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# The Effect of Various Kind of Live Feeds to Digestive Enzymes Activity of Coral Trout *Plectropomus leopardus* (Lacepède, 1802) Larvae

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**Abstract**
Digestive enzyme activity is a biological indicator of feed utilization by the larvae. The purpose of this study was to determine the digestive enzymes activity of coral trout larvae which fed (A) copepods, (B) copepods and rotifers and (C) rotifers. Biochemical analysis and SDS PAGE was performed to determine the enzymes activity and capability of protein hydrolysis on larvae and live feeds. The result indicates that the various kind of live feeds influence to digestive enzymes activity of coral trout larvae. Larvae B at 30 days after hatching had higher activity of protease, amylase and lipase ($P<0.05$) than larvae A and C. Larvae A and B had higher capability of protein hydrolysis than larvae C. Copepods had higher digestive enzymes activity ($P<0.05$) and higher capability of protein hydrolysis than rotifers. Therefore, feeding of copepods which combined with rotifers to coral trout larvae is strongly recommended.

**Keywords:** Digestive enzymes activity, copepods, rotifers, coral trout, larvae.

## 1. Introduction
Coral trout *Plectropomus leopardus* (Lacepède, 1802) is one of highly economical commodities of marine fisheries [1, 2]. Therefore, this species is a very prospective marine fish commodity to be cultured in Indonesia [3]. So far, the culture of coral trout is still facing a high mortality rate that generally occurs in the larval stage [4]. This is due to the higher level of difficulty in rearing of coral trout larvae than the other kinds of groupers.
In general, there are several factors that influence to the successful of larvae rearing. Feed is one of the key success of larvae rearing [5, 6] because feed is source of energy and materials for growth and survival of larvae. Zooplankton is the initial feed to the marine fish larvae because its relatively small size is suitable to the small size of larval mouth which starts to take the exogenous feed [7]. Zooplankton also supposedly contains digestive enzymes that make it more digestible to the larvae [8, 9].
Rotifers (*Brachionus rotundiformis*) is commonly used as the initial live feed for marine fishes larvae [10], including grouper [11]. Another kind of live feed is copepods that currently being developed for marine fish larvae [12]. Copepods are abundant in sea water, including in Indonesia waters [13, 14] and consumed by many of marine fishes larvae [15]. Copepods contain highly nutrient which are essential for growth of marine fish larvae [16].
Feed will have a significant role in the larval growth process if the feed nutrients can be absorbed by the larval body cells. Digestive enzyme activity is a biological indicator to determine the ability of larvae to digest the feed [17]. Therefore, protease, amylase and lipase are the biological indicator of the larval ability to digest protein, carbohydrate and fat contained in the feed. Digestive enzyme activity influenced by several factors, such as age of larvae [18], substrate [19], kind of feeds and feed composition [20, 21, 22].
Based on those, the feed and digestive enzymes are the important factors that considered for the success of coral trout culture. Hence, the purpose of this study is to determine the digestive enzymes activity of coral trout larvae which fed different kinds of live feeds. This study is expected to recommend the kinds of live feeds which have positive effect to digestive enzymes activity of coral trout larvae.

## 2. Materials and Methods

### 2.1 Location of study

Larval rearing was done at Institute for Mariculture Research and Development (IMRAD), Bali, Indonesia. Enzymatic analysis performed at Biological Laboratory and Chemical Laboratory of IMRAD. SDS-Page electrophoresis performed at Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia.

### 2.2 Source of eggs and larval rearing

Coral trout eggs were obtained from natural spawning of domesticated coral trout broodstocks in concrete tanks at IMRAD. Fertilized eggs were selected to be used in the study. Larvae rearing was done in hatchery using three concrete tanks volume 6000 L each and equipped by aeration system.

Phytoplankton *Nannochloropsis oculata* was added to each tanks started in the morning at 2 Days after Hatching (DAH) larvae. During the rearing period, larvae were fed live feeds according to the treatment being tested. Besides, larvae were fed micro pellets as the artificial feed started at 8 DAH, Artemia naupli at 25 DAH and mysid at 35 DAH. Larval feeding time was twice a day at 8-9 am and 2-3 pm.

