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Research Article

Effects of dietary protein levels on growth performance, amino and Fatty acids of juvenile sandfish, *Holothuria scabra* (Jaeger, 1833)

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Abstract

The experiment was carried out for 120 days to determine the optimal dietary protein requirement for the growth of sandfish, *Holothuria scabra*. The experiment used 12 plastic containers sized 59×47×37 cm, and each container stocked 30 individual hatchery-produced sandfish juveniles (initial weight= 2.70±0.01 g). Experimental diets with different levels of protein, i.e., 10% (A), 20% (B), and 30% (C) as a treatment; it was as dry pellet with 3 mm diameters. Sandfish were fed with experimental diets once in the afternoon at 2 % biomass. Results of the experiment showed that sandfish fed a 20% protein diet has the highest final weight (5.10±0.02 g), specific growth rate (SGR; 0.53±0.01 % day^{-1}), and feed efficieny (FE; 62.48±0.13) and is significantly different compared to other treatments (p<0.05). On the other hand, feeding with different dietary protein levels did not influence the survival rate greatly (p>0.05), ranging from 85.00-95.83%. The total amino acid content of sandfish juveniles in treatment B was highest (6.62%) and followed by C (6.27%) and A (4.75%). Likewise, the highest fatty acid content was also found in treatment B (0.440%) followed by treatments A and C of 0.416%; 0.332% respectively. Results of the present study found the best feed was treatment B at 20 % protein content. Moreover, based on statistical analyses, it is suggested that dietary protein requirement for maximum growth of juvenile sandfish is 16%.

Keywords: Amino acid, Fatty acid, Growth, Protein level, Sandfish

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Introduction

The need for sandfish, *Holothuria scabra* (Jaeger, 1833) products is increasing because it was not only used as food (Rasyid *et al*. 2020) but are also currently growing towards phytopharmacology use (Sroyraya *et al*., 2017; Nursid *et al*. 2021; Wulandari *et al*. 2022). The increase in fishing has led to a sharp decline in the natural population of sandfish (Purcell, 2010). Therefore, it is necessary to develop aquaculture for the sustainability of sandfish. Recently, technology for sandfish juvenile production in hatcheries has been successfully developed. However, the growth of sandfish in the nursery is still slow due to relying solely on natural feed such as benthos produced in the nursery tank (Sembiring *et al*. 2015). Meanwhile, benthos production depends on environmental conditions, such as lighting and nutrient supply, and will grow better during the dry season.

Feed is a critical limiting factor in sandfish's hatchery and grow-out activities. The application of formulated feed has been tested in sandfish cultivation and could support the growth of sandfish (Giri *et al*. 2017). Xia *et al*. (2012) stated that different sizes of sea cucumbers require different types of seaweed and nutritional needs. Therefore, it is necessary to determine the optimum protein requirement for juvenile sandfish as an initial step in developing artificial feed to support sandfish culture development.

Some research on the protein requirement for sea cucumbers has been done. Bai *et al.* (2016) reported feed with 11% protein and 2.8% lipid provides the best growth and efficiency for sea cucumber, *Aposthicopus japonicus*. On the other hand, Seo *et al*. (2011) and Wu *et al.* (2015) reported that sea cucumber grows well with feed containing 20.0% protein and 4.38% lipid, respectively. However, information on the protein level in feed to support good growth of the sandfish juvenile is still limited.

Some research on feed ingredients for sea cucumber has been conducted (Seo *et al*. 2011; Wu *et al*. 2015; Martinez-Milian and Olvera-Novoa, 2016). Further research was reported by Shi *et al*. (2013); Kurnianto *et al*. (2021), who found that feeding only by macroalgae or microalgae for sea cucumber showed slow growth. According to Orozco *et al*. (2014), sandfish digest nutrients from animal feed sources better than vegetable sources. Therefore, in the present study, animal protein of fish meal was used because the fish meal has high palatability, mineral composition, fatty acids, and essential amino acids (Novriadi, 2019). Research on protein needs in feed for sandfish is very limited, and its results even varied. Thus this study aims to find the optimum protein content in the diet to promote good growth and survival rate of sandfish juveniles, and high protein and amino acid content in the body tissue is relevant.

