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Enrichment potential of *Brachionus plicatilis* with fish Oil and squid oil for the acceleration of the early giant Grouper development (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*)

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ABSTRACT

The development of early stadia giant grouper larvae is a problem that often occurs in grouper hatcheries. The highly DHA and EPA content in feed is very necessary to the grouper larvae. However, the natural feed that is given, namely *Brachionus plicatilis*, has low EPA and DHA content. This study aims to determine the development of early stage grouper abstinence by giving *Brochionus plicatilis* enriched with a combination of fish oil and squid oil. This study used an experimental method and the test animals were divided into two groups, namely control (feed *B. plicatilis* without enrichment plus *Nannochloropis sp.*) and treatment (feed *B. plicatilis* by enriching the combination of fish oil and squid oil plus *Nannochloropis sp.*). Statistical test used Independent-samples, T-Test. The acceleration of the grouper larvae development in the treatment group, especially in the dorsalis spina and ventral spines formed earlier on the seventh day (D7) compared to the control group larvae of the newly formed control group on the eighth day (D8). In addition, it was found that enrichment of *B. plicatilis* with fish oil and squid oil could accelerate the growth of absolute length (L) of the flak grouper larvae by 3.27 ± 0.17mm. The administration of *B. plicatilis* which was given squid oil and fish oil as larvae feed that made the early stages growth of the grouper larvae faster in several stages. In addition, the absolute lengths of larvae that are fed with enrichment are longer than the larvae that are fed without enrichment.

Key words : *Giant grouper, B. plicatilis enrichment, Squid oil, Fish oil, Early larval stage*

Introduction

Grouper is a leading Indonesian fishery commodity and has high demand reaching 35,000 tons per year with a price range of US $ 25 - US $ 125. BPS also noted that the contribution of live grouper exports in 2016 reached 1.11% of the total export value of fisheries commodities. Total value of live grouper exports in 2016 reached 32.18 million US $ (Kementrian Kelautan and Perikanan Republik Indonesia, 2013). However, there are still many problems in breeding process. The most common problem encountered in hatchling grouper fish is the slow development of the initial larval stage. This is caused by the low content of EPA and DHA in the natural food that was given, namely *Brachionus*

plicatilis, whereas EPA and DHA are important components for the growth of giant grouper larvae.

The development of grouper larvae can be divided into 4 phases, such as; i) yolk sac phase, which starts from hatching until the egg yolk runs out; ii) the pre-flexion phase, which starts from the yolk absorbed completely until the spine is formed; iii) the flexion phase, which starts from the formation of the dorsal spine and ventral spine to reduce the spine; iv) the post-flexion phase, which starts from the loss or reduction of the spine until it becomes juvenile.

One of the nutrients that are highly needed by marine fish larvae is fat (Halver and Hardy, 2002). The main constituent of fat is fatty acids (Purwaningsih *et al.*, 2014). Seawater fish larvae require long unsaturated fatty acids or Highly Unsaturated Fatty Acids (HUFA) of more than 4% (Léger *et al.*, 1986). HUFA that is needed by sea water fish larvae are Eiocosa Pentanoid Acid (EPA) and Docosa Hexanoid Acid (DHA) (Tocher, 2003). EPA and DHA are very instrumental in the development of grouper larvae.