### 2.3 Study design and treatment.

The study was done by completely randomized design consisted of 3 treatments and 3 replications. Two kinds of live feeds, i.e. copepods and rotifers as the treatment tested. The treatments detail was (A) copepods, (B) copepods + rotifers and (C) rotifers.

### 2.4 Supply of live feeds

Copepods collected from brackish water pond. Copepods were given to larvae every day at 2 until 13 DAH and afterwards were given every two days. Rotifers harvested from mass cultured and were given to larvae every day at 2 until 40 DAH.

### 2.5 Preparation and digestive enzyme assays

The activity of three kind of digestive enzymes, i.e. protease, amylase and lipase were determined in this study. Digestive enzyme activity in this study was expressed as the total activity defined as Units per larvae (U/larvae) based on the whole body of larvae [23]. Larvae at 1, 3, 10, 20 and 30 DAH were used as the samples.

Whole body of larvae samples were collected from larval rearing tanks, rinsed in distilled water and kept frozen at -80 $^{o}$C. The number of samples varied from 3 to 350 larvae depended on the larval body size. Higher number of samples was required for younger larvae than the older ones in order to obtain the accurate samples. Before assays, samples were extracted, centrifuged at 8000 rpm for 5 minutes at 4 °C. All of the assays were made in duplo.

Protease assays was performed as described by [24] with slight modification. Casein was used as substrate and tyrosine as standard. Briefly, 100 µl samples were incubated for 10 min in 0.65% casein diluted in phosphate buffer (50 mM; pH 7.5). After addition of 10% TCA, it was centrifuged at 8000 rpm for 5 min at 4 °C. Then, 500 µl of the supernatant was added $Na_2CO_3$ and Folin and kept for 10 min in room temperature. The absorbance of samples was read at λ 578 nm within 10 min. One unit of protease expressed 1 mM tyrosine released by 1 gram of samples per minute.

Amylase assay was performed as described by [25] with slight modification. Starch and maltose were used as substrate and standard, respectively. Into 1% starch was added 100 µl sample then it was incubated for 30 min at room temperature. Subsequently, it was heated in boiling water for 15 min, added 900 µl cold aquabidest and measured the absorbance λ 540 nm within 10 minutes. Amylase activity was expressed as mg of maltose released per gram of samples per minute [26].

Lipase assay was performed by the modified method of [27]. Samples were incubated in vegetable oil and 0.05 M Acetate Buffer in pH 5.6, homogenized by automatic stirrer at room temperature for 60 min. Subsequently, Acetone: Ethanol (1:1) and 1% Phenolphthalein was added to the solution and then titrated with 0.01 M of KOH. One unit of lipase was described as the enzyme in 1 gram of samples that released fatty acids per minute.

### 2.6. Estimation of protein digestion

The capability of protein hydrolysis of samples was estimated by Sodium Dodecyl Sulfate-Polyacrylamide (SDS-PAGE) gel electrophoresis referring to [28] with slight modification. Larvae 30 DAH from each treatments, copepods and rotifers were used as the samples. Samples were extracted in Phosphate Buffer Saline pH 7.4 and centrifuged at 5000 g for 5 min at 4 °C. The supernatant were collected and frozen at -80 °C.

Protein standards (BioRad #161-0374) were used as both the marker and substrate. Samples were incubated in the substrate for 0 min, 15 min, 1.5 h and 3 h. Furthermore, sample buffer was added in a ratio of 1: 1 to sample volume and then heated at 95 °C for 5 min. Electrophoresis was performed on the Mini-Protean II, using two gels consisted of 10% separating gel (0.375 M of Tris-HCl, pH 8.8) and 4% stacking gel (0.125 M of Tris-HCl, pH 6.8). Gels were put into the electrode running buffer pH 8.3. As much as 15 µl of samples were put into each well. Electrophoresis was performed at 100 V for 1 to 1.5 h. Furthermore, the gels were stained with Coomasie Blue dissolved in destainer (Methanol: Acetic: Distilled water, 4:1:5) for 24 h. During the staining, gels were placed on Rocking Platform Mixer (Ratek Instruments, Australia). Gels were destained for 20-30 min and kept in 10% acetic acid.