Materials and methods

Experimental diets

The experimental diets were formulated to contain different dietary protein levels, namely: A (10%), B (20%), and C (30%), as treatment. Table 1 presents all feed ingredients used in feed formulation, such as *Ulva* meal, soybean meal, lap lap algae meal, rice flour, fish oil, vitamin and mineral mix, filler, and carboxymethyl cellulose (CMC) as feed binder (Wealthy, China) with minimum 95% particles pass 80 mesh. All ingredients were mixed well, and added some water to get approximately 40-50 % moisture content. The dough was extruded through a 3 mm die plate to make pelleted diet. The feeds were then oven dried in an oven at 60ºC and then stored in the refrigerator.

Table 1: Ingredient, proximate composition, and apparent digestibility coefficient of experimental diets

Ingredients	Experimental diets (%)		
	A	**B**	**C**
Fish meal[1)]	6.0	22.0	37.0
Ulva meal[2)]	10.0	10.0	10.0
Soybean meal	3.5	3.5	3.5
Lap lap algae meal	27.25	23.25	19.25
Rice flour	27.25	23.25	23.25
Fish oil	3.8	2.4	1.0
Vitamin mix[3)]	1.0	1.0	1.0
Mineral mix[4)]	1.0	1.0	1.0
Filler	16.2	9.6	-
Carboxymethyl cellulose (CMC)	4.0	4.0	4.0
Total	100	100	100
Proximate composition			
Moisture (%)	7.31	6.66	7.23
Crude protein (% dry matter)	9.83	19.11	29.14
Lipid (% dry matter)	4.43	4.76	5.46
Ash (% dry matter)	26.77	24.93	18.2
Fiber (% dry matter)	1.6	1.25	1.97
Nitrogen free extract (NFE) (% dry matter)	50.06	43.29	37.96
Energy (Kcal g^{-1})	30.01	33.64	37.91

[1] Commercial fish meal (dry matter, %): crude protein 65.17%, lipid 9.63%, ash 37.77%, fiber 7.10%, NFE 40.77%.

[2] Ulva meal (dry matter, %): crude protein 11.63%, lipid 1.08%, ash 14.35%, fiber 0.01%, NFE 1.00%.

[3] Vitamin mix: Vit A 5000 UI/g; Vit D3 1200 UI/g; Vit E 75 UI/g; Vit K3 23.5 mg/g; Vit B1 15 mg/g; riboflavin 20 mg/g; Vit B6 20 mg/g; Vit B12 0.01 mg/g; pantothenic acid 45.1 mg/g; niacin 100 mg/g; folic acid 7 mg/g; biotin 0,2 mg/g.

[4] Mineral mix (mg/g): $CuSO_4.5H_2O$ 20; $FeSO_4.H_2O$ 50; ZnO 200; $Ca(IO_3)2$ 7.5; MnO_2 50; $CoCO_3.H_2O$ 0.05; Na_2SeO_3 0.8.

Experimental setup

The experiment used 12 plastic containers measuring 59×47×37 cm. Each container was filled with sand as a substrate at a three cm thickness. Each container was equipped with aeration and flow-through water systems at 1 L min^{-1} to maintain water quality. Cleaning the surface of the sand substrate from feces and uneaten feed was carried out daily by siphoning, and every week the sand substrate was removed, washed, and then returned to the container.

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The sandfish juveniles used in this study (initial weight=2.70±0.01 g) were obtained from the hatchery of the Gondol Institute for Mariculture Research and Fisheries Extension (IMRAFE). Thirty sandfish juveniles/containers were randomly distributed to plastic containers and adapted in advance to experimental diets for two weeks.

