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Scientific Article

Inclusion of protein hydrolysate extracted from bycatch in the diet of the Malaysian giant prawn: effects on physiology

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ABSTRACT

Bycatch refers to all the animals caught with species of economic interest. These economically unattractive animals are later discarded at sea. Protein hydrolysate with antioxidant potential was extracted by our research group from two main fish species (*Paralonchurus brasiliensis* and *Micropogonias furnieri*) of the shrimp fishing bycatch fauna of the São Paulo coast, Brazil. This study tested the inclusion of different concentrations of hydrolysate (0.0, 2.5, 5.0, and 10.0%) in the diet of *Macrobrachium rosenbergii*. Survival, growth, and physiological processes (ingestion, defecation, hepatosomatic index, O:N ratio, metabolism, and ammonia excretion) were assessed. The inclusion of hydrolysate did not affect crucial parameters for aquaculture, such as survival, growth (about 2% in relation to initial biomass), intake, and mechanisms related to obtaining and using energy (hepatosomatic index and protein as main type of energy substrate oxidized). Metabolism and nitrogen excretion were reduced (~70%) in all treatments with hydrolysate, suggesting lower energy requirements for digestion and absorption of nutrients, as well as the optimization of animal protein use. We recommend the inclusion of 2.5% hydrolysate for future work to test the antioxidant capacity of hydrolysate in *M. rosenbergii*. This concentration level does not alter important physiological parameters and is cost-effective.

Keywords: Shrimp farming; *Macrobrachium*; Accompanying fauna; Bioprospecting marine.

Inclusão de hidrolisado proteico extraído do bycatch na dieta do camarão-gigante-da-malásia: efeitos na fisiologia

RESUMO

Bycatch refere-se a todos os animais capturados com espécies de interesse econômico. Esses animais economicamente pouco atrativos são posteriormente descartados no mar. Nosso grupo de pesquisa extraiu um hidrolisado proteico com potencial antioxidante das duas principais espécies de peixes (*Paralonchurus brasiliensis* e *Micropogonias furnieri*) da fauna acompanhante da pesca do camarão do litoral de São Paulo, Brasil. Este estudo testou a inclusão de diferentes concentrações de hidrolisado (0,0, 2,5, 5,0 e 10,0%) na dieta de *Macrobrachium rosenbergii*. Sobrevivência, crescimento e processos fisiológicos (ingestão, defecação, índice hepatossomático, razão O:N, metabolismo e excreção de amônia) foram avaliados. A inclusão de hidrolisado não afetou parâmetros cruciais para a aquicultura, como sobrevivência, crescimento (2% em relação à biomassa inicial), ingestão e mecanismos relacionados à obtenção e ao uso de energia (índice hepatossomático e proteína como principal tipo de substrato energético oxidado). O metabolismo e a excreção de nitrogênio foram reduzidos (~70%) em todos os tratamentos com hidrolisado, sugerindo menores requisitos de energia para digestão e absorção de nutrientes, bem como a otimização do uso de proteína animal. Recomendamos a inclusão de 2,5% de hidrolisado para trabalhos futuros para testar a capacidade antioxidante do hidrolisado em *M. rosenbergii.* Esse nível de concentração não altera parâmetros fisiológicos importantes e possui melhor custo-benefício.

Palavras-chave: Carcinicultura; *Macrobrachium*; Fauna acompanhante; Bioprospecção marinha.

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INTRODUCTION

Bycatch and *accompanying fauna* are terms used for species that are fished along with others of economic interest. Bycatch is discarded mainly because it has no economic value and comprises diverse animals (Werner et al., 2015). This process of accidental capture can cause ecological changes in the food chain because of decreased marine diversity and financial losses for fishermen (Lewison et al., 2004; Thorne et al., 2019). Shrimp trawling is the main contributor to *bycatch* discards because of its unselective and aggressive nature (Bochini et al., 2019). A possible alternative to reducing the effort and environmental impact of fishing is the use of bycatch as a promising alternative source of bioactive compounds and nutraceuticals in animal feed (Camargo et al., 2021). One important area to research for these biomolecules is marine bioprospecting.

