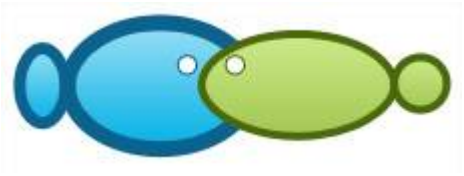

Growth performance and immune response of tropical abalone (*Haliotis squamata*) fed various animal protein sources

[1]Asep Ridwanudin, [2]Dien A. Anggorowati, [2]Idham S. Pratama, [3]Ibnu Sahidhir, [3]Arsyad Sujangka, [1]Lusi H. Suryaningrum, [3]Sulasy Rohmy

[1] Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia; [2] Research Center for Marine and Land Bioindustry, BRIN, Lombok, Indonesia; [3] Research Center for Fishery, BRIN, Cibinong, Bogor, Indonesia. Corresponding author: A. Ridwanudin, ase_rid@yahoo.com

Abstract. The utilization of protein in aquafeed is vital to producing fish and other aquatic organisms. Traditionally, fish meal (FM) has been the primary source of protein in aquafeed. However, there is a growing need to explore alternative sources of protein. The present study aimed first to investigate the potential of alternative animal protein sources, such as shrimp head meal (SM) and meat and bone meal (MBM), to replace FM in the diet of tropical abalone *Haliotis squamata*, and second to evaluate their effects on the growth of the organism. Four experimental diets were formulated, including a control diet containing FM, SM, MBM, and a mixture of fish meal, shrimp head meal, and meat and bone meal (MIX). The experimental diets were fed to triplicate groups of abalone once a day at a level of 1.5% of their total body weight. Two hundred and forty abalone with an initial average weight of 3.55±0.02 g and a shell length of 28.59±0.12 mm were distributed into 12 rectangular plastic baskets at a density of 20 abalone per basket. After twelve weeks of feeding, the final weight, weight gain, specific growth rate, final shell length, and shell length gain of abalone fed the FM diet were not significantly different ($p > 0.05$) from those fed the other diet treatments. Dietary treatments also did not affect the total haemocyte count (THC) of abalone. These findings suggest that SM or MBM could partially or entirely replace FM in the abalone feed.

Key Words: feed, fish meal, meat and bone meal, shrimp head meal.

Introduction. Aquaculture has emerged as the fastest growing food production sector globally, supplying approximately 47% of the world's seafood demand. With an ever-increasing population, aquaculture is well suited to meet the protein requirements of humans. Over the last five decades, global fish production has exhibited a steady increase, with an average annual rise of 3.2% in food fish supply, surpassing the world population growth rate of 1.6%. Notably, the per capita apparent fish consumption worldwide has surged from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO 2012). The global aquaculture industry has experienced exponential growth, leading to the emergence of cost-effective and species-specific commercial diets. This trend has been driven by the need to optimize production processes. Feed expenses, which constitute more than half of the total production costs, are particularly essential in aquaculture (Bullon et al 2023).

Abalone is an important seafood commodity, and an important source of income for many coastal communities to be farmed. The success of abalone farming depends on many factors, including diet, which plays a critical role in growth and health. Abalone, members of the family Haliotidae, are single-shelled marine molluscs classified under the phylum Mollusca, class Gastropoda, and genus *Haliotis*. They are characterized by their flattened shell, which features a mother-of-pearl lining and a row of respiratory pores extending from the shell's left anterior margin and closing posteriorly as growth proceeds. The abalone body follows the typical molluscan body plan, comprising head-foot and visceral mass portions (Venter et al 2018). There are approximately 90 known

species of abalone that are widely distributed throughout tropical and temperate water. Around 15 of these species are cultivated in various countries, including Australia, China, Japan, Korea, New Zealand, Philippines, South Africa, and Taiwan. These gastropods are entirely herbivorous and exhibit nocturnal grazing behaviour (Sales & Janssens 2004).

The emergence of abalone farming has spurred scientific research into various aspects of abalone physiology, including digestive physiology, animal feed science principles, feeding behavior, and optimizing of diet utilization under intensive culture conditions. While some abalone farms rely on macroalgae as a food source, there is an increasing need for nutritionally complete feeds due to limited algae supply, logistical challenges associated with harvesting, transporting, and storing algae, and the potential for higher yields and faster growth with the improvement of artificial diet formulations. Artificial feeds have been utilized in abalone culture in Japan and China for several years and are currently being developed in various other countries, including Australia, Canada, Chile, Korea, Mexico, New Zealand, South Africa, Thailand, North America, the Philippines, and Iceland (Sales & Janssens 2004). Hence, the search for alternative protein sources has become necessary due to the recent decline in the availability and sustainability of fish meal (FM).

