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Biochemical Composition and Digestive Enzyme Activity of *Anguilla bicolor* McClelland 1844 on Reproductive Phase

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ABSTRACT. The critical problems in eel cultivation are slow growth due to low digestibility and high feed conversion. This shows the need to understand the biochemical composition of eel to obtain information about the physiological condition, energetic adaptation, habits, nutritional value, and industrial uses. Nutritional data are also essential to develop a suitable processing method that enables eel to be consumed throughout the year while respecting state-imposed limitations to protect the species. Despite the significant contribution, there has been no data about the biochemical composition and the digestive enzyme activity of eel species such as *Anguilla bicolor* McClelland 1844, during the reproductive phase. Therefore, this study aimed to evaluate the biochemical composition of *A. bicolor* McClelland and the digestive enzyme activity during the reproductive phase. The experiment was conducted through a survey on eels in the reproductive phase with an average weight and length of yellow (106.00 ± 38.1 g and 42.00± 5.78 cm) and silver eel (362.89 ± 88.93 g and 59.86 ± 7.39 cm) respectively. The results showed that Nitrogen Free Extract (NFE) did not substantially alter between different phases, while the percent protein, lipid, ash, and fiber had significant variation ($p<0.05$). The activity of pepsin differed in various phases ($p<0.05$), while the activity of trypsin, amylase, and lipase of *A. bicolor* did not vary ($p>0.05$). The yellow eel had a higher body protein composition and pepsin activity than the silver eel, although the biochemical composition and other enzyme activities were similar. In summary, the biochemical compositions, and digestive enzymes of *A. bicolor* varied depending on their phases. Moreover, further studies were recommended to understand the biochemical composition of *A. bicolor* and digestive enzyme activity during the reproductive phase.

Keywords: *Anguilla bicolor,* Digestive enzyme activity, Proximate analysis

INTRODUCTION

Anguilla sp. is catadromous, thriving in 2 different habitats during the life phase. This eel species is found in freshwaters and migrate to the sea during growth through estuarine habitats (salty water). The life cycle of eel fish consists of 5 phases namely leptocephalus, glass, elver, yellow, and silver eel. Leptocephalus live in the sea and metamorphose into glass eel, which is ready to migrate from seawater to freshwater to mature (silver eel) and return to the sea for spawning (Tsukamoto et al., 2011; Lin et al., 2012; Churcher et al., 2014; Nowosad et al., 2014; Cresci et al., 2017; Chai & Arai, 2018). Juveniles (elver and yellow eel) live in freshwater, while silver eel thrives in an seawater at spawning. Small-sized eel is found in shallow waters with abundant aquatic plants, while large species are found in medium and deep oceans with few aquatic plants (Laffaille et al., 2005).

The populations of eel in the world, such as *A. anguilla* and *A. japonica*, have experienced a very sharp decline due to overfishing, environmental damage, and the presence of reservoirs limiting migration to spawn. The influencing factors lead to a decrease in population (Yokouchi et al., 2014), which is not accompanied by high productivity of cultivation because eel is challenging to mature in aquaculture conditions. This shows the need for information regarding the reproductive character of eel in aquaculture conditions, serving as the basis for gonad maturity (Rachmawati et al., 2022).

As a unique species, *A.bicolor* McClelland has a distinct life cycle, starting from leptocephalus, glass, elver, yellow, and silver eel (Tesch, 2008; Arai & Chino, 2022). Elver and yellow eel live in freshwater during the growth phase and migrate to the sea after puberty to spawn as a silver eel. In its development, leptocephalus migrates to freshwaters and experiences metamorphosis into glass eel (Tesch, 2008; Arai, 2022).

Globally, there are 19 species of eel, where 13 are tropical (Kumai et al., 2020) and 7 are found in Indonesia, namely *A. celebesensis, A. interioris, A. bengalensis bengalensis, A. marmorata, A. borneensis, A. bicolor* and *A. bicolor pacifica* (Arai & Abdul Kadir, 2017; Chai & Arai, 2018; Arai, 2022). The tropical eel is considered the ancestor of the sub-tropical eel (Arai, 2022). *Anguilla bicolor* McClelland is a tropical eel found in Indonesian waters

(Rachmawati & Sistina, 2020), with economic value. However, the population tends to decrease due to the high demand for eel in the community.

Eel has not been successfully spawned on a cultivation scale, as aquaculture activities primarily focused on growing out in the growth phase. Therefore, information regarding the movement of digestive enzymes in the growth phase is needed to provide an overview of nutritional needs for effective cultivation activities (Fekri et al., 2018).