Feeding and sampling

The experiment was conducted for 120 days trial. Experimental diets were given once daily in the afternoon for as much as 2% of the sandfish biomass. Every two weeks, 20 sandfish juveniles were taken randomly from each container to measure their length and weight. At the end of the experiment, the specific growth rate (SGR), survival rate (SR), and feed efficiency (FE) of sandfish were calculated as follows:

SGR (%/d) = 100 × (ln[final weight] – ln[initial weight])/number of days of experiment

SR (%) = 100 × (final/initial numbers of sandfish juvenile in each container)

FE = 100% × [(final weight + weight of the dead sandfish juvenile) – initial weight]/dry weight of feed consumption during the experiment

Sample collection and chemical analysis

A total of twenty juvenile sandfish were taken out from each treatment at the end of the experiment, dried in a freeze dryer, and then ground to become a fine powder for proximate, amino acid, and fatty acid analysis. The protein content of feed and juveniles was determined using the Kjeldahl method (González-Wangüemert *et al*. 2018), and lipid content using the Bligh and Dyer method (1959). Analysis of moisture by the oven-drying method described by Andriamanamisata and Telesphore (2019) and the ash content using the AOAC method (1990). The amino acid composition of sandfish juveniles was analyzed using High-Performance Liquid Chromatography (HPLC) with thermo scientific column ODS–2 hyersyl (AOAC 2005 item 969.33/HPLC). Fatty acid composition analysis following the procedure described by the AOAC Official Method 991.39 (AOAC, 2012).

Apparent Digestibility measurement

The digestibility test for three experimental diets was carried out separately from experimental setup. Feed formulation followed as in Table 1 but replacing 1% rice flour with chromium oxide (Cr_2O_3, Sigma-Aldrich, USA) as digestibility indicator. The experimental container uses were three polycarbonate tanks, 25 L volumes equipped with a flow-through water system, and aeration. Thirty sandfish juveniles (initial weight=2.58±0.36 g) were introduced into each tank for digestibility determination. Sandfish juveniles adapted to experimental diets for one week before feces were collected. Furthermore, periodic collection of feces was carried out using slow exposure to prevent the dissolution

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of nutrients in the feces (Bai *et al*. 2016). Feces were collected daily and immediately dried in the oven (70°C), then stored in the freezer until enough to analyze. Chromium concentrations in feed and feces were analyzed according to Takeuchi (1988). The digestibility of diet dry matter and protein was calculated based on the formula Bai *et al*. (2016):

$$ADD\ (\%) = 100 \times (1 - MD/MF);\ ADP\ (\%) = 100 \times [1 - (MD \times AF)/(MF \times AD)],$$

Where: ADD: Apparent digestibility of dry feed matter; ADP: Apparent digestibility of feed protein; MD and MF are the chromium content in feed and feces successive; AD and AF are the protein levels in feed and feces successively.

Water quality

Water quality such as temperature and acidity (pH), dissolved oxygen (DO), ammonia (NH_3) and nitrite (NO_2) were measured using pH meter (Eutech expert pH, Thermo Scientific), DO meter (Pro20, YSI) and Spectrophotometer (UV mini-1240, Shimadzu), respectively.

Data analysis

Statistical analysis was carried out to determine the difference in SGR, SR, FE, and feed digestibility. The Normality test is performed with the Kolmogorov Smirnov non-parametric one-sample test, while the homogeneity test is performed with the Levene test. Normal and homogeneous distributed data is then tested differently by One-Way ANOVA with post hoc Tukey HSD at a significance of $p<0.05$. Data of SGR was used to determine the optimum dietary protein level for maximum growth of juvenile sandfish using polynomial regression (Robbins *et al*. 2006).

Ethical Statement

The handling of sandfish juveniles during the experiment and prior to analysis was performed in accordance with the principles of animal use and welfare (B/11/UN.14.2.9/PT.01.04/2022) by Udayana University Animal Ethics Commmittees.

Results

Growth performance and survival of sandfish juvenile

The growth pattern of sandfish juveniles fed with experimental dietary protein levels for 120 days of the experiment is presented in Figure 1. Sandfish juveniles fed experimental diets A and B began to increase from 4 weeks until the end of the study, while the growth of sandfish in treatment C was much lower.