Protein is an important nutrient for all living organisms, including aquatic animals such as abalone (Kaushik & Seiliez 2010). It is used for growth and maintenance, particularly in the early stages of organisms, and also for reproduction in the sexual maturation stage (Radhakrishnan et al 2020). The requirement for dietary protein in an aquatic organism is influenced by several factors, such as growth rate, the quality of ingredients (protein digestibility and amino acid profiles), and the digestible energy of the diet (Matsumoto 2002). Moreover, protein is a nutritionally valuable constituent in the diets of both aquatic and terrestrial animals, but its procurement can entail significant costs. FM, a common protein source, is frequently utilized for this purpose (Tacon & Metian 2008). The FM is not only a good source of indispensable amino acids, but also contains some trace minerals, particularly calcium, phosphorous, magnesium, and potassium (Hardy 2010). It is also good in palatability due to its chemo-attractant properties such as glutamic acid content (Hertrampf & Piedad-Pascual 2000).

However, the increasing demand of FM for the aquaculture industry is not in line with increasing FM production due to the unstable supply of marine captured fisheries as a raw material to produce it. Consequently, the inclusion of FM in aquafeed becomes uneconomical due to the increasing price of FM (Hardy 2010; Kaushik & Seiliez 2010). Findings of alternative protein sources are a main priority in fish nutrition research to reduce the dependency on FM. Alternative ingredients to replace FM in the aquafeed industry must have some characteristics such as low level of non-soluble carbohydrate, anti-nutrient factors, high protein content, balance in amino acid composition and also reasonable in palatability (Gatlin III et al 2007). Some alternative ingredients which are originated from plant sources that have been used are seaweed (Suryaningrum et al 2017; Suryaningrum & Samsudin 2020; Nafiqoh et al 2021), sugarcane bagasse (Suryaningrum et al 2021; Suryaningrum & Samsudin 2021), rice bran (Ries et al 2020), wheat bran (Das et al 2021), cassava (Mahanama et al 2021). The use of plant sources ingredients is limited by several factors such as inconsistency in quality, discontinuity and the presence of crude fiber, which do not exist in ingredients originated from animal sources.

Among alternative protein sources, animal protein sources such as shrimp head meal (SM) and meat and bone meal (MBM) are interesting ingredients as the alternative for FM due to their high content of protein, high amounts of lysine, methionine and choline, and also rich in highly unsaturated fatty acid (HUFA). Moreover, MBM also contains high amounts of vitamin B, calcium and phosphorous (Hertrampf & Piedad-Pascual 2000). However, MBM also holds some disadvantages, including high ash content due to the presence of bone and inorganic matter, and also inconsistent quality which depends on the freshness of raw material and processing method (Bureau et al 2000). Some previous studies have successfully reported in partial substitution of FM by MBM in fish and shrimp diets (Tan et al 2005; Moutinho et al 2017). Moreover, replacing FM with animal protein sources in some fish species have also been reviewed by Luthada-

Raswiswi et al (2021). However, the information regarding the utilization of animal protein sources, mainly SM and MBM in the formulated diet of abalone is scarce. Therefore, in this study, we aim to investigate the effects of various animal protein sources on the growth performance and immune response of tropical abalone *Haliotis squamata*. Specifically, we will examine the differences in growth rate, feed conversion ratio, survival rate, and immune response among abalone fed with diets containing different types of animal proteins. Our study will provide valuable insights into the nutritional requirements of tropical abalone and the potential benefits of using different types of animal proteins in aquaculture.

Material and Method

Experimental diets. In this study, four experimental diets were devised (Table 1), all of which incorporated seaweed meal from *Gracilaria* sp. as a key component. The experimental diets featured different animal protein sources, namely fish meal (FM), shrimp head meal (SM), meat and bone meal (MBM), and a combination of FM, SM, and MBM (MIX), which were employed to substitute fish meal either partially or entirely. A FM diet was employed as a control. Additionally, soybean meal was included in the diet formulations to satisfy protein requirements. Lipid sources were derived from cod liver and soybean oils, whereas wheat flour served as the carbohydrate source. To enhance the diet's cohesiveness, sodium alginate and carboxymethyl cellulose (CMC) were incorporated as binders.