A crucial factor affecting juvenile survival is the adequacy of nutrients, depending on the effectiveness of ingestion, digestion, and assimilation of diets that have essential nutrients (Gisbert et al., 2019). This shows the need to understand the biochemical composition of eel to obtain information about the physiological condition, energetic adaptation, habits, nutritional value, and commercial application. Nazemroaya et al., (2015) stated that the availability of nutrients for all biological functions of eel was influenced by the metabolic process of digestion.

Regarding the digestive enzyme activity of eel in cultivation conditions, several studies have been conducted but the results cannot be used as a reference for their nutrient requirements. Therefore, information about the development of the digestive tract and nutrition is needed during the growth phase to optimize diet, feeding patterns, and improve the quality of larval culture as well as productivity (Khoa et al., 2019).

The physiology of eel digestive system has been explored, with a focus on the activity of digestive enzymes. However, these studies have only investigated the digestive enzyme activity of 2 species, namely *A. bicolor* and *A. marmorata*. The results showed the influence of environmental factors such as salinity (Luo et al., 2015), laboratory maintenance (Mulyani et al., 2016), the silvering process *of A. anguilla* (Gurkan et al., 2022), as well as feed supplementation on *A. rostrata* (Arai, 2020) and *A. japonica* (Kuo et al., 2022). This showed the need to provide elevated feeding levels during the juvenile phase of elver and yellow eel to support growth and development. According to Yufera et al. (2018), quality feeding is closely related to the growth of these species, serving as the most critical factor in enhancing productivity in cultivation activities.

The activity of eel digestive enzymes is influenced by type and developmental phase (Yufera et al., 2018), food availability, environmental factors (Zaefarian et al., 2018), and feed supplementation (Mostafaloo et al., 2020). Previous studies have shown that the activity of digestive enzymes can decrease with a change in incubation temperature, although the trend varies based on species and the tissue being analyzed (Hidalgo et al., 1999).

Gisbert et al. (2011) conducted a study on *A. anguilla* and showed that eel of varying sizes had varying enzymatic activities. Smaller size species showed higher protease and lipase-specific activity, while larger sizes had increased amylase-specific activity. The results provided valuable information as a basis for formulating suitable feed for varied sizes of *A. japonica*, ensuring best nutritional value and cost-effectiveness (Murashita et al., 2013)

The measurement of trypsin activity is considered proper for assessing actual feeding and digestion processes in eel juvenile's growth, serving as the most critical proteolytic enzyme in this stage of development. This is due to the high demand for amino acids to support new tissue formation and rapid growth, with trypsin being the primary digestive enzyme in fish larvae (Chiu et al., 2002; Kurokawa et al., 2002).

Information on eel natural habitat is essential, despite significant studies on the somatic index and digestive enzyme activity of eel in feed and culture treatments (Mulyani et al., 2016). This is essential to obtain the appropriate representation of the actual feed requirements. Although observations of enzyme activity in the natural habitat of eels have been made during the silver phase (Taufik et al., 2017), more data are still needed in the growth phase. The information is highly significant, precisely representing nutritional and energy requirements, which are essential for their cultivation and survival.

Although Wijayanti and Setiyorini, (2018) have explored the biochemical composition of eel fish using different rearing media, there is no data on the reproductive phase, biochemical composition, and digestive enzyme activities. Currently, no information is available regarding the biochemical composition and digestive enzyme activity of *A.bicolor* McClelland 1844 during the reproductive phase. Therefore, this study aimed to evaluate the biochemical composition of *A. bicolor* McClelland and the digestive enzyme activity during the reproductive phase.

EXPERIMENTAL SECTION

Sample Preparation

The study was conducted through a survey of eel in the reproductive phase, with an average weight and length of yellow eels (106.00 ± 38.1 g and 42.00± 5.78 cm) and silver eel (362.89 ± 88.93 g and 59.86 ± 7.39 cm), respectively. A survey method with random sampling was used, where eel was obtained from fisherman in Cilacap, and taken to the Laboratory of Animal Physiology using an open transportation system. Furthermore, eel was accommodated in a fiber aquarium (2 x 3 x 1 m3) and satisfied for 1 x 24 hours during data retrieval. The samples were acclimatized for 1 day before surgery for morphological and physiological observations. The observed variables were body weight, length, and the biochemical composition, as well as digestive enzyme activity. The biochemical composition parameters included water content, dry weight, lipid, protein, crude fiber, ash, and NFE (Nitrogen Free Extract). The

digestive enzyme activity measured included pepsin, trypsin, lipase, and amylase.