The results showed that the protein content in the feed had a noticeable influence on the final weight and specific growth rate of sandfish juveniles but did not significantly affect their survival (Table 2).

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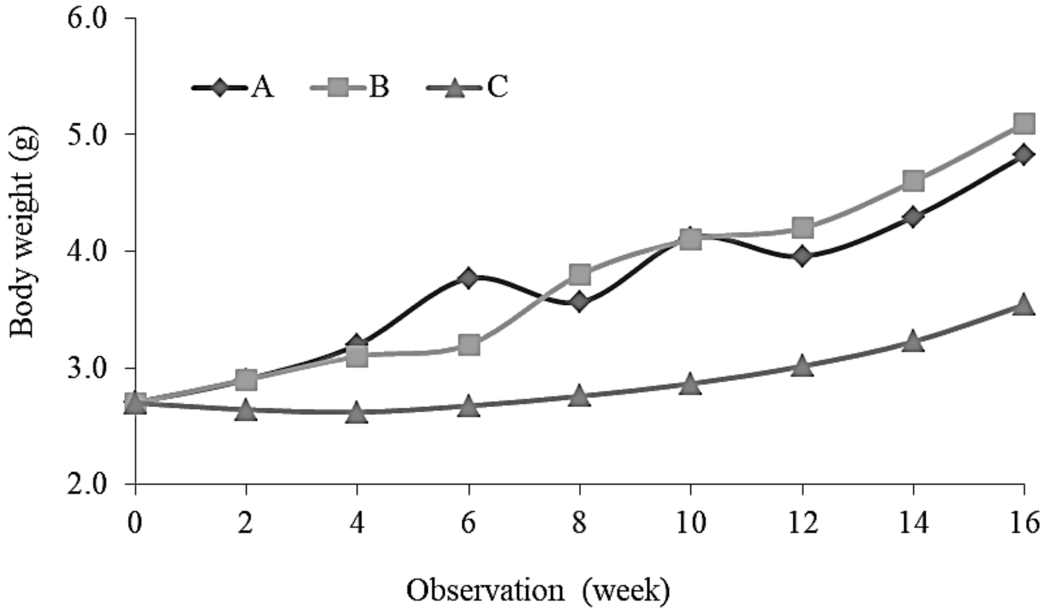

Figure 1: Growth patterns of sandfish juveniles fed experimental diets for 120 days.

Table 2: Final weight, specific growth rate, survival rate, and feed efficiency of sandfish juveniles fed the experimental diets for 120 days.

Parameters	Experimental diets		
	A	B	C
Final weight (g)	4.83±0.07^{b}	5.10±0.02^{c}	3.54±0.14^{a}
Specific growth rate (% day^{-1})	0.48±0.01^{b}	0.53±0.01^{c}	0.15±0.04^{a}
Survival rate (%)	95.00±0.30^{a}	95.83±0.33^{a}	95.00±0.30^{a}
Feed efficiency	47.16±0.24^{b}	62.48±0.13^{c}	19.18±0.43^{a}

Values in the same row followed by the same superscript letter are not significantly different (p>0.05).

Sandfish juvenile feeding with experimental diet B have a final weight of 5.10±0.02 g and a specific growth rate of 0.53±0.01 % day^{-1} and was significantly different compared to treatments A and C (p<0.05). The survival rate of sandfish juveniles in this experiment was relatively high, ranging from 85.00 to 95.83 %, and was not significantly different among treatments (p>0.05). Most of the mortality occurred at the beginning of the experiment due to adaptation to the new rearing environment.

The relationship between the treatment of protein content in feed and SGR was polynomial regression, as shown in Figure 2, and the optimum value of protein content in the feed was 16%.

Feed efficiency

Different levels of dietary proteins exerted a noticeable influence on feed efficiency (Table 2). Juveniles fed on experimental diet B gave significantly higher feed efficiency (62.48±0.13) compared to treatments A and C (p<0.05).