Table 1

Formulation (g $100g^{-1}$) of the experimental diets for abalone *H. squamata*

Ingredients	*FM*	*SM*	*MBM*	*MIX*
Fish meal	18	-	-	6
Shrimp head meal	-	18	-	6
Meat and bone meal	-	-	18	6
Soybean meal	20.5	20.5	20.5	20.5
Seaweed meal	23	23	23	23
Sodium alginate	7	7	7	7
Wheat flour	20	20	20	20
Cod liver oil	1.5	1.5	1.5	1.5
Soybean oil	1.5	1.5	1.5	1.5
Vitamin mixture[1]	3	3	3	3
Mineral mixture[2]	4	4	4	4
Carboxymethyl cellulose	1.5	1.5	1.5	1.5

[1]Vitamin mixture composition (unit kg^{-1}): vitamin A - 60,000,000 IU; vitamin D3 - 12,000,000 IU; vitamin E - 75,000 mg; vitamin K3 - 10,000 mg; thiamine - 10,000 mg; riboflavin - 30,000 mg; pyridoxine - 20,000 mg; cyanocobalamin - 100 mg; biotin - 100 mg; nicotinic acid - 5,000 mg; pantothenic acid - 54,000 mg; folic acid - 5,000 mg; [2]Mineral mixture composition (g $100g^{-1}$): NaCl - 1; $MgSO_4.7H_2O$ - 15; $NaH_2PO_4 \cdot 2H_2O$ - 25; KH_2PO_4 - 32; dicalcium phosphate - 20; $FeCl_3$ - 2.5; $ZnSO_4 \cdot 7H_2O$ - 0.4; Ca-lactate - 3.85; CuCl - 0.03; $AlCl_3.6H_2O$ - 0.01; $MnSO_4.H_2O$ - 0.2; $CoCl_2.6H_2O$ - 0.01.

All dry ingredients were first ground into a fine powder in a hammer mill, and then analyzed for the proximate composition (Table 2).

Table 2

Proximate composition (g $100g^{-1}$) of ingredients for abalone *H. squamata*

Component	*Fish meal*	*Shrimp head meal*	*Meat and bone meal*
Dry matter	89.82	85.29	92.89
Crude protein	34.64	21.46	54.63
Crude lipid	3.49	9.97	11.45
Ash	22.55	12.34	18.21

The experimental diets were then prepared by thoroughly mixing those dry ingredients with cod liver and soybean oils at a ratio of 1:1 until homogenous. Sixty percent of distilled water was then added to the premixed diet to produce a stiff dough. The dough was pelleted by shaping it into a ±2 mm-thick sheet, then cut into 10 x 10 mm flakes by hand. The flakes were steamed for 3 min and then oven-dried by dry air mechanical convection oven (Memmert Wisconsin Oven) at 60ºC. After drying, the flakes were stored in the refrigerator at 4ºC until used. Prior to the feeding trial, the experimental diets were analyzed for the proximate composition (Table 3).

Table 3

Proximate composition (g $100g^{-1}$) of the experimental diets for abalone *H. squamata*

Ingredients	*FM*	*SM*	*MBM*	*MIX*
Dry matter	90.81	94.15	93.79	95.04
Crude protein	22.13	19.21	25.32	19.03
Crude lipid	2.77	4.41	4.73	3.79
Ash	20.04	17.86	19.31	16.79

Feeding trial. Juvenile *H. squamata* abalone were procured from the Marine Cultivation Hall under the Ministry of Marine Affairs and Fisheries Republic of Indonesia (KKP) in Sekotong, West Lombok. The abalone were transported to the Laboratory of Cultivation, Research Center for Marine and Land Bioindustry, National Research and Innovation Agency (BRIN) in Pemenang, North Lombok. Prior to initiating the feeding trial, the juvenile abalone were acclimated to the laboratory conditions. The abalone were fed *ad-libitum* with fresh seaweed *Gracilaria* sp. during the acclimation period. The abalone were divided into triplicate groups for each dietary treatment. A total of 240 juvenile abalone, with an initial average body weight of 3.55±0.02 g and an initial shell length of 28.59±0.12 mm, were randomly distributed into 12 rectangular plastic baskets with 20 abalone in each. The experimental diets were fed to the abalone for 12 weeks from November 29th, 2021 to February 21st, 2022, with feeding done by hand once daily (15:00h) at 1.5% of the total body weight. Prior to feeding, uneaten feed and faeces were siphoned off every day. All abalone were individually weighed every four weeks after 24 hours of fasting to determine growth. During the feeding trial, the abalone were housed in twelve rectangular plastic baskets (400 x 310 x 220 mm; length x width x height) arranged in the concentrate raceway (50 x 55 x 300 cm; length x width x height). The raceway was equipped with a seawater recirculation system that operated at a flow rate of 60 L min^{-1}, and aeration was provided to maintain sufficient dissolved oxygen levels. The raceway was cleaned weekly by replacing the seawater in the system. Water quality parameters were measured thrice a week, including temperature and salinity.