Marine bioprospecting involves the search for biomolecules and biologically active compounds of the fauna and flora, which could be used in the development of products for various sectors, such as aquaculture, pharmaceutical industry, and biotechnology (Jain & Tailor, 2020). On the southeast coast of Brazil, shrimp fishing mainly targets the seabob shrimp (*Xiphopenaeus kroyeri*) and pink shrimp species (*Penaeus brasiliensis* and *Penaeus paulensis*) (Perroca et al., 2022). Our research group attempted to identify the main bycatch species on the São Paulo coast, Brazil, and conducted bioprospecting for biologically active molecules (Camargo et al., 2021).

As a result of this study, protein hydrolysate was extracted from the muscle and skin of the two most abundant bycatch fish species (*Paralonchurus brasiliensis* and *Micropogonias furnieri*). This protein hydrolysate has a high degree of hydrolysis and can be used as a nutritional and nutraceutical supplement. Camargo et al. (2021) demonstrated the ability of this protein hydrolysate to sequester peroxyl radicals and peptides to act as electron donors, stabilizing free radicals, and, therefore, having antioxidant potential. Protein hydrolysates, when added to the diet of farmed animals, can play an antioxidant role, promote the animals' increased growth, and improve their immune and intestinal parameters (Hlordzi et al., 2022; Suma et al., 2023; Wei et al., 2023). Adding protein hydrolysates with antioxidant properties to the diet can slow down the oxidation of macronutrients and cellular components (Aklakur, 2018).

The Malaysian giant prawn, *Macrobrachium rosenbergii*, is one of the five most cultivated crustacean species in the world (FAO, 2022). According to the Food and Agriculture Organization (FAO, 2022), approximately 294 thousand tons of this species were produced in 2020, which is equivalent to 2.6% of the world's farmed crustaceans. Commercial feed is not available for this species, but commercial feed for marine shrimp is often used, which is expensive for producers (Nik Sin et al., 2021). One striking feature of *M. rosenbergii* biology is the presence of three morphotypes among males with different hierarchical roles in the ponds: blue males (BC), small males (SM), and orange clawed male (OC), which have a higher growth rate compared to the others (Aziz et al., 2018; Xue et al., 2021).

In this study, we evaluated the effects of different concentrations (0.0, 2.5, 5.0, and 10.0%) of protein hydrolysate extracted from the bycatch by Camargo et al. (2021) and included in the diet on the survival, growth, and various physiological processes (ingestion, defecation, hepatosomatic index, metabolism, and ammonia excretion) of *M. rosenbergii*. This study is important because, before assessing the antioxidant effects of protein hydrolysate, it is essential to examine whether its inclusion in the diet affects key aspects of the animal's physiology.

MATERIAL AND METHODS

Collection and acclimation of animals in the laboratory

Male adults of the OC morphotype of *M. rosenbergii* (approximately 10 g) were found in ponds at the Aquaculture Center of Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Jaboticabal, SP, Brazil (21°14'19.7"S 48°17'36.4"W). All animals used in experiments were in the intermolt phase. The animals were transported in boxes containing water from the collection site and constant aeration to the Sustainable Aquaculture Laboratory, located at the UNESP, campus of the São Paulo State coast (23°58'16.1"S 46°23'40.5"W). The animals were acclimatized to laboratory conditions for four days in individual aquariums containing 20 L of fresh water under constant aeration and natural photoperiod.