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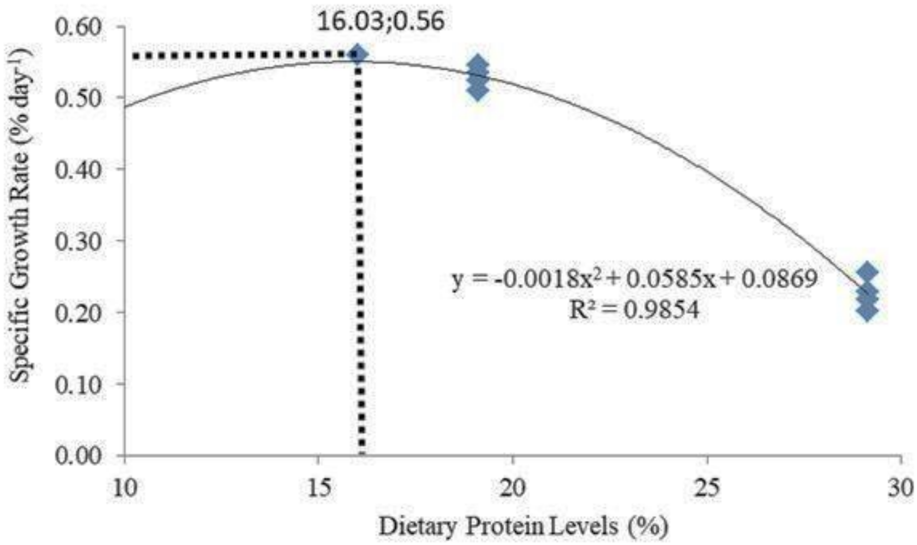

Figure 2: Relationship between dietary protein level and specific growth rate of sandfish juveniles.

Whole-body tissue composition of sandfish juveniles

The proximate composition of sandfish juveniles after being fed with experimental diets for 120 days showed that juveniles fed with experimental diet B had a higher protein content (13.37%) and provided optimal growth, successively followed by treatment C (11.96%) and A (11.07%) (Table 3).

Table 3: Proximate whole-body composition of sandfish juveniles (% dry matter) fed with experimental diets for 120 days.

Experimental diets	Protein	Lipid	Ash	Fiber	NFE
A	11.07	4.18	72.93	1.42	10.40
B	13.37	3.52	73.01	0.92	9.17
C	11.96	1.92	75.04	1.00	10.09

Total amino acid of sandfish juveniles fed with experimental diet B was higher (6.62%) and followed by C (6.27%) and A (4.75%) (Table 4).

Total fatty acid of sandfish juveniles fed with experimental diet B was higher (0.440%) compared to those juveniles fed with experimental diet A (0.416%) and experimental diet C (0.332%) (Table 5).

Apparent Digestibility

Apparent digestibility coefficients of dry matter and protein of sandfish fed the experimental diets are presented in Table 6.

Water quality

The results of water quality measurements during the study were still in the optimum range for the rearing of sandfish juvenile (Table 7).

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Table 4: Amino acids whole-body composition of sandfish juveniles fed the experimental diets for 120 days (% of dry matter).

Amino acids	Experimental diets		
	A	B	C
Essential AA			
Methionine	0.15	0.19	0.19
Valine	0.21	0.28	0.28
Phenylalanine	0.52	0.66	0.58
I-leucine	0.18	0.25	0.25
Leucine	0.33	0.46	0.48
Threonine	0.26	0.35	0.35
Lysine	1.10	1.42	1.36
Histidine	0.05	0.07	0.10
Non-essential AA			
Arginine	0.16	0.26	0.27
Aspartic acid	0.44	0.65	0.56
Glutamic acid	0.46	0.74	0.63
Serine	0.26	0.34	0.36
Glycine	0.28	0.47	0.37
Alanine	0.18	0.31	0.28
Tyrosine	0.14	0.18	0.20
Total Amino Acids	4.75	6.62	6.27

Table 5: Fatty acids whole-body composition of sandfish juvenile fed with the experimental diets for 120 days (% of dry matter).