Sample collection and analysis. Three abalone from each basket were selected randomly for haemolymph sampling, both before and after the trial. Haemolymph was collected from the pedal sinus of the abalone using a sterile syringe equipped with a needle, and a total of two sterile microtubes were used to pool the collected haemolymph. The samples were then placed on ice to prevent cell clumping and agglutination. The haemolymph samples were later analyzed to determine the total haemocyte count (THC). According to Xue et al (2008), the THC for each replicate was determined. Briefly, undiluted haemolymph (200 µL) was added to a Neubauer haemocytometer (Erma Inc., Tokyo, Japan) and counted under a microscope (Olympus CX43, Shinjuku-ku, Tokyo, Japan).

The ingredients and experimental diets were ground with a laboratory mill prior to the proximate analysis. Crude lipid, crude protein, moisture and ash were analyzed in triplicates with the following; crude lipid content was extracted using chloroform:methanol (2:1, v/v) according to Folch et al (1957), crude protein was calculated by multiplying nitrogen (N) content by 6.25 using Kjeldahl method, ash content by combusting the sample in the muffle furnace at 560ºC for 8h, and moisture

content was analyzed by drying the sample in the oven at 105ºC until a constant weight obtained.

Weight gain (WG), specific growth rate (SGR), shell length gain (SL) and survival rate (SR) of abalone were calculated according to Ansary et al (2019) as follows:

$$WG\ (\%) = 100 \times (\text{final weight, g} - \text{initial weight, g}) / (\text{initial weight, g})$$
$$SGR\ (\%\ day^{-1}) = 100 \times (\ln \text{final weight} - \ln \text{initial weight}) / (\text{feeding days})$$
$$SL\ (\%) = 100 \times (\text{final length, mm} - \text{initial length, mm}) / (\text{initial length, mm})$$
$$SR\ (\%) = 100 \times (\text{final number of abalone}) / (\text{initial number of abalone})$$

Statistical analysis. Data were shown as mean±SD, then analyzed by one-way analysis of variance (ANOVA) in SPSS version 20.0 (IBM® SPSS® Statistics, New York, USA). Tukey's HSD test was further applied to identify the differences in means values. All statistical analysis were performed at the level of 5% of significance ($p < 0.05$).

Results. SR of abalone after 12 weeks of feeding trial was high (100%), and no significant differences ($p > 0.05$) were found among dietary treatments (Table 4). Moreover, there were no significant differences ($p > 0.05$) in the final weight of abalone fed the dietary treatments, ranging from 7.00 to 7.59 g. The WG and SGR of abalone were also not significantly affected ($p > 0.05$) by the inclusion of various animal protein sources. WG ranged from 96.94 to 113.21%, while SGR ranged from 0.79 to 0.90 (Table 4).

Table 4

Weight growth performance and survival rate of abalone *H. squamata* for 12 weeks

Ingredients	*FM*	*SM*	*MBM*	*MIX*
Initial weight (g)	3.55±0.04	3.55±0.02	3.55±0.03	3.56±0.02
Final weight (g)	7.03±1.34	7.00±1.79	7.24±0.41	7.59±1.54
Weight gain (%)	97.95±36.14	96.94±50.08	103.60±9.67	113.21±42.35
SGR (% day^{-1})	0.81±0.21	0.79±0.29	0.86±0.06	0.90±0.23
Survival rate (%)	100	100	100	100

Values are shown as mean±SD (n = 3 tanks/diet). No significant differences were found among dietary treatments ($p > 0.05$). SGR = specific growth rate.

Similar to weight performance, shell performance of abalone fed the experimental diets were unaffected by dietary treatment ($p > 0.05$). Shell performances, including final shell length and SL of abalone are presented in Table 5. The final shell length ranged from 34.80 to 35.81 mm, and SL of abalone ranged from 21.47 to 25.47%.