Experimental design

The shrimp were distributed into four groups, with seven replicates per treatment (n = 7/group), one animal per aquarium (20 L). They were fed a diet (Table 1) produced by the company Camarões Brasil with different concentrations of hydrolysate (0.0, 2.5, 5.0, or 10.0%). The animals were fed an amount equivalent to 5% of their individual biomass (Chen & Chen, 2003), given once a day (8 a.m.), and removed after 3 hours (11 a.m.), over a span of 34 days (four days of acclimation and 30 days of experimentation). Partial water exchanges were conducted as needed, with complete water changes occurring every five days.

Experimental diets

The diets were formulated based on fish meal, corn, and wheat bran, and 31% of crude protein (Table 1). Diet processing

Table 1. Ingredients and proximate composition of the experimental diets (%).

	Ingredients			
	H0%	**H2.5%**	**H5%**	**H10%**
Fish meal	30,00	26,50	22,00	16,00
Poultry viscera meal	8,00	7,60	8,50	7,00
Wheat bran	19,00	19,00	19,00	19,00
Corn	25,00	25,00	25,00	25,00
Wheat flour	10,50	11,00	11,50	12,00
Cellulose	4,00	4,50	5,00	6,50
Dicalcium phosphate	2,00	2,20	2,25	2,50
Premix	0,50	0,50	0,50	0,50
Hydrolysed	0.00	2,50	5,00	10,00
Bromatological composition				
Dry mass %	97,13	97,19	96,80	96,70
Crude protein %	31,93	30,89	30,78	30,88
Ethereal extract %	6,10	5,42	5,26	4,65
Ash %	6,19	6,40	6,10	6,26
Crude Fiber %	2,17	2,12	2,17	1,95
Gross energy (kcal•kg^{-1})	4.158,95	4.156,27	4.135,91	4.161,61
Energy:Protein	13,02	13,45	13,43	13,47
Calcium %	2,38	2,55	2,58	2,87
Phosphorus %	1,13	1,20	1,22	1,35

H0%: control; H2.5%: 2.5% hydrolysate; H5%: 5% hydrolysate; H10%: 10% hydrolysate.

was conducted by Signor et al. (2011). The diets were oven dried at 55°C for 24 h and fractionated to obtain granules with diameters of about 1 mm. Four types of diets were prepared with different concentrations of protein hydrolysate (H0; H2.5; H5 and H10%). The calculations and adjustments for formulating the diet were carried out in collaboration with the company Camarões Brasil.

Protein hydrolysates

The hydrolysate used was the same as that produced by Camargo et al. (2021). The samples were obtained in the region of Ubatuba, SP, Brazil, packed in plastic bags and kept in a freezer (-20°C). For enzymatic hydrolysis, the samples were thawed in a refrigerator (4°C; 24 h), then homogenized in a blender, with two volumes of distilled water (w/v), followed by heating (80°C; 20 min), to inactivate endogenous enzymes. The hydrolysis was started using the enzyme Alcalase 2.4 L (Novozymes, Bagsvaerd, Denmark) at pH 8 and the enzyme Protamex (Sigma Aldrich, MO, United States of America) at pH 7, with a temperature of 50°C for both. The enzyme/substrate ratio was at 2% (w/w) for both enzymes. The hydrolysis processes were performed to reach the maximum degree of hydrolysis (DH), becoming constant after 5 h of hydrolysis, and the reaction was terminated by deactivation of the enzyme by heating (80°C for 20 min). Then, samples were centrifuged (20 min at 16,300 × g), and supernatants were frozen (-80°C). The composition of the hydrolysates varied with average values of moisture content (78.8 to 79.6%), crude protein levels (16.7 and 16.8%), ash content (1.8 and 2.0%) and lipids (0.3 to 0.5%).

Evaluation of the effects of different concentrations of hydrolysates in the diet on the physiology of Macrobrachium rosenbergii

Survival

The survival of the animals was checked during the 30 days of experiments three times a day: at 8 a.m., 2 p.m. and 8 p.m. (Yu et al., 2023).