### 2.7. Statistical analysis

Statistical analysis was performed by SPSS 21 software. One way analysis of variance was used to test the significant differences among treatments at level 95%. When significant differences were detected, analysis continued by Duncan Multiple Range Test to determine the statistical differences among treatments.

## 3. Results

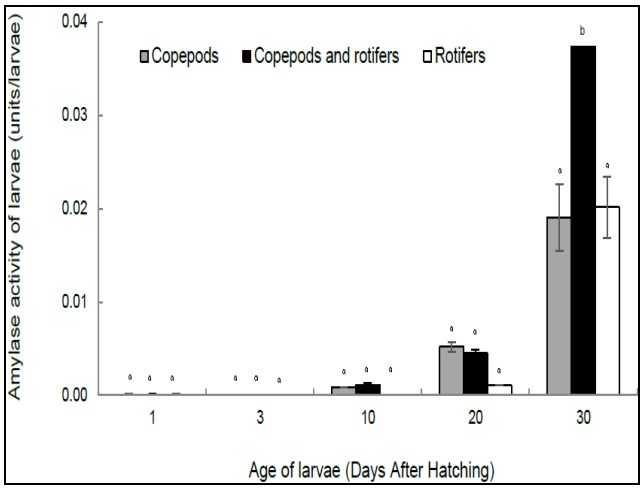
### 3.1. Digestive enzymes activity of larvae

Protease activity at 1 and 3 DAH larvae in all treatments were still very low and almost invisible (Fig. 1). The activity started to increase from 10 to 30 DAH. Protease activity of 30 DAH larvae fed copepods (A), copepods and rotifers (B) and rotifers only (C) were 0.059 ± 0.032 U/larva, 0.417 ± 0.027 U/larva and 0.135 ± 0.023 U/larva, respectively. The result indicates at 30 DAH larvae B had higher protease activity ($P<0.05$) than those of larva A and C.


Values above bars followed by the same superscript are not significantly different (P>0.05)

**Fig 1:** Protease activity of 1-30 DAH coral trout larvae fed copepods and rotifers

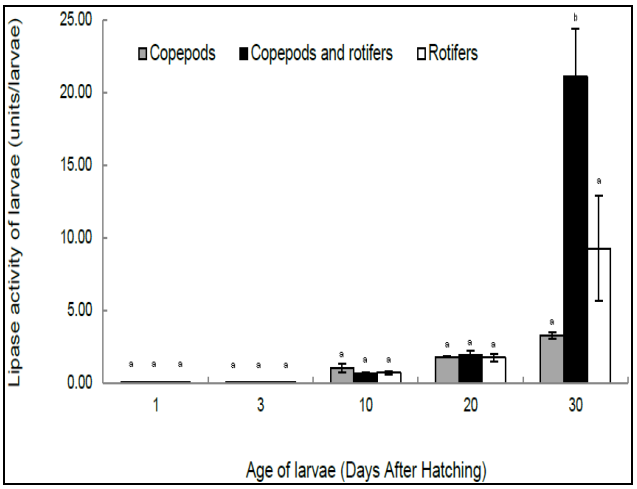
Amylase activity at 1 and 3 DAH larvae in all treatments were still very low, so they were almost invisible (Fig. 2). Amylase activity started to be observed at 10 DAH on larvae A and B, while at 20 DAH on larvae C. The level of amylase activity at 30 DAH on larvae A, B and C were 0.019 ± 0.004 U/larva, 0.038 ± 0.000 U/larva and 0.020 ± 0.003 U/larva, respectively. This result indicates that the level of amylase activity at 30 DAH on larvae B was higher than those of larvae A and C (P<0.05).