Table 5

Shell growth performance of abalone *H. squamata* for 12 weeks

Ingredients	*FM*	*SM*	*MBM*	*MIX*
Initial shell length (mm)	28.59±0.16	28.65±0.13	28.545±0.17	28.60±0.05
Final shell length (mm)	35.09±0.36	34.80±0.87	35.81±0.69	35.47±0.43
Shell length gain (%)	22.76±0.80	21.47±2.81	25.47±2.27	24.02±1.49

Values are shown as mean±SD (n = 3 tanks/diet). No significant differences were found among dietary treatments ($p > 0.05$).

In addition, the THC of abalone in the present study varied significantly ($p < 0.05$) following the administration of different protein types compared to pre-treatment. Notably, the highest THC levels were recorded in specimens fed with MBM protein sources, with a value of 331.17×10^4 cells mL^{-1} (Figure 1). However, the immune response did not differ significantly across the various dietary treatments ($p > 0.05$).

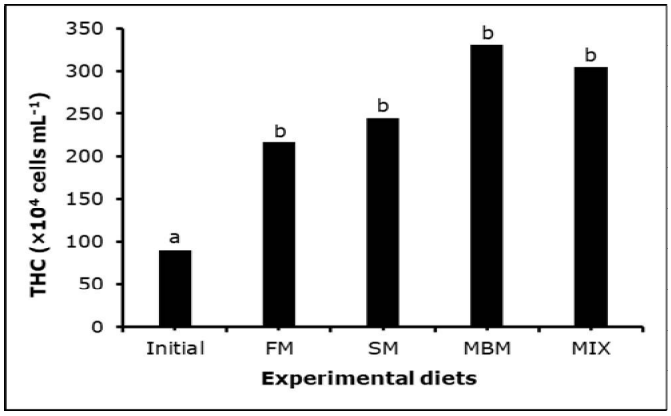

Figure 1. Total haemocyte count (THC) (10^4 cells mL^{-1}) of abalone *H. squamata*.

Moreover, the results of the water quality parameter analysis also indicated that all of the measured parameters during the dietary treatment remained within the optimal range (Table 6).

Table 6

Water quality parameters during for 12 weeks

Temperature (ºC)	*Salinity (ppt)*	*pH*	*DO (ppm)*
26-28	30-33	8-8.5	5-7

Discussion. Protein represents the most indispensable nutrient for aquatic organisms, being a fundamental constituent of their formulated diets. In general, protein intake by aquatic animals is aimed at procuring amino acids, which are critical for their growth performance and metabolic processes (Fall et al 2023). No retardation effect on abalone growth by replacing FM with other protein sources in the present study was similar with some previous studies. For instance, the combination of abalone viscera silage and soybean meal could totally replace FM in the diet without negative effects on the growth of abalone *Haliotis fulgens* (Gunzmán & Vianna 1998). FM could also be replaced by tuna by-product meal up to 75% in the diet of abalone *H. discus* (Jung et al 2016). Similarly, the tunic meal of sea squirt could substitute for FM up to 80% without decreasing the growth performance of abalone *H. discus* (Choi et al 2018). Plant protein sources could also replace FM in the abalone diet. Yu et al (2022a) reported that soybean meal could replace 50% of dietary FM without influencing the growth of abalone *H. discus hannai*. A higher replacement level of FM was found by the inclusion of 75% enzyme-treated soybean meal in the diet of abalone *H. discus hannai* (Yu et al 2022b). Moreover, corn gluten meal could also replace up to 75% of dietary FM in the diet of abalone *H. discus hannai* (Wu et al 2022).

In the present study, although statistically not significant, SGR of abalone fed with MBM diet was numerically 4.4% lower than SGR of abalone fed with MIX diet, but 6.2% higher than SGR abalone fed with FM diet. Moreover, SGR of abalone fed with SM diet was also 2.5% lower than SGR of abalone fed with FM diet (Table 4). It has been known that FM is commonly used as a suitable protein source for abalone (Britz 1996). However, the crude protein content of FM diet in this study was 14.4% lower and 15.2% higher than the crude protein content in MBM and SM diets, respectively (Table 3). Thus, from our findings, it could be assumed that the growth performance of abalone is correlated with protein content in the diet. Ma et al (2020) reported that deficiency and excessive protein level in diet have a negative effect on the growth of abalone *H. discus hannai*. Furthermore, low intake of protein (< 15.11%) has also been reported to decrease the growth of abalone (Ma et al 2021).