Measurements of Body Length and Body Weight

Body length and weight measurements were conducted after eel was unconscious to reduce movement. Eel was sedated using eugenol at a dose of 5 ppm for 30 minutes until fainting (Rachmawati & Sistina, 2020). The body weight was measured with a technical scale, while the body length was measured using a ruler. The measured body length was the total length from the head's tip to the tail's end.

Observing the body's skin color is essential to determine the obtained eel development phase (stadia). The part of the body with the observed skin color is the abdomen. According to (Palstra & van den Thillart, 2010), the abdomen of juvenile has yellow skin, and the core of mature eel is silver

Preparation of The Biochemical Composition Analyses

Eel body without visceral organs is divided into 3 parts. One-third of the body, the abdomen, is placed on a petri dish and covered with aluminum foil. The sample is put in the oven at 60°C until dry and the biochemical composition analysis is conducted at the Laboratory of Nutrition Science, Faculty of Animal Science, UNSOED.

Preparation of Enzyme Activity Analyses

Digested organ isolation and preparation of enzyme crude extract

Samples of digestive fish organs were isolated and used to measure the activity of proteases such as pepsin and trypsin. Foregut (stomach) and hindgut samples measured pepsin-like and trypsin-like activity. The digestive organs were crushed with an electric homogenizer and homogenized using a cold buffer of 0.05 M Tris-HCl (pH 7.5) with 1:8 (w: v). The homogenate obtained was collected in a 1.5 mL Eppendorf tube and centrifuged at 12000 rpm (temperature 4 °C) for 15 minutes. The supernatant obtained as a crude extract of the enzyme was collected in a 1.5 mL Eppendorf tube and stored in a freezer at -80 °C before being used to measure enzyme activity. The measurement of the dissolved protein content in the enzyme extract was determined by the Lowry method using albumin as the standard (Susilo et al., 2022). The protein content of this enzyme extract could be used to calculate the specific activity of the enzyme.

Measurement of pepsin and trypsin-like activities

The pepsin and trypsin-like activities were measured by Folin-Cioclateu's method with casein as a substrate (Rungruangsak and Utne 1981, Susilo et al., 2022) using buffer solution 60 mM HCl (pH 2) for pepsin and 0.1 M Tris-HCl buffer solution (pH 7,6) for trypsin. The amount of tyrosine produced was calculated from the standard tyrosine curve, while the pepsin and trypsin-like activities were expressed as U (µmol. minute-1) mg-1 protein.

Measurement of lipase activities

Lipase activities were measured using the p-Nitrophenylpalmitate (p-NPP) hydrolysis method modification by Susilo et al. (2020). Approximately 0.1 M phosphate buffer (pH 7.0) and 0.1 M Tris-HCl (pH 8.1) were the buffers used. The reaction mixture, which included 200 µL of p-NPP substrate, 25 µL of enzyme extract, and 575 µL of buffer, was incubated for 30 minutes at 37°C. This was followed by adding 400 µL of 0.1 M Na_2CO_3 reagent to halt the reaction after incubation. With the exception of adding the enzyme extract after the 0.1 M Na_2CO_3 reagent, the same process was used on the blank. After 15 minutes of centrifuging the reaction mixture at 10,000 rpm, the supernatant absorbance was measured at 410 nm. The specific activity of lipase was measured in units (U) and protein was expressed as Abs.h-1.mg-1. The reaction mixture was diluted in double-distillate water at 1:3 ratios. Subsequently, the reaction mixture was measured for absorbance at 540 nm.

Measurement of amylase activities

Amylase activities were measured using 3,5-Dinitrosalicylic acid (Susilo et al., 2020) and the starch substrate. Subsequently, 0.1 M phosphate buffer (pH 7.0) and 0.1 M Tris-HCl (pH 8.1) were the buffers used. A substrate (200 µL), buffer (275 µL), and enzyme extract (25 µL) made up the reaction mixture, which was incubated at 37°C for 15 minutes before 500 µL of 1% DNS reagent was added to stop the process. Subsequently, the reaction mixture spent 5 minutes in boiling water. With the exception of the enzyme extract that was added following the administration of a 1% DNS reagent, the same process was performed on the blank. The reaction mixture was diluted in double-distillate water at 1:3 ratios. The amylase-specific activity was expressed as a unit (U) after the reaction mixture's absorbance at 540 nm

Statistical Analysis

The quantitative data obtained were analyzed by one-way analysis of variance (One-way ANOVA).