Values above bars followed by the same superscript are not significantly different (P>0.05)

**Fig 2:** Amylase activity of 1-30 DAH coral trout larvae fed copepods and rotifers

Lipase activity at 1 and 3 DAH larvae in all treatments were still very low as low as protease and amylase (Fig. 3). Lipase activity could be observed at 10 DAH larvae and the level on larvae A, B and C were 1.015 ± 0.269 U/larva, 0.675 ± 0.079 U/larva and 0.706 ± 0.112 U/larva, respectively. Afterwards the level was increased until 30 DAH larvae. The level of lipase activity at 30 DAH on larvae A, B and C became 3.266 ± 0.255 U/larva, 21.090 ± 3.302 U/larva and 9.276 ± 3.647 U/larva, respectively. It means that larvae B had higher level of lipase activity at 30 DAH than those of larvae A and C (P<0.05).

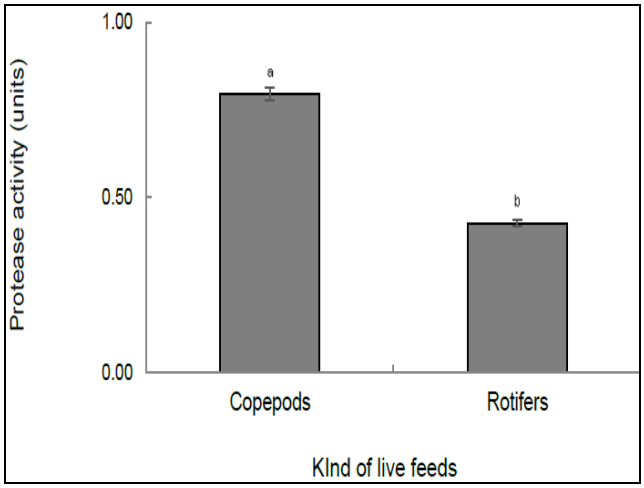


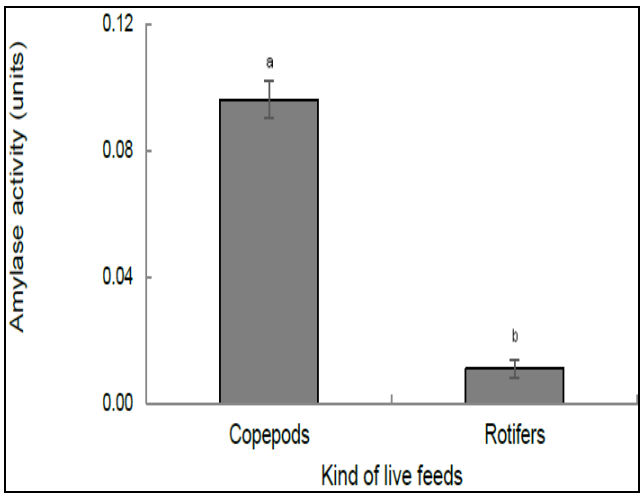

Values above bars followed by the same superscript are not significantly different (P>0.05)

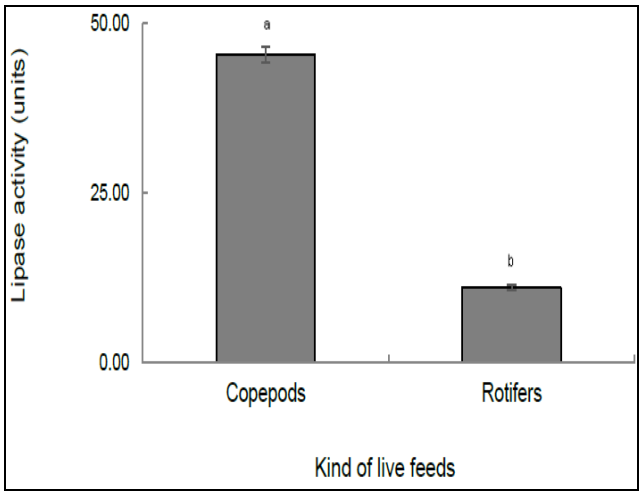
**Fig 3:** Lipase activity of 1-30 DAH coral trout larvae fed copepods and rotifers

### 3.2. Digestive enzyme activities of zooplankton

Zooplanktons used in this study, both copepods and rotifers, also had digestive enzymes activity (Fig. 4), but the activity of protease, amylase and lipase of copepods was higher than those of rotifers (P<0.05).