Ingestion, egestion and growth rates

Ingestion rate was calculated by the difference between the mass of the food offered and the mass of the uneaten food (Augusto & Valenti, 2016). Any remaining uneaten food was siphoned out of the aquariums after 3 h, dried in a 60°C oven for 48 h, and weighed (Mettler Toledo, 1 µg). The leaching rate (Soares et al., 2021) was calculated in control aquariums without animals. Feces were siphoned from the tanks throughout the day, placed on plates, dried in a 60°C oven for 48 h, and weighed using analytical balance (Metler Toledo, 1 µg). The growth was assessed by weighing the animals (Marte, AS 2000C) after the acclimation period and at the end of the 30-day experiment.

Metabolism and ammonia excretion

Oxygen consumption was evaluated in closed respirometric chambers equipped with an oximeter and monitor (YSI models 53 and 5905, respectively) (Augusto & Masui, 2014; Augusto et al., 2020). The animals were starved for 24 h to reduce the calorigenic effect of food before being individually transferred to the respirometry chambers. A 30-min acclimation period in acrylic chambers with appropriately sized water and aeration was crucial to minimize animal stress. Initial oxygen consumption measurements were taken after acclimation, with final measurements 4 h later. Control chambers without animals were used to calculate oxygen consumption. Following oxygen consumption determination, the animals were killed by freezing, weighed for wet mass, then dried in an oven for 48 h, and weighed again for dry mass. Ammonia excretion was measured from water samples obtained from the respirometric chambers at the end of each oxygen consumption experiment. Ammonia concentration was determined using colorimetry (Hach, 2500 spectrophotometer) (Koroleff, 1983).

Hepatosomatic index and O:N atomic ratio

The hepatopancreas of euthanized shrimp was dissected, weighed, dried, and reweighed. The hepatosomatic index was assessed using Eq. 1 (Yu et al., 2023):

$$\text{Hepatosomatic index (\%)} = 100 \times \text{Wet hepatopancreas weight (g)} / \text{Wet body weight (g)} \quad (1)$$

The O:N atomic ratio was calculated by dividing the oxygen consumed (mol) by the ammonia excreted (mol) (Mayzaud & Conover, 1988). O:N ratios between 3 and 16 suggest protein oxidation, while values between 50 and 60 indicate a mix of proteins and lipids, and ratios above 60 suggest lipid dominance.

Statistical analysis

Variables were subjected to Kolmogorov–Smirnov and Levene's tests to evaluate normality and homoscedasticity, respectively. Variables that adhered to normality and homoscedasticity were subjected to one-way analysis of variance. Significant differences were further analyzed using the Student–Newman–Keuls' test. Non-normally distributed data underwent the Kruskal-Wallis' test. A significance level of $p \leq 0.05$ was used for all analyses, conducted using the SigmaStat 3.5 program.

RESULTS

Survival

Animals from all treatments showed no mortality during the experimental period, with a 100% survival rate.

Table 2. Mean daily rates of ingestion (C), growth (P), defecation/ingestion relation (F/C), hepatosomatic index (HSI), and atomic ratio (O:N) in *Macrobrachium rosenbergii* fed different concentrations of protein hydrolysate during 30 days. Values are mean± standard error (n = 7)*.

	H0%	H2.5%	H5%	H10%
C/WWi (%)	1.65 ± 0.20	1.70 ± 0.05	1.70 ± 0.04	1.56 ± 0.04
C (mg WW·day^{-1})	170 ± 0.03	210 ± 0.02	190 ± 0.01	210 ± 0.01
P/WWi (%)	1.60 ± 0.72	2.16 ± 1.08	0.99 ± 0.67	3.31 ± 1.60
F/C (%)	4.17 ± 0.53^{a}	4.21 ± 0.14^{a}	3.45 ± 0.31^{ab}	2.51 ± 0.21^{b}
HSI	6.07 ± 0.52	6.84 ± 0.63	5.77 ± 0.72	5.51 ± 0.39
O:N	7.88 ± 2.03	10.86 ± 2.73	6.03 ± 1.74	10.21 ± 1.98