EPA and DHA is long chain polyunsaturated fatty acids that play an important role in the largest structural component of the phospholipid membrane that regulates membrane fluidity and ion transport (Chapkin *et al.*, 2008). The need of HUFA increases in the grouper larval development stage because it is widely used in the formation of cell membranes and tissues (Leaver *et al.*, 2008). *B. plicatilis* as a natural feed that is often given to grouper larvae has relatively small amount of HUFA, especially EPA and DHA, hence, the nutritional quality of fatty acids is very low (Watanabe, 1993). *B. plicatilis* that is cultured with bread yeast or Nannochloropsis has a low nutritional content. The content of *B. plicatilis* essential fatty acids fed with bread yeast gives a low nutritional value of only 1% EPA and 0.1% DHA (Watanabe *et al.*, 1983), while enrichment with Nannochloropsis only has an EPA value of 0.94%-1.46% and DHA in very small amounts. The low content of essential fatty acids especially EPA and DHA can be increased by utilizing *B. plicatilis*. *B. plicatilis* is a non-selective filter feeder, which take all the food around it without selection. Thus, the type of feed given during *B. plicatilis* culture greatly influences its nutritional value. By utilizing these properties, the nutritional value of *B. plicatilis* can be improved by enrichment. One of the enrichment in *B. plicatilis* can be done by giving oil emulsions that are rich in EPA and DHA content. Fish oil and squid oil are rich of essential fatty acids such as EPA and DHA. *B. plicatilis* enriched by lemuru fish oil has an EPA content of 8.8% and DHA 0.1%. The content is higher than *B. plicatilis* enriched by *Nannochloropsis salina, Isochrysis galbana, Chlorella marina,* and *Saccharomyses cerevisiae*. Enrichment of *B. plicatilis* with squid oil contains 9-12% EPA and 2-3% DHA. The high content of EPA and DHA is caused the most squid lipids in the form of phospholipids which contain lots of essential fatty acids. Enrichment of *B. plicatilis* with a combination of fish oil and squid oil is assumed to increase the content of essential fatty acids needed in the development of grouper larvae. Some of them, namely fish oil and squid oil, are oils that are rich in EPA and DHA content. If fish oil and squid oil are added to enrich *B. plicatilis*, it is suspected that it can increase the EPA and DHA content to help accelerate the development of grouper larvae. This study aims to determine the development of the initial stage of the grouper abstraction by giving *Brochionus plicatilis* enriched with a combination of fish oil and squid oil.

Method

This method used an experimental method with the experimental design used t-test. The sample is divided into 2 treatments, namely P0 given *B. plicatilis* without enrichment and *Nannochloropsis sp*. and P1 given *B. plicatilis* by enriching the combination of fish oil and squid oil plus *Nannochlorosis sp.* The independent variable in this study was the administration of *B. plicatilis* enriched in a combination of fish oil and squid oil. The dependent variables in this study were the speed of development (width of the mouth opening, eye diameter, dorsalis spine length and ventral spine length) abstraction grouper larvae and growth in length (L) abstraction grouper larvae.

Research Material

The materials used in this study included D-0 grouper larva that was origin from Bali, Indonesia. Natural feed was the form of *B. plicatilis* and *Nannochloropsis sp*. Seawater of 30-31 ppt salinity as a medium for maintenance of grouper larvae. Chlorine, detergents, and chlorine were as treatment materials or sterilization tools and maintenance

media. Sodium thiosulphate function neutralized the chlorine or chlorine content in the device or media that has been treated. Checking the water with chlorine test, if yellow indicated the media is not neutral and if clear or no color indicated the media is neutral.

Enriching agents used included fish oil and squid oil. Duck egg yolk as an emulsifier which functions to keep oil grains suspended in water. Aquades was as dispersing media (Winarno, 1984). 5% formalin solution used to preserve (Kohno, 1998) and kill grouper larvae when observed through a microscope.

Manufacture of Fish Oil and Squid Oil Emulsions

Fish oil and squid oil cannot be given directly as an enrichment of *B. plicatilis* so it must be mixed with other ingredients such as duck egg yolk through an emulsion process. The ingredients needed were 10 mL of duck egg yolk, 40 mL of distilled water, 25 mL of fish oil, and 25 mL of squid oil. Then, the emulsion was stored in the refrigerator and can be used with thawing first.

Provision of *B. plicatilis*

The initial stage is filling of the structure given by the seeds of *Nannochloropsis sp*. with a density of 107 cells/mL. Then, fill 25% of seawater from the bottom of the tub. After that, *B. plicatilis* was spread with a density of 50 ind/mL. Harvesting of *B. plicatilis* from mass culture was carried out partially on the third day after stocking.