RESULTS AND DISCUSSION

Biochemical Composition

The biochemical composition of eel in the yellow and silver phases varied, tending to increase with increasing body size (**Table 1**).

Protein

Protein is an essential component during fish growth and development. In this study, protein levels tended to decrease with increasing body size or stage. Yellow eel had a higher protein content than silver eel, namely 16.99 ± 1.04%, which decreased to 13.20 ± 2.12%. Eel protein levels were significantly different between phases ($P<0.05$). In the growth phase, namely in the yellow eel, body protein levels are higher than in the silver phase. This is because there is an increase in protein requirements during growth (Damusaru et al., 2019). Protein retention is a

parameter of feed use efficiency, which is very important in eel cultivation.

Based on the results, there are no significant differences between lipid content, NFE, Ash, Fiber, and body moisture with the reproduction phase ($p<0.05$). This is because eel observed in the study was late yellow and early silver. Therefore, changes in the biochemical composition particularly fat, still need to be significant.

Generally, yellow eel in the growth phase has lower fat content which is ready to migrate. This is because body fat content is an important parameter to determine the level of gonad maturity of eel, serving as an essential part of preparing for migration (Arai and Abdul Kadir., 2017; Hagihara et al., 2019). The biochemical composition of eel varies depending on species, age, diet, feeding frequency, migration, sex, and temperature (Desta et al., 2019). One reason is that as fish grow, their body's dry matter and lipid contents increase, decreasing feed protein and energy deposition rate (Azevedo et al., 2004). Fish growth is based on protein and fat deposition, which is closely related to the digestion and absorption of nutrients (Zhao et al., 2012). The digestive capacity of eel is governed by digestive and brush border enzymes in the intestine (Wu et al., 2011).

Lipid

Eel fat content was not significantly different between phases ($P>0.05$). Generally, fat content of eel will increase along with the level of gonad maturity and preparation for migration (Saito et al., 2015; Capocciani et al., 2018). Based on the fat content value, silver eel in this study with an average body size of 362.89 ± 88.93 g had no mature gonads and were grouped into the early phase. According to Okamura et al., 2007, based on body color, eel is grouped into early yellow (Y-1), late yellow (Y-2), early silver (S-1), and late silver eel (S-3). Silvering is the last metamorphosis in eel life cycle, which induces morphological and physiological modifications in yellow eel (sedentary stage) (Amerand et al., 2017).

As eel grows and prepares for long migration, there is a need to accumulate many lipids, according to Boëtius and Boétius (Parzanini et al., 2021). Optimal trophic and environmental conditions allow favorable lipid accumulation for gonad development, facilitating sexual maturation and complete migration (Gurkan et al., 2022). This difference in lipid content can be related to the reproductive phase, as eels may stop feeding during migration and rely on their lipid stores for energy.

Silvering is more flexible than generally presumed (Gong et al., 2017) and can be influenced by several trophic and environmental factors (Palstra, 2010). Information on the gonadal phase and morphological indices is necessary for better selection and the release of sexually mature individuals who are ready to migrate, as requested by the E.U. directive (Sudo *et al.,* 2022).

Digestive Enzymes Activity

Eel physiological state influences the feed's digestibility, including the presence of enzymes and feed particles in the digestive tract, which is converted into energy and body tissue. Enzymes in the body are responsible for the digestibility of feed, serving as a source of energy and growth of body tissues. Food that enters the digestive tract is digested into simple micro-sized compounds, where protein are hydrolyzed into simple forms or peptides, fats into glycerol, fatty acids, and carbohydrates into simple sugars (Halver & Hardy, 2003).

The digestive enzyme activity of yellow and silver eel varies, with the highest being trypsin and the lowest being amylase (**Table 2**). However, the enzyme activities of pepsin, trypsin, and lipase were not significantly different between the yellow and silver phases ($P>0.05$). Protease enzyme activity generally increases during development and decreases in the adult or reproductive phases. In this study, silver phase was still in the pre-pubertal period, showing the significant role of enzyme activity of trypsin and pepsin. Based on the comparison, the amylase activity of eel in this study was deficient ($p<0.05$). Amylase activity in carnivorous fish is generally low, showing values of 2.56 ± 0.81 mU/mg and 4.95 ± 3.29 mU/mg. The activity of fish digestive enzymes is influenced by the type of fish, developmental phase (Yufera et al., 2018), food availability, environmental factors (Zaefarian et al., 2018), and supplementation of feed (Mostafaloo et al., 2021). A study conducted on *A. anguilla* by Gisbert et al. (2011) showed that fish of varying sizes had varying enzymatic activities. Small fish have higher protease and lipase-specific activity, while large size increases amylase-specific activity. This is because the growth phase of fish requires more protein than the adult. The results can be used as a basis for formulating suitable feeds for varied sizes of *A. japonica*, ensuring best nutritional value and cost-effectiveness (Murashita et al., 2013).