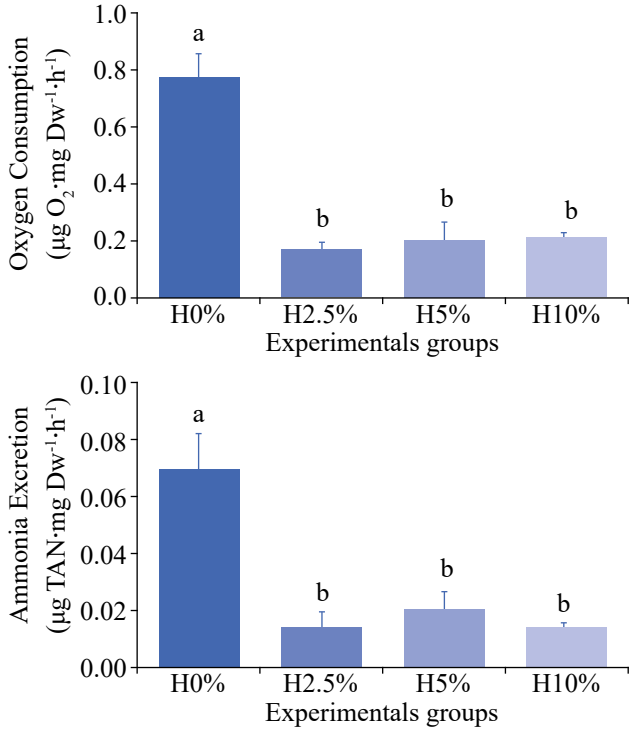
*Values with different letters in the same line differ statistically; WW: wet weight; WWi: initial wet weight; H0%: control; H2.5%: 2.5% hydrolysate; H5%: 5% hydrolysate; H10%: 10% hydrolysate.

Ingestion, growth and egestion rates in Macrobrachium rosenbergii

The daily rates of ingestion, growth, egestion, respiration, and excretion in *M. rosenbergii* fed with a diet containing different concentrations of hydrolysate are presented in Table 2. The ingestion and growth rates of animals fed with a diet containing hydrolysate were similar to those of the control animals. A statistically unproven tendency towards increased growth rate was observed in the H10% treatment (p = 0.54). The egestion rate (F/C%) of animals fed with diet containing 10% protein hydrolysate was reduced by approximately 40% compared to the control animals and those fed with diet containing 2.5% hydrolysate.

Hepatosomatic index and O:N atomic ratio

The hepatosomatic index was unaffected by the inclusion of hydrolysate in the diet (Table 2). Atomic ratio calculations of O:N suggest that the animals primarily oxidize proteins, regardless of the presence of hydrolysate in the diet (Table 2).



*Different letters indicate statistical differences; H0%: control; H2.5%: 2.5% hydrolysate; H5%: 5% hydrolysate; H10%: 10% hydrolysate.

Figure 1. Oxygen consumption (µg O2 mg·DW^{-1}·h^{-1}) and ammonia excretion (µg TAN·mg DW^{-1}·h^{-1}) in *Macrobrachium rosenbergii* fed with diet containing different concentrations of protein hydrolysate*.

Metabolism and ammonia excretion

Oxygen consumption and ammonia excretion are shown in Fig. 1. Oxygen consumption is reduced by up to 70% in animals fed with a hydrolysate-containing diet, irrespective of the inclusion concentration. Ammonia excretion decreased by 74% in animals fed a diet containing protein hydrolysate compared to the control animals (H0%).

DISCUSSION

The present study revealed that the inclusion of protein hydrolysate with antioxidant potential in the diet of *M. rosenbergii* did not alter important parameters for aquaculture, such as survival, ingestion rate, growth, hepatosomatic index, and energy substrate utilization. However, it did lead to a decrease in certain physiological parameters (defecation rate, oxygen consumption, and ammonia excretion).