Enrichment of *B. plicatilis*

The density of harvested *B. plicatilis* is 150 ind/mL. The enrichment process of *B. plicatilis* with fish oil and squid oil emulsions at a dose of 0.5 ml/L enrichment media. Enrichment was carried out for 12 hours every day (Akbary *et al.*, 2011). In addition, *B. plicatilis* in the control treatment (P0) and enrichment treatment (P1) also added *Nannochloropsis sp*. with a density of 20-30 × 10^6 cells /mL (Redjeki, 1999). The administration of *Nannochloropsis sp*. served as *B. plicatilis* food for 12 hours of enrichment. This can keep *B. plicatilis* alive for 12 hours of the enrichment process.

Rearing Grouper Larvae

Stages in the maintenance of larvae include preparation of the container, feeding both natural and artificial, and water quality management media maintenance. Spread eggs as much as 500,000/tub. The grouper larvae are kept in larval rearing tanks with a capacity of 30 tons that have been sterilized.

There are two treatments given to *B. plicatilis* as grouper larvae feed in the control treatment (P0) *B. plicatilis* without enrichment. Treatment one (P1) *B. plicatilis* was enriched with emulsion of fish oil and squid oil as much as 0.5 mL/L of media yield of *B. plicatilis* for 12 hours.

The administration of *Nannochloropsis sp*. in grouper larval rearing tanks carried out since D2 with a density of 300-500 × 10^5 cells/mL. Apart from being a feed for B. plicatilis, *Nannochloropsis sp*. in grouper larval rearing tanks also acted as green water (Ismi *et al.*, 2012). The use of *Nannochloropsis sp*. as green water formed the color of the water to be dimmer, so that the grouper larvae do not cluster (Ismi, Asih and Kusumawati, 2014).

B. plicatilis was given when grouper larvae are D-2 to D-15. At the beginning of administration, the density of *B. plicatilis* given was 1-3 ind/mL. In D-3 to D-8 the density of *B. plicatilis* was maintained at 5 ind/mL, in larvae D-8 to D-15 the density of *B. plicatilis* was increased to 10-15 ind/mL. The frequency of administration of *B. plicatilis* was once a day at 08.00 a.m.

The grouper larval rearing tank was maintained static until D-7. Initially, water exchange was limited to only around 10% / day D-7 to D-12, to avoid sudden changes in water quality. Substitution of water gradually increased to 20% / day, when given artificial feed D-13 to D-15. From around D-12, grouper larvae droppings, dead grouper larvae, and food that accumulate at the bottom of the tank must be siphoned out at least once a day to maintain water quality. Initially, only one-quarter of the bottom of the tank was siphoned every day. This was done gradually increasing until all parts of the tank are siphoned every day.

Research Parameters

The main parameters in this study were the speed of development of mouth opening width, eye diameter (Glamuzina *et al.*, 1998), dorsalis spine length and ventral spine length, and specific growth rate of the grouper abbot larvae length. The total length was measured from the most anterior part of the mouth to the most posterior caudal fin (Glamuzina *et al.*, 1998). The mouth opened was calculated by the length of the lower jaw multiplied by tan (50^)

(Dabrowski and Bardega, 1984). The eye diameter was the longest diameter of the eye. Dorsal spine length measured from the base to the tip of the first radius of the dorsal fin (Moyle and Cech, 2004), whereas the length of the ventral spine was measured from the base to the tip of the first radius of the abdominal fin (Moyle and Cech, 2004).

Larvae samples were taken randomly using 3 dropper pipettes from each tub. Sampling was done every morning on D-3 to D-15 because grouper larvae generally consume *B. plicatilis* at that age. The larvae taken were placed on a concave object glass and turned off with 5% formalin to then be observed its development using a microscope equipped with an ocular micrometer at a magnification of 40 times.

Growth measurement aimed to determine the magnitude of larval growth during maintenance. Growth of absolute length larvae of grouper fish was calculated by measuring the total length of larvae at the end of maintenance minus the total length of larvae at the beginning.

Supporting parameters in this study were observations of water quality media for maintenance of grouper larvae. Water quality parameters observed were temperature, DO, pH, and salinity. Observation of temperature, DO, pH and salinity was carried out three times a day at 07.00, 12.00, and 16.00.