Although the highest crude protein content was found in MBM diet, the highest final weight, WG and SGR were found in abalone fed with the MIX diet in this study. A combination of animal protein sources in the MIX diet might contribute to balanced amino

acid profiles in the diet, and then effectively improve the weight performance of abalone. A similar finding has also been reported by Cho (2010) who found that WG of abalone *H. discus hannai* fed diets containing single protein sources such as poultry meal, corn gluten meal, silkworm pupae meal, and meat and bone meal was poorer than that abalone fed with the diet containing a combination of different protein sources. Bautista-Teruel et al (2003) also found that a combination of animal and plant protein sources in the diet enhanced the growth of abalone *H. asinina*.

Weight and shell length performances of abalone fed with an SM-based diet were numerically lower than other diet treatments in this study (Tables 4 and 5). SM contains a high amount of chitin, as a major component of the exoskeleton of crustaceans (Hertrampf & Piedad-Pascual 2000). Moreover, some fish species have been reported incapable to digest chitin due to the absence of chitinolytic enzymes (Fines & Holt, 2010). Furthermore, Hertrampf & Piedad-Pascual (2000) also suggested that the inclusion of SM in the diet for herbivorous fish species was no more than 10%. Thus, decreasing the growth of abalone fed with SM diet might be associated with lowering diet digestibility in this study.

Haemocytes in molluscs are versatile constituents of the hemolymph that assume multiple roles in various aspects of life, encompassing immune response, bio-mineralization, cell-cell communication, and regenerative processes (Machałowski & Jesionowski 2021; Fall et al 2023). Large differences in the number of circulating haemocytes in the haemolymph are seen when mollusk species are compared (Machałowski & Jesionowski 2021), and such differences depend on both environmental and other significant factors, including the age of the animal, parasite infection, water content in the haemolymph, general condition of the organism, diet, and seasonal state (active or hibernating) (Adamowicz & Bolaczek 2003). Abalone fed with MBM as a protein source showed a higher THC and SL, although the difference was not statistically significant compared to other treatments. The MBM protein source is likely to be efficiently absorbed and utilized to produce a greater number of haemocytes and promote shell development. Haemocytes play a critical role in various physiological functions such as wound healing, mineral ion and nutrient transportation, calcium-rich deposit and calcium-binding protein transport, metabolite excretion, osmoregulation, and gas exchange (Hong et al 2019). However, some of these functions lead to a considerable reduction in the number of circulating hemocytes, which must be replenished by hematopoietic processes. Therefore, the regulation of hematopoietic processes that control the proliferation and differentiation of these specialized cells is essential not only for immune responses but also for the survival of the animal as a whole (Pila et al 2016). Subsequent years of research unveiled the pivotal role of hemocytes in biomineralization (Machałowski & Jesionowski 2021). The evaluation of water quality parameters during the treatment period revealed that the conditions in the rearing tanks were within the optimal range. As a result, environmental factors did not influence feeding behavior or food intake by the abalone.

Conclusions. We clarified that the inclusion of animal protein sources, including fish meal, shrimp head meal and meat and bone meal, either singularly or in combination, in the diet did not affect weight and shell performances, as well as total haemocyte count of abalone *Haliotis squamata*. However, the combination of fish meal, shrimp head meal and meat and bone meal is recommended to achieve the maximum growth performance of abalone. This study will have important implications for the abalone farming industry. By investigating the effects of animal protein sources on the growth performance and immune response of the tropical abalone, we hope to provide valuable information for the aquaculture industry and contribute to the sustainable production of this species. In addition, this study may contribute to our understanding of the nutritional requirements of abalone and inform future research in this area.

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Conflict of interest. The authors declare that there is no conflict of interest.

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Authors:
Asep Ridwanudin, Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia, e-mail: ase_rid@yahoo.com
Dien Arista Anggorowati, Research Center for Marine and Land Bioindustry, National Research and Innovation Agency (BRIN), Lombok, Indonesia, e-mail: anggorowati.da@gmail.com
Idham Sumarto Pratama, Research Center for Marine and Land Bioindustry, National Research and Innovation Agency (BRIN), Lombok, Indonesia, e-mail: idham.sumarto@gmail.com
Ibnu Sahidhir, Research Center for Fishery, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia, e-mail: ibnusahidhir@yahoo.com
Arsyad Sujangka, Research Center for Fishery, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia, e-mail: ar.sujangka@gmail.com
Lusi Herawati Suryaningrum, Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia, e-mail: lusi006@brin.go.id; lusihera@gmail.com
Sulasy Rohmy, Research Center for Fishery, National Research and Innovation Agency (BRIN), Cibinong, Bogor, Indonesia, e-mail: sulasy_rohmy@yahoo.com

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