Values above bars followed by the same superscript are not significantly different (P>0.05)

**Fig 4:** Enzymes activity of copepods and rotifers

### 3.3. Estimation of protein hydrolysis

The larvae from all treatments had the capability of protein hydrolysis and occurred at 25-75 KDa of molecular weight (Fig. 5). The protein hydrolysis of larvae A and B occurred within 15 minutes incubation. There were a lot of hydrolyzed protein by larvae A and B after 3 hours incubation however there was still a lot of protein which has not been hydrolyzed by larvae C. This indicated that larvae A and B had higher capability of protein hydrolysis than those of larvae C. Related to digestive enzyme activity, it could be qualitatively estimated that the larvae A and B had higher protease activity than those of larvae C.

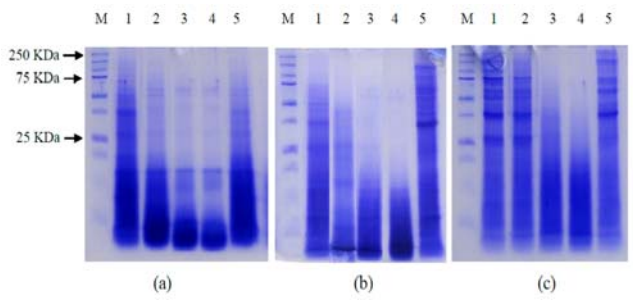



**Fig 5:** Capability of protein hydrolysis of 30 DAH larvae performed by SDS-Page gel electrophoresis. (a) Larvae fed copepods; (b) Larvae fed copepods and rotifers; (c) Larvae fed rotifers. M: Marker; 1: Sample + substrate without incubation; 2: Sample + substrate incubated 15 minutes; 3: Sample + substrate incubated 1.5 hours; 4: Sample + substrate incubated 3 hours; 5: Sample without substrate without incubation.

The protein hydrolysis of copepods and rotifers occurred within 15 minutes after incubation (Fig. 6). Copepods could hydrolyze 25 kDa molecular weight of protein within 1.5 hours incubation however the same molecular weight of protein on rotifers hasn't yet fully hydrolyzed after 3 hours. This indicated that copepods had higher qualitatively capability of protein hydrolysis than rotifers. Therefore it could be estimated that copepods had a higher protease activity than rotifers.

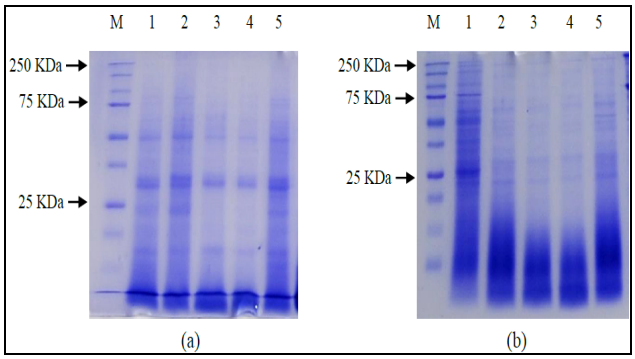



**Fig 6:** Capability of protein hydrolysis of copepods and rotifers performed by SDS-Page gel electrophoresis. (a) Copepods; (b) Rotifers. M: Marker; 1: Sample + substrate without incubation; 2: Sample + substrate incubated 15 minutes; 3: Sample + substrate incubated 1.5 hours; 4: Sample + substrate incubated 3hours; 5: Sample without substrate without incubation

## 4. Discussion

This study showed that in the beginning of their life, 1 and 3 DAH coral trout larvae had low level of digestive enzymes activity, including protease, amylase and lipase. There wasn't yet difference level of larval digestive enzymes activity among treatments. The digestive enzymes activity was increased by the increasing of larval age. The low level of digestive enzyme activity in the early stages of larvae also mentioned before [29, 30]. This is due to the incomplete formation of larval digestive tract [31], which correlated with the low level of digestive enzymes secretion [32]. Additionally, larvae didn't consume much feed in the early stage of their life, means the low availability of substrates for digestive enzyme, leading to the low level of enzyme activity. The substrate will greatly affect to digestive enzyme activity of larvae [19].