Ingestion rate and growth

Protein hydrolysates are used in aquaculture because they can optimize growth (Gunathilaka et al., 2020), the immune system (Suratip et al., 2023), and digestibility; have antioxidant capacity (Gunathilaka et al., 2021); and help reduce the use of fishmeal (Gao et al., 2021). However, some protein hydrolysates can deter growth or alter the palatability and attractiveness of feed for animals (Alves et al., 2020; Ha et al., 2022). Therefore, they have to be appropriately evaluated before introduction into the animals' diet (Soares et al., 2020; Zhou et al., 2016).

This study showed no difference in intake rates (approximately 200 mg WW·day^{-1}) and growth (2%) between animals fed control feed and feed containing protein hydrolysate extracted from bycatch, which is a positive result. The observed ingestion rate was similar to shrimp *Macrobrachium amazonicum* in cinnamon claw morphotype (198 mg DM·day^{-1}) (Augusto & Valenti, 2016), however in *Penaeus vannamei* the feed intake was higher (410 mg DM·day^{-1}) (Pallaoro et al., 2016), although this result may suggest that is a species-specific response. The ingestion rate, as well as diet type, temperature, and salinity, could influence the growth rate of crustaceans (Ibrahim et al., 2023; Wangari et al., 2021).

In the present study, the animals grew by 2%. No change was observed between control animals and those fed a diet containing hydrolysate, despite a tendency toward an increase in the H10% treatment. Studies show that *M. rosenbergii* males can weigh between 30 and 180 g and that this variation is mainly due to the evaluated morphotype (Karplus et al., 2005; Ranjeet et al., 2002). In our experiments, all the animals were OC, the morphotype

with the highest growth rate among the population (Aziz et al., 2018; Ibrahim et al., 2023). Augusto & Valenti (2016) identified that the growth of *M. amazonicum* varied between 2 to 16%. They attributed this result to the type of morphotype studied and the differential energy channeled into this function.

Egestion rates

The defecation rate of *M. rosenbergii* decreased by 40% when the shrimp were fed a diet containing 10% protein hydrolysate, but it remained unchanged for the other concentrations. This result may be related to an improvement in nutrient absorption by the intestinal epithelium or the retention of feces in the digestive tract of the animals in the H10% treatment. The optimization of nutrient absorption may be associated with the composition of the hydrolysate, which may be a source of smaller peptides and free amino acids, enabling better absorption in the intestinal epithelium (Siddik et al., 2021; Soares et al., 2020). In *P. vannamei*, tests with tuna and shrimp hydrolysates showed better digestibility of the feed when compared to the use of squid liver meal. This was probably because of substantial low-molecular-weight dietary compounds, peptides, and soluble nitrogen, as well as the possible increase in the activity of digestive enzymes (Gunathilaka et al., 2022). In the marine fish *Paralichthys olivaceus*, the addition of hydrolysates from tilapia and shrimp led to improvements in the morphology of the intestinal epithelium followed by an increase in growth rate (Gunathilaka et al., 2020). The hypothesis of feces retention in the digestive tract of *M. rosenbergii* is less likely because the lack of nutrients, water, and ions could affect the growth even the survival of the animals.

Hepatosomatic index and energy substrate

The hepatopancreas of decapods stores energy, secretes digestive enzymes, and absorbs nutrients (Vogt et al., 2019). Changes in this organ occur when the demand for stored energy increases and/or when the diet and intake rate are insufficient (He et al., 2022; Ramaglia et al., 2018). The present study showed no change in the hepatosomatic index of *M. rosenbergii*. This is a positive result for the use of protein hydrolysate in the species' diet and indicates that stored energy reserves remained unchanged. Additionally, the atomic O:N ratio indicated that the species mainly oxidizes proteins as an energy source, regardless of the treatment. The atomic O:N ratio can be used to assess diet quality and depends on protein content, ingestion rate, digestibility, and environmental factors (Pillai & Diwan, 2002; Rajaram et al., 2022). Crustaceans have a significant capacity to digest proteins of animal origin; in certain species of shrimp, the digestibility of amino acids can exceed 92% (Cruz-Suárez et al., 2009). The use of protein as an energy substrate has already been reported in *P. vannamei* fed fresh fish (Coelho et al., 2019) and in *M. amazonicum* fed commercial feed (Augusto & Masui, 2014).