According to Tola et al. (2019), the results of protease activity measurements show the secretion of digestive enzymes. As shown in **Table 2**, the pepsin activity of yellow eel averaged 137.52±44.31 mU/mg, which was higher than silver eel at 86.82±56.73 mU/mg. The activity of protease enzymes in yellow eel was relatively low compared to silver *A. bicolor,* which ranged from 1.510 U/mg to 4.970 U/mg protein in culture conditions (Taufik et al., 2017). The protease enzyme activity of small rasbora (1.68 g ± 0.2) and large fish (3.6 g ± 0.5) was 0.115 U/mg protein ± 0.01 and 0.103 U/mg protein ± 0.06, respectively. This difference caused by feed treatment during cultivation was a significant factor in increasing enzyme activity. Protease activity in rasbora fish was not affected by the body size and developmental stage but by feed, temperature, and pH (Mazumder et al., 2018; Susilo & Rachmawati, 2020). During the growth phase, the protease activity

Table 1. The biochemical composition of eel in the yellow and silver types varies which increases with increasing body size.

No.	Parameter	Yellow Eel	Silver Eel
1	Body weight (g)	106.00 ± 38.1	362.89 ± 88.93
2	Body length (cm)	42.00 ± 5.78	59.86 ± 7.39
3	Protein (%)	16.99 ± 1.04	13.20±2.12
4	Lipid (%)	11.11±9.15	11.75±4.67
5	NFE (%)	1.74±1.86	0.97±0.56
6	Fibre (%)	0.27±0.22	0.51±0.16
7	Ash (%)	7.12±2.46	4.79±1.03
8	Moisture (%)	67.67±9.1	72.29±6.95

Table 2. The digestive enzyme activity of *Anguilla bicolor* in different phases is varied.

No.	Parameter	Yellow Eel	Silver Eel
1	Pepsin activity (mU/mg)	137.52±44.31	86.82±56.73
2	Trypsin activity (mU/mg)	475.18±221.94	429.82±364.88
3	Lipase (mU/mg)	15.27±5.94	22.09±11.14
4	Amylase (mU/mg)	2.56±0.81	4.95±3.29

of eel tended to increase with the stage of development, while amylase and lipase decreased. According to Mulyani et al., (2016), lipase and amylase activity tended to be high at the beginning of the development stage and decreased gradually.

Similar to other animals, the activity of digestive enzymes in *A.bicolor* can vary depending on age, sex, diet, and environmental conditions (Gurkan et al., 2023). As eel grows, the digestive system also develops and matures, affecting the activity of digestive enzymes. Previous studies have shown that the action of digestive enzymes, such as trypsin and chymotrypsin, can increase during the first phase of *A. bicolor* growth, indicating an increased demand for protein digestion as the eel grows. However, the specific changes in digestive enzyme activity during the entire growth phase of *A.bicolor* still need to be better understood and vary depending on individual eel and environmental factors.

Body size can influence the activity of digestive enzymes (Hana et al., 2021). The protease activity of toman fish (*channa micropeltes*) was higher in the smallest fish, followed by larger species. This suggests that protease and amylase activity generally increase with age. (Pratama, 2020). Total protease activity in the *Channa aurantimaculata Musikasinthorn* (Pubali et al., 2022) increased with increasing fish growth phase, and conversely, amylase activity was higher in fish larvae compared to the juvenile and adult phases.

Amylase activity in eel is not influenced by body size but by different locations along the digestive tract. Protease and amylase activities may also differ between fish digestive tract segments. According to Taufik (2017), protease is found throughout the digestive tract of eels, including the stomach, hepatopancreas, and intestines. Protease activity in yellow eel phase eel fish is higher than silver.

CONCLUSIONS

In conclusion, this study showed that the biochemical composition and digestive enzymes varied depending on phase. NFE and ash did not have substantial variation between phases, while protein, lipid, ash, and fiber showed significant differences. The yellow eel had a higher body protein composition and pepsin activity than the silver eel, while the biochemical composition and other enzyme activities were similar. Moreover, further studies should be carried out to understand the biochemical composition of *A.bicolor* and digestive enzyme activity during the reproductive phase.

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