Fatty acids	Experimental diets		
	Diet-A	Diet-B	Diet-C
Butyric acid, C4:0	0.016	0.014	0.008
Caprilic acid, C8:0	0.002	0.011	0.011
Capric acid, C10:0	0.008	0.010	0.007
Lauric acid, C12:0	0.006	0.006	0.005
Myristic acid, C14:0	0.065	0.025	0.020
Palmitic acid, C16:0	0.073	0.082	0.060
Stearic acid, C18:0	0.060	0.098	0.069
Oleic acid, C18:1n9	0.011	0.012	0.009
Linoleic acid, C18:2n6	0.011	0.012	0.009
Arachidic acid, C20:0	0.008	0.012	0.008
Eicosanoic acid, C20:1	0.020	0.021	0.011
Heneicosanoic acid, C21:0	0.016	0.020	0.013
Behenic acid, C22:0	0.009	0.015	0.009
Docosadienoic acid, C22:2	0.035	0.034	0.033
Nervoic acid, C24:1	0.033	0.035	0.029
Total n-3	0.021	0.010	0.012
Total n-6	0.021	0.024	0.020
Total fatty acids	0.416	0.440	0.332

Table 6: Apparent digestibility coefficients (%) for dry matter and protein of juvenile sandfish fed with the experimental diets for 2 months.

Apparent Digestibility Coefficients (ADC) (%)	Experimental diets		
	Diet A	Diet B	Diet C
ADC of dry material	65.38±0.99^{a}	68.05±0.18^{b}	68.17±0.22^{b}
ADC of protein	61.01±1.12^{a}	70.51±0.20^{c}	67.37±0.19^{b}

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Table 7: Water quality of sandfish juveniles fed with the experimental diets for 120 days.

Parameters	Experimental diets		
	A	B	C
Temperature (°C)	28.59±0.78	28.56±0.81	28.53±0.79
DO (ppm)	5.49±0.23	5.58±0.21	5.56±0.19
pH	8.34±0.07	8.37±0.07	8.37±0.06
NH_3 (ppm)	<0.01	<0.01	<0.01
NO_2 (ppm)	<0.003	<0.003	<0.003

Discussion

The growth performance of sandfish juveniles was clearly expressed as related to protein content in experimental diets. In this study, the SGR of sandfish juveniles significantly increased from 0.48±0.01% day^{-1} to 0.53±0.01% day^{-1} when the dietary protein diet increased from 10% to 20%. However, increasing dietary protein to 30% did not support increasing the SGR of juvenile sandfish anymore, even resulted lower

Although the 30% diet protein content showed higher protein, lipid, and energy values than the other two treatments, its growth rate was the lowest. Figure 1 show that the growth of sandfish juveniles in treatment C was lower than in treatment A, which had 10% protein content. A negative growth response was seen in sea cucumber *A. japonicus* when fed with protein content in a diet above the optimum level (Seo and Lee, 2011; Bai *et al*. 2016). The result indicates that the sandfish juveniles do not require diet with high-protein content. Bai *et al*. (2016) reported that cells have a specific limit in storing protein. When it reaches this limit, there is not enough energy available for the deamination and excretion of excess amino acids that are absorbed from the breakdown of protein from a high-protein diet. Therefore, a protein content of 20% is required to support better growth of sandfish juveniles. Moreover, based on polynomial regression analysis, the optimum protein requirement for maximum growth of juvenile sandfish in the present study was 16 % in the diet (Fig. 2).

Nutrient utilization in animals may be evaluated by digestibility measurement. The apparent digestibility of feed dry matter (65–68 %) and protein (61-70 %) (Table 6) of sandfish juveniles in this study were similar to a previous study (Giri *et al*. 2017). But according to Orozco *et al*. (2014), when sandfish fed with formulated feed contain shrimp and mussel powder, the digestibility protein reach even higher 88.7% and 84.8%, respectively.