Metabolism and excretion of ammonia

Diet can affect oxygen consumption mainly because of changes in the catabolism and anabolism of nutrients (González-Penã & Moreira, 2003). The oxygen consumption of *M. rosenbergii* fed a control diet (0% hydrolysate) reached levels similar to those shown in other studies with shrimp of the *Macrobrachium* genus (Mantoan et al., 2021; Moreira et al., 1983; Wang et al., 2003). Although the inclusion of protein hydrolysate reduced the oxygen consumption of *M. rosenbergii*, this reduction did not affect important parameters for aquaculture, such as survival, growth, and energy reserves stored in the hepatopancreas.

It is possible that the reduction in oxygen consumption observed in *M. rosenbergii* is due to a lower energy requirement for digestion and absorption of nutrients. In the marine shrimp *Penaeus monodon*, the lower oxygen consumption observed in animals fed with feed compared to those fed with fresh diet was attributed to the fact that feed is more easily digestible (Rajaram et al., 2022). Changes in oxygen consumption could affect ATP-dependent physiological mechanisms. However, based on the parameters evaluated here, we did not observe such effect.

Ammonia is the main nitrogenous excreta of crustaceans from the catabolism of free amino acids that are in excess in the diet, being used as osmolytes in osmoregulation or as an energy substrate in cellular respiration. Here we observed that ammonia excretion decreased when *M. rosenbergii* was fed protein hydrolysate. According to Rajaram et al. (2022), low ammonia excretion is a physiological indication of the favorable nutritional quality of shrimp diet. It is possible that the inclusion of protein hydrolysate in the feed optimized the use of proteins by the animals or improved the quality of proteins for this species, and, therefore, digestibility. Additionally, the results can be considered positive for the cultivation of *M. rosenbergii* because nitrogen excretion involves energy expenditure for the species (Augusto & Masui, 2014; Augusto et al., 2020) and the increase in NH_3 in the effluent from shrimp farming (Ahmad et al., 2021).

CONCLUSIONS

We conclude that the inclusion of protein hydrolysate derived from bycatch in the diet of *M. rosenbergii* does not affect important cultivation parameters, suggesting the potential for its use in the species' diet. Additionally, the reduction in

oxygen consumption and ammonia excretion suggests that the addition of the hydrolysate optimized the diet. Future studies should assess the antioxidant role of this protein hydrolysate extracted from bycatch under stress conditions imposed during the cultivation of *M. rosenbergii*. In this regard, we propose the inclusion of 2.5% hydrolysate as promising because it requires a lower concentration of hydrolysate, optimizing costs.

CONFLICT OF INTEREST

Nothing to declare.

DATA AVAILABILITY STATEMENT

All data were generated or analyzed in this study.

AUTHORS' CONTRIBUTION

Conceptualization: Louzã, A. ; Camargo, T. R.; Rodrigues, C. G.; Augusto, A.; **Data curation:** Louzã, A. C.; **Formal analysis:** Louzã, A. C.; Rodrigues, C. G.; Borges, E. P.; Ramaglia, A. C.; **Funding acquisition:** Augusto, A.; **Supervision:** Augusto, A.; **Writing – original draft:** Louzã, A. C.; Augusto, A.; **Writing – review & editing.:** Louzã, A. C; Borges, E. P.; Ramaglia, A. C.; Augusto, A.; **Final approval:** Louzã, A. C.

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