The digestive enzyme activity was increased at 10 DAH larvae and afterwards in all treatments. The digestive enzyme activity on the others fish larvae such as Eurasian perch (*Perca fluviatilis*) [33], red drum (*Sciaenops ocellatus*) [34] and Gilthead Sea Bream (*Sparus auratus*) [35] also increase as does coral trout larvae in this study. Thus digestive enzyme activity patterns occur in coral trout grouper larvae in this study are similar to the digestive enzyme patterns of others fish larvae.

Digestive enzyme activity increased quite high at 30 DAH larvae. The secretion of digestive enzyme could be triggered by increasing the amount and quality of the feed consumed [36]. Furthermore, [37, 38] also pointed out that the kinds of feed will also affect to the digestive enzyme secretion.

Digestive enzyme activity at 30 DAH on larvae B was higher than those of larvae A and C. It is assumed due the use of two kinds of live feeds to larvae. Copepods are known to have high level of fatty acids [39, 40] while rotifers have high level of amino acids [41] so that the availability of substrate on larvae B was higher than those on larvae A and C. Copepods also have higher digestive enzymes activity than rotifers [42].

Digestive enzymes in larvae could be come from endogenous enzyme or exogenous enzyme derived from live feeds [43]. The exogenous enzymes generally vary because it depends on the kinds of feed consumed by the larvae. Exogenous enzyme can be an activator to increase enzyme activity by activating zymogens [44] or producing proteases [45]. Digestive enzyme activity in common carp (*Cyprius carpio*) grass carp (*Ctenopharyngodon idella*) [44], *Coregonus* sp. [46], turbot (*Scophthalmus maximus*) [42], roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) [47] and green catfish (*Mystus nemurus*) [48]

are believed derived from the contribution of zooplankton live feed which they were consumed. On the other hand, live feeds are not contributed to larval digestive enzymes activity such as on larvae of striped bass [49], Japanese sardine (*Sardinops melanotictus*) [50], *Solea senegalensis* [51], African catfish *Clarias gariepinus* [52], haddock (*Melanogrammus aeglefinus*) and Atlantic cod (*Gadus morhua*) [53]. In this study, both copepods and rotifers have the digestive enzymes activity, but the enzymes activity on copepods is higher than rotifers, either quantitatively or qualitatively. However the exogenous enzymes on copepods and rotifers are supposed don't have a contribution to digestive enzymes activity of larvae because the patterns of protein hydrolysis in larvae at all treatments occur more rapidly than that in the zooplankton. In another words it can be said that the enzymes activity which were measured in the larvae is an endogenous enzymes activity. However, the interaction between the copepods and rotifers may contribute to the increasing of digestive enzymes secretion in coral trout larvae through a mechanism that can't be described in this study yet.

As it has been known, there are several methods that can be used to analyze digestive enzyme activity in fish. The present study analyzes enzymes activity quantitatively and qualitatively. SDS-PAGE electrophoresis is one of the molecular analysis methods that can be used to identify the presence of an enzyme [54] or to determine the activity of an enzyme in hydrolysis [28]. Besides, [34] suggested using a biochemical analysis as a quantitative analysis and one of the qualitative analysis in order to obtain more accurate results. In the present study, analysis was performed quantitatively by biochemistry and qualitatively by SDSPAGE electrophoresis. Hence, this study is expected to provide accurate results.

Based on the results of this study, it is concluded that coral trout larvae fed copepods and rotifers have higher digestive enzymes activity than those of larvae fed copepods or rotifers only. Therefore, feeding copepods and rotifers are highly recommended in the rearing of coral trout larvae.

## 5. Conflict of Interests

There is no conflict of interest among the authors for the publication of this research article.

## 6. Acknowledgements

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