Data Analysis

The observing results of the development speed of grouper abstraction larvae were explained descriptively. Growth data for absolute long grouper larvae were analyzed by Independent Samples T-Test by the SPSS of Windows program. Supporting data in the form of a range of water quality values for grouper larvae maintenance were revealed descriptively.

Results

The results of the grouper larvae growth for 15 days

Table 1. The Growth of Grouper Larvae

Larvae aged (D-)	Growth	
	P0	P1
D-1	New larvae hatch and cannot swim actively, they still have egg yolk and globule oil, the eyes have not been pigmented and the mouth not open yet.	New larvae hatch and cannot swim actively, they still have egg yolk and globule oil, the eyes have not been pigmented and the mouth not open yet.
D-3	The larvae begin to swim actively, egg yolk and globule oil are completely absorbed, the pectoral fins are formed, the eyes have pigmented and the mouth is open, the average eye diameter is 0.227 ± 0.006 mm.	The larvae begin to swim actively, egg yolk and globule oil are completely absorbed, the pectoral fins are formed, the eyes have pigmented and the mouth is open, the average eye diameter is 0.248 ± 0.015 mm.
D-4	Total length of 2.706 ± 0.048 mm.	Total length of 2.867 ± 0.101mm.
D-5	The total length is 2.867 ± 0.101 mm.	The total length is 2.975 ± 0.023 mm.
D-6	The black spot of dorsal spina candidate has not been seen.	Spina dorsalis prospective black dots have been seen.
D-7	Black dots of dorsal spine candidates have been seen in the dorsal section.	Dorsal spine formed with a length of 0.033 ± 0.014 mm and ventral spine with a length of 0.425 ± 0.066 mm.
D-8	Dorsal spine formed with a length of 0.033 ± 0.014 mm and ventral spine with a length of 0.358 ± 0.014 mm.	Spina dorsalis has pigmented.
D-9	Spina dorsalis has pigmented.	The dorsal spina lengthens the dorsal section.
D-15	The total length of larvae increased to 4.872 ± 0.547 mm.	The total length of larvae increased to 5.792 ± 0144 mm.
Average L (mm)	2.20 ± 0.43^{a}	3.27 ± 0.17^{b}

Note: Different superscripts on the same line showed differences ($P < 0.05$). The average L was measured at D3 - D15.

can be seen in Table 1. Results showed that grouper larvae given enrichment of *B. plicatilis* with L P1 of 3.27 ± 0.17 mm were longer than P0 (without enrichment) which was only 2, 20 ± 0.43 mm.

Water quality

Water quality parameters measured during the study were temperature, pH, DO, and salinity. The results of water quality measurements were carried out on each treatment as many as two replications every day at 07.00, 12.00 and 16.00. Data on water quality range for maintenance of grouper larvae during the study can be seen in Table 2.

The value of water quality media for maintenance of grouper larvae during the study was within the normal range for the development of grouper larvae. Water temperature during the study ranged from 29.0 °C - 31.3 °C. Water pH during the study ranged from 8.15 - 8.64. DO water during the study ranged from 5.4 to 6.8 ppm. Salinity of water during the study was worth 31 ppt.

Discussion

First day (D-1) grouper aberration larvae in the study has the following morphology: the body was still transparent, the skin has not been pigmented, the mouth has not opened, the eyes have not been segmented yet, the intestine was straight (not filled), the anus was still closed, the apical tip of the caudal fin was still rounded, pectoral fins, dorsal fins, anal fins, and ventral fins have not been differentiated. At this age, the larva grouper larvae still have egg yolks and oil bubbles that were used as a food source.

At the age of D-3 the yolk has been absorbed, the mouth can open, the stomach begins to contract, the anus has opened, and the eye pigment was clear. The eye pigment was very important for the grouper larvae to see and find food. Pigtail grouper larva eye pigments begin to form at the age of D-3 with different diameters at P0 and P1. Eye pigmentation indicated that the grouper larvae were ready to search for prey / food. At this stage, the grouper larvae were in the first critical phase because the larvae undergo an adaptation process that came from the endogenous feeding phase to exogenous feeding. At this time, grouper larvae have to get food from the outside because the egg yolk has run out. This was consistent with previous research (Setyadi, 2008), that the critical period of fish larvae occurred in the transition period between the expiration of egg yolk and the time to start taking food from outside.

