:3n-6 | 0.20±0.01 | 0.19±0.01 | | |
| 14 | 20:1 | 0.25±0.01 | 0.22±0.01 | | 0.53 |
| 15 | 18:3n-3 | 0.5 ±0.00 | 0.5±0.01 | 0.97 | 0.28 |
| 16 | 20:2 | 0.06±0.00 | 0.08±0.01 | 0.04 | 0.25 |
| 17 | 22-0 | 0.22±0.00 | 0.2±0.01 | 0.26 | 0.23 |
| 18 | 20:3n-6 | 0.04±0.00 | 0.05±0.01 | | 0.14 |
| 19 | 20:5n-3 | | 0.33±0.01 | | 2.15 |
| 20 | 22:6n-3 | | 0.32±0.01 | | 12.43 |
| | Total n-3 | 0.5±0.00 | 1.15±0.02 | 0.97 | 14.84 |
| 8 | Total n-6 | 26.89±1.24 | 22.54±2.22 | 28.48 | 3.63 |

**Table 4: The water quality of media in *M. macrocopa* mating culture using substitution rice bran suspension feed with fishmeal suspension.**

| No | Parameter | Results |
|---|---|---|
| 1 | Temperature (°C) | 28-30 |
| 2 | pH | 7.5-8 |
| 3 | hardness (mg/L $CaCO_3$) | 55 |
| 4 | Dissolved oxygen (mg/L) | 4.0 -2.3 |
| 5 | Total Ammonia (mg/L) | 0.250-1.50 |

## Discussion

*M. macrocopa* mating culture using a 70-hour-old female child produces more ephippia than a 48-hour-old child. This suggests that *M. macrocopa* females at 70 hours have been characterized as sexual females. However, one female *M. macrocopa* produces only one ephippia grain. *M. macrocopa* mating culture using a 48-hour-old female with a female sex ratio (9:30) and with substitution of rice bran suspension feed with fishmeal suspension of 30% and 45% yields *M. macrocopa* ephippia with hatching degree of 55.7-55.9% while *M. macrocopa* mating culture using a 70-hour-old female child with the same feed produced ephippia with a lower hatching

degree of 46.7-46.8%. This indicates that the increase in the quality of the mother feed has a significant effect on the degree of ephippia hatching compared to previous studies, which are still below 20%.

The rice bran contains 6.25-6.85% high linoleic acid (Faria *et al.,* 2012). In comparison, fishmeal has fatty acid content of EPA 0.1% and DHA 0.21% (Stevanato *et al*., 2008). The total concentration of n-3 ephippia from *M. macrocopa* mating culture using rice bran suspension substitution with 30% fishmeal suspension increased to 1.15%; otherwise, the concentration of n-6 fatty acid (22.54%) was lower than ephippia result from a culture with rice bran suspension feed (Table 18). Omega-3 fatty acids play an essential role in the development and functioning of neurogenesis of the brain, as well as cell division and embryonic development (Beltz *et al.,* 2007; Watters *et al.,* 2012), where the diet of omega-3 fatty acids, omega-6 affects neurogenesis in the brain (*Homarus americanus*) and the growth rate associated with the number of cells entering the S phase (Koopman and Siders, 2013). In addition, the presence of omega-3 and omega-6 has been shown to increase egg hatchability and survival as in artemia, fish and crustaceans (Prusinska *et al.,* 2015; Kangpanich *et al.,* 2016).

Cladocera cannot synthesize linoleic and α-linolenic acids, and both fatty acids are essential for cladocerans (Persson and Vrede, 2006; Lomthaisong and Sanoamuang, 2012). Some species of Cladocera are reported to be able to convert α-linolenic acids to EPA and DHA, with abilities varying between species (Masclaux *et al.,* 2012). Low α-linolenic acid in rice bran (Faria *et al.,* 2012) limits the conversion process. The linoleic acid can be converted to arachidonic acid (Chang *et al.,* 2012).

Egg production and egg hatching are linked to EPA availability in feed as well as EPA reserves from mains. The degree of hatching of eggs is also influenced by the concentration of DHA and linolenic acid in egg cells (Jonasdottir *et al.,* 2009), which is influenced by the quality of feed received by the mother that produces ephippia (Putman *et al.* 2015). PUFA promotes the growth and metabolism of various animal species, including embryonic development in ephippia (Arulvasu and Munuswamy, 2009). Cladocera requires high PUFAS fatty acid, especially EPA, to produce ephippia eggs, where the EPA concentration in ephippia eggs (2.4 μg/mg dry weight) is higher than in subcutaneous eggs (0.01 μg/mg dry weight) (Abrusan *et al*., 2007).

Feed supplementation with low PUFA content with EPA or using algae containing high EPA can increase the production of ephippia *Daphnia magna* (Jonasdottir *et al.,* 2009), and the degree of hatching ephippia *D. magna*. Ephippia from *M. macrocopa* mating culture using 100% rice bran suspension feed produces ephippia with a hatching degree of 30-31%. Substitution of rice bran suspension with fishmeal suspension by 30% in *M. macrocopa* culture yielded ephippia with hatching degree, which is not different from the

substitution rate of rice bran suspension with 45% fishmeal suspension. In line with the (Sperfeld and Wacker, 2011) study, the increased EPA concentration in the *M. macrocopa* ephippia in fish suspension substitution is thought to cause the increased degree of ephippia penetrant penetration.

Female-male sex ratio of *M. macrocopa* affected the number of ephippia containing two eggs. *M. macrocopa* male behavior in mating is different from *Daphnia*. *Moina* males only attack and copulate with sexual females in the condition of synthesizing eggs in the ovary and have not been released in the brooding room. The mating time of *Daphnia* and *Moina* lasts for 8-10 minutes (Freedberg and Taylor, 2007). The mating of *Moina. branchiata* consists of three phases which begin with the male capture of sexual females and followed by male movements to position themselves up to perpendicular to the sexual female, and then copulation lasts for 16-25 seconds (Forro, 1997).

Freedberg and Taylor (2007) state that the ephippium of *Moina* sexual females will produce ephippia without eggs or damage if not fertilized. According to Conde *et al*. (2011), the female-male sex ratio of *Moina australiensis* is 4:5. The results of this preliminary study and this study show that the sex ratio of male and female sexual *M. macrocopa* is 9:30 resulting in ephippia containing two eggs of 100%. This is because the production of ephippia *M. macrocopa* is not simultaneous, from four days to six days, so that males can mate/couple more sexual females. Fertilized ephippia eggs have a greater chance of hatching.

Substitution of rice bran suspension with fishmeal suspension of 30-45% in *M. macrocopa* mating did not increase ephippia production but increased the degree of hatching of higher ephippia *M. macrocopa*. Substitution of rice bran suspension with fishmeal suspension by 30%, increasing the concentration of n-3 fatty acids in ephippia eggs *M. macrocopa.*

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