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Effect of dietary chitosan on the growth, survival, and prophenoloxidase of male freshwater prawns *Cryphiops (Cryphiops) caementarius*

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ABSTRACT

This research aimed to evaluate the effect of dietary chitosan on the growth, survival, and prophenoloxidase of male freshwater prawns *Cryphiops (Cryphiops) caementarius*. Seventy-two prawns were acclimated and allocated into four dietary treatments: a control group and three experimental diets supplemented with 1, 2, and 4 g chitosan/kg of diet. Growth parameters were similar ($P > 0.05$) among dietary treatments with a higher weight gain (21%) observed in the 2 g of chitosan/kg diet. Prawns' survival (55–72%) was similar ($P > 0.05$) among dietary treatments, although it was biased by deaths caused by incomplete ecdysis syndrome. The highest prophenoloxidase levels in hemocytes (66.28%) were those from the prawns fed on 1 g of chitosan/kg of diet. On the other hand, hemocytes with lower prophenoloxidase content (12.52% and 19.36%) were observed in prawns fed on 2 and 4 g of chitosan/kg of diet, respectively, however it is likely that their prophenoloxidase levels were negatively affected by the high concentration of nitrites ($\geq$ 0.33 mg/L) detected in the culture water. This study is the first to demonstrate that low concentrations of chitosan in the diet of *C. (C.) caementarius* increase prophenoloxidase levels and thus it could be used in the aquaculture practice of this resource to stimulate its immune system.

1. Introduction

Chitin is an important polysaccharide and the most abundant on the planet after cellulose and is present in the exoskeleton cuticle of arthropods and insects (Dassanayake et al., 2018). Deacetylation of chitin with an alkali, results in chitosan (Ahyat et al., 2017), whose higher solubility determines its quality (Renuka et al., 2019). Chitosan is an edible, biocompatible, biodegradable, non-toxic, and safe natural polymeric material (Wang et al., 2017) that promotes growth and health in fish (El-Naggar et al., 2021; Mohan et al., 2023; Stanek et al., 2023) and crustaceans (Zhu et al., 2010; Niu et al., 2013a; Mohan et al., 2023). For instance, dietary chitosan was reported to increase growth and maintain high survival rates in the shrimps *Penaeus vannamei* (Niu et al., 2011) and *P. monodon* (Niu et al., 2013b). In the crayfish *Procambarus clarkii* chitosan was found to produce an increase in the total number of hemocytes and greater activity of prophenoloxidase and dismutase oxidase (Zhu et al., 2010). In *P. monodon*, chitosan also displays antioxidant activity and improves the ability to resist stress due to low oxygen content (Niu et al., 2013b). Furthermore, Zhu et al. (2010) reported antibacterial and antimycotic properties of dietary chitosan in *P. clarkii*.

In crustaceans, the nonspecific or innate immune response is the first line of defense against pathogens (Kumar et al., 2017), where hemocytes in circulating hemolymph are directly involved in cellular responses, such as recognition, phagocytosis, coagulation, and encapsulation (Liu et al., 2021; Kumar et al., 2023). Crustaceans possess granular, semigranular, and hyaline hemocytes (Söderhäll, 2016). Prophenoloxidase (proPO) is localized in the granules of the hemocytes of crustaceans (Johansson and Söderhäll, 1985) and its main function is melanization to prevent the proliferation of pathogens (Boonchuen et al., 2021; Kumar et al., 2023), which constitutes an important component of the cellular defense reaction of crustaceans.

The freshwater prawn *Cryphiops (Cryphiops) caementarius* Molina, 1782 is the only prawn species native to the coast of Peru that has high value and commercial importance (Pinazo et al., 2021), and where the highest population density is found in the Ocoña, Majes-Camaná, and Tambo rivers (Wasiw and Yépez, 2015). The extraction of this resource

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is oriented to the domestic market only and during 2019 reached 1124 t (PRODUCE, 2020).