Apart from eye development, the development of the mouth opening was also very important in the development phase of the giant grouper larvae. The rapid development of mouth opening will affect the ability of giant grouper larvae to catch food. The size of the mouth opening was larger than the size of *B. plicatilis* which ranges from 0.08 to 0.12 mm, thus the larvae can catch its food because it matched the size of its mouth opening.

The dorsal spine and ventral spine on P1 form earlier on the seventh day (D-7) than on P0 that only forms on the eighth day (D-8). This was shown in Table 3, at the age of D-7, the dorsal spina P1 appeared to be prominent and formed earlier while the dorsal spina P0 was only formed at D-8. In addition, at the age of D-7, the ventral spine P1 has formed, whereas the ventral spina P0 was only formed at D-8. The formation of dorsal spine and ventral spine larvae of the grouper abstraction in this study was faster than in previous studies (Ch'ng and Senoo, 2008) where new dorsal spines formed at the age of D-9 while ventral spina was only seen at D-9 to D-10. The difference in the initial development of the dorsal spine and the ventral spina can be caused by differences in the nutrients contained in the feed given. The dorsal spine and ventral spine will reach a maximum length at the age of D-20 after hatching (Ch'ng and Senoo, 2008). After reaching the maximum length, the spine will reduce and turn into hard fin radii (Bulanin *et al.*,

Table 2. Data of Water Quality Enrichment Media of Grouper Larvae during Study

Parameter	Value		Standard value
	P0	P1	
Temperature (°C)	29.0 – 31	29.1 - 31.3	28-32
pH	8.15 - 8.64	8.28 - 8.57	7.5 – 8.6 (Ch'ng and Senoo, 2008)
DO (ppm)	5.4 - 6.8	5.6 - 6.7	5 – 6 (Chu *et al.*, 2016)
Salinity (ppt)	31	31	31 – 32 (Ch'ng and Senoo, 2008)

2018). The fingers of the hard fin on the dorsal fin and the abdominal fin are characteristic of fish from the family of serenades.

In the phase of grouper larvae where dorsal spines and ventral spines have formed, indicated that the larvae have entered the second critical phase. In this study, the second critical phase of the P1 grouper larvae occurred at the age of D-7, however in P0 it only occurred at D8. In this critical phase larvae already need feed with more complex nutritional content. This was consistent with study that during the growth of fish, especially in the younger phase requires more nutrient content in previous food (Effendi, 2004). The faster the dorsal and ventral spines were formed, the faster the larvae pass through the second critical phase, the faster the process of metamorphosis.

Growth was the increase in length or weight over time. Based on the result of the Independent-Samples, T Test analysis the absolute length (L) larvae growth of grouper revealed significantly different results. This examined that the enrichment of *B. plicatilis* with a combination of fish oil and squid oil influenced the growth of absolute length of the grouper larvae. The absolute length growth is illustrated in Table 3, where the absolute length growth P1 (with enrichment) was faster than P0 (without enrichment).

The enrichment of fish oil and squid oil combination has accelerated the growth of absolute length of the grouper larvae in P1 compared to P0. This happened because the enrichment has improved the nutritional quality of *B. plicatilis* consumed by the grouper larvae. These nutrients, especially EPA and DHA, can support faster metabolic processes and have an impact on better growth in larva grouper larvae.

Based on the results of the study, *B. plicatilis* with enriched combination of fish oil and squid oil have higher EPA and DHA content than *B. plicatilis* without enrichment. *B. plicatilis* enriched with a combination of fish oil and squid oil had a percentage of EPA and DHA respectively 15.60% and 4.56%, whereas *B. plicatilis* without enriching the percentage of EPA and DHA were lower, i.e. 12.92% and 2.26%. The higher EPA and DHA content in P1 was influenced by the addition of a combination of fish oil and squid oil emulsion given at the time of *B. plicatilis* enrichment.