Analysis of variance showed that the increase in protein content in the feed was not always accompanied by an increase in growth. According to Seo and Lee (2011), these observations suggest that aquatic animals could increase the digestibility and absorption rate of dietary protein by increasing protease activity and adapting to different dietary protein levels. However, when the protein is increased

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even higher (30%) the protease activity was inhibited, means the protein level in the feed was exceed the amount required for growth. Therefore, the proper protein level in the diet is essential during cultivation. The diet containing 30% protein in the present study showed the body weight of juvenile sandfish only increased by 0.84 g, and the feed efficiency was very low (19.18±0.43) compared to the experimental diet of 20% protein with a feed efficiency value (62.48±0.13).

Although the feed protein content is different, the juvenile tissue proximate was not different. The same result was also reported in *A, japonicus,* at high levels of protein in the diet, do not change its proximate composition (Seo and Lee 2011; Seo *et al*. 2011). The ash content in this study ranged from 72.93-75.04% and was relatively high compare to juvenile *I. badionotus* fed with different animal feed of 61.81-63.68% (Zacarias-Soto and Olvera-Novoa, 2015). High ash content in this study might be due to the whole body was analyzed at the end of the study. Ridwanudin *et al*. (2018) states that the sandfish from natural catches, cultivation and different sizes provide different proximate composition.

Biochemically it is proven that the whole-body composition of sandfish juveniles fed with an experimental diet of 20% protein has higher protein content than other treatments. Fed with 10% protein content, juvenile is still growth, while at 30% protein content, growth was very low. Slightly different as Zacarias-Soto and Olvera-Novoa (2015) found in *I. badionotus* fed with protein content up to 37.7%, its body protein content still increased to 23.7%. Likewise, the amino acid content found in sandfish fed with the experimental diet of 20% protein is higher (6.62%) than in experimental diets of 30% protein (6.27%) and 10% protein (4.75%). The dominant amino acids content were lysine, phenylalanine, aspartic acid, and glutamic acid. Similar result was found by Rasyid *et al*. (2021) in cultured *sandfish*, where threonine was the primary essential, followed by leucine, phenylalanine, lysine, valine, isoleucine, histidine, and tryptophan. Additionally, Ridhowati and Asnani (2015) and Rahael *et al*. (2019) also reported that glutamate, glycine, aspartic acid, and lysine are the dominant amino acids in dried natural sandfish. Base on feed protein and dominant amino acids content is in balance, its utilization could support for *A. japonicus* juvenile growth (Xia *et al*. 2015). In line with Sun *et al*. (2004), reported that weight gain in *A. japonicus* was maximal when the diet was rich in threonine, valine, leucine, phenylalanine, lysine, histidine and arginine, which are essential amino acids for *A. japonicus*.

At the end of the experiment, the dominant fatty acids in the whole body were palmitic acid (C16:0); myristic acid (C14:0); stearic acid (C18:0); docosadienoic acid (C22:0); nervoic acid (C24:1), and eicosanoic acid (C20:1). This was similar to the results reported by Ridhowati and Asnani (2015). Fawzya *et al*. (2015) also reported that palmitic fatty acid was the

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dominant fatty acid in the *Holothuria fuscogilva* and reach 32% of total fatty acids (Wen *et al*., 2010). Stearic acid (18:0) and oleic acid (18:1n-9) is a fatty acids that tend to be accumulated by the *I. badionotus* which is fed a diet with excess fat content (Zacarias-Soto and Olvera-Novoa, 2015). Meanwhile, *A. japonicus* which was fed feed with high fat content tend to accumulate linolenic and oleic fatty acids (Seo and Lee, 2011). This information indicates that the fatty acid profile of sea cucumbers is affected by the fat content of the feed.

In addition to good nutritional content in the feed, environmental factors and water quality can also affect the growth of sandfish juveniles. Sandfish juveniles will remodel protein to grow if the environmental conditions and water quality follow their natural habitat. From the observations of water and substrate quality during the experiment, it turned out that differences in feed protein content did not affect water quality.

The finding in the present study suggested that an artificial diet containing 16% diet protein could support good growth, proximate and amino acid content, and feed efficiency of juvenile sandfish. Further study is needed to improve feed efficiency by balancing essential amino acids in the diet to meet the amino acid requirement of sandfish.

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