Giant grouper was a hybrid grouper that results from a cross between tiger grouper (*Epinephelus fuscoguttatus*) female and giant grouper (*Epinephelus lanceolatus*) male that live in sea waters. Fish that live in marine waters do not have the activity of the delta 5-desaturase enzyme as those in freshwater fish, so that marine fish were in dire need of long-chain polyunsaturated fatty acids such as EPA and DHA in their feed (Peng *et al.*, 2003).

Faster growth of grouper larvae in P1, due to the enrichment of *B. plicatilis* with a combination of fish oil and squid oil can increase the content of fatty acids, especially EPA and DHA needed in the development of grouper larvae. Fat that enters the body of the fish will be hydrolyzed by the lipase enzyme into fatty acids and glycerol (Afrianto and Liviawaty, 2005). In this hydrolysis process, lipases were aided by bile acids and lecithin. The process of hydrolysis occurs along the digestive tract, especially in the intestine. These fatty acids will be absorbed by the intestinal mucosal enterocytes and into the blood. Most of the absorbed fat was transported to the liver. Fat was converted into phospholipids and transported by blood to cells and tissues. These fatty acids, specifically EPA and DHA, were the largest structural component in the phospholipid membrane that regulated membrane fluidity and ion transport (Chapkin *et al.*, 2008). Hence, the existence of EPA and DHA can support the smooth metabolism of cells and tissues, thus it impacts on the rapid development of larva grouper larvae.

Other factors besides feed influenced the development of early stadia grouper larvae were water quality media maintenance. The condition of water quality of the maintenance media showed that conditions were still suitable for the maintenance of the grouper larvae. Water quality observed included temperature, pH, DO, and salinity.

Temperature of grouper larvae rearing media influences the process of hatching organ development in which the process of embryogenesis and early organogenesis occurs. Water temperature during rearing influenced the metabolic rate of aquatic animal metabolic processes that were also poikilothermic which also impacts on the development process of larval organs produced. The initial development of fish was strongly influenced by the temperature at which the eggs. The temperature during the study in both treatments ranged from 29.0 - 31.3 ^{o}C. This temperature was still in the range of temperatures that can be feasible in the development of bleach grouper larvae. This was consistent with the statement that the temperature 28–32 °C was the

water quality standard for grouper hatcheries.

The pH content in each treatment ranged from 8.15 to 8.64. This was consistent with the previous one, that the pH value for the maintenance of larva grouper larvae was 7.5 - 8.6 (Ch'ng and Senoo, 2008). Aquatic pH influences the pH of blood plasma which can negatively influence larval development. A low pH affected the oxygen consumption of grouper larvae in decomposing alkaline properties in water. This can be seen from the difficulty of the gills in the process of taking oxygen and interfering with the transportation of oxygen in the blood, causing metabolic processes to be disrupted (Saleh *et al.*, 2013).

Dissolved oxygen content during the study ranged from 5.4 - 6.7 ppm. This condition was very supportive for maintenance of larva grouper larvae. The measurement results revealed that the higher the temperature, the lower the dissolved oxygen (DO). This was because an increase in temperature causes an increase in the speed of metabolism and respiration of aquatic organisms, thus resulting in an increase in oxygen consumption which results in a decrease in the value of oxygen content in water. DO requirements in raising grouper larvae range from 5-6 ppm, so it can be said that the oxygen content during this study can meet the needs of dissolved oxygen needed by the grouper larvae (Chu, 2016).

Salinity was one of the determining factors in the development of grouper larvae. The ideal salinity for maintenance of larva grouper larvae was 31 - 32 ppt (Ch'ng and Senoo, 2008). During the study, there were no significant changes in salinity. Salinity of maintenance media water was 31 ppt.

Conclusion

Enrichment of *B. plicatilis* with a combination of fish oil and squid oil can accelerate the development of the grouper larvae larvae, especially on the development of dorsalis spina and ventral spina that formed earlier in D7 than the larva grouper larvae with the administration of *B. plicatilis* without enrichment newly formed in D8. The enrichment of *B. plicatilis* with the combination of fish oil and squid oil can accelerate the growth of absolute length of the grouper larvae by 3.27 ± 0.17 mm.

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