In Peru, the cultivation of *C. (C.) caementarius* is performed by local families and the used diets still need to be improved. Besides, nutrition research in this prawn species is recent and mostly aimed at using food inputs to increase growth and maintain high survival with the use of biological silage (Terrones and Reyes, 2018), soy lecithin (Acosta et al., 2018), common salt (Ramírez et al., 2018) and zeolite (Senmache and Reyes, 2020). However, the use of chitosan in the diet of *C. (C.) caementarius* has not been yet investigated, despite the results from past studies reporting that it stimulates growth and immune response in other crustacean species (Zhu et al., 2010; Niu et al., 2013a; Mohan et al., 2023). Therefore, the present research aimed to evaluate the effect of different concentrations of dietary chitosan on the growth, survival, and prophenoloxidase levels of hemocytes of male freshwater prawn *C. (C.) caementarius*.

2. Material and methods

2.1. Formulation and preparation of diets

The basal diet (Table 1) was previously formulated by Reyes-Avalos (2016) with modifications in the level of common salt (Ramírez et al., 2018), zeolite (Senmache and Reyes, 2020), and paprika flour (Díaz et al., 2020). The soybeans and corn were crushed in a manual mill. The soybean meal was roasted until it turned into a mustard color. The paprika fruit was deveined, washed, dried in an oven at 60 °C for 3 h, and then pulverized in a mill. All the flours were sieved (250 μm) and mixed with zeolite, vitamin complex, and sorbic acid. The oils, soy lecithin, and Butylated hydroxytoluene were diluted in a water bath (40 °C) and then incorporated into the mixture. The molasses and common salt were kept in separate containers and diluted with hot water before they were added to the mixture. The ingredients were mixed for 30 min and then kneaded for 40 min with hot water until a compact dough texture that did not stick when pressed by hand was achieved. The filaments (3 mm Ø) of the food were obtained with a manual press, then they were dried in an oven (60 °C for 24 h), cut (5 mm in length), and stored in airtight bags until used.

The experimental diets were prepared from the basal diet (control diet) whose dry pellets were crushed in a manual mill to be supplemented with 1, 2, and 4 g of chitosan/kg of diet. Chitosan was of low molecular weight (Cat. 448869, 76% degree of deacetylation, Sigma-Aldrich) (Table 2). Processing was performed as in the basal diet but starting with kneading.

Table 1
Ingredients of basal diet composition (dry basis).

Ingredients	g/kg
Fish meal	300.00
Soybean meal	210.00
Corn flour	154.50
Paprika flour	2.50
Fish oil	20.00
Soybean oil	5.00
Corn oil	5.00
Soy lecithin[a]	10.00
Rice powder	200.00
Molasses	27.00
Zeolite	40.00
Common salt	20.00
Vitamins and minerals[b]	3.00
Butylated hydroxytoluene	1.00
Sorbic acid	2.00

[a] Purified soy lecithin (1200 mg soft capsules with phosphatides ≥ 250 mg).

[b] Each 100 g contains Vitamin B1 (Thiamine) 500 mg, Vitamin B2 (Riboflavin) 1200 mg, Vitamin B6 (Pyridoxine) 900 mg, Vitamin B12 (Cyanocobalamin) 1000 μg, Biotin 2 mg, Nicotinamide 2000 mg, Calcium pantothenate 1000 mg, Sodium chloride 20000 mg, Potassium chloride 8000 mg, Magnesium sulfate 1200 mg.

2.2. Proximal chemical analysis

The chemical analysis of the diets (Table 2) was determined at the *Laboratorio Física Químico Ambiental Perú S.A.C.*, according to the Peruvian Technical Standard (NTP, for its acronym in Spanish), which included crude protein (NTP 205.005/79), crude fat (NTP 205.006/80), crude fiber (NTP 205.002/79), and ash (NTP 205.004/79). For carbohydrates, the formula by difference was used: 100 - (% protein + % fat + % fiber + % ash). Diets were analyzed in duplicate.

2.3. Prawn collection and acclimation, and selection

One hundred prawns were collected from the Pativilca River (10°39′48″ S, 77°39′20″ W) (Lima, Peru) in October 2022 with the help of local fishermen, using the manual diving collection method. For transport to laboratory facilities, each prawn was placed in a perforated plastic cup (200 mL), which was conditioned in 50 L containers with water and aeration at a density of 50 prawns per container. Land transport lasted 4.5 h. The collected prawns were acclimated in their respective transport vessels, which were conditioned in three aquariums (60 L) at 20 prawns per aquarium, and they were provided with basal diet for eight days. Species-level identification of the collected prawns was performed with a taxonomic key (Méndez, 1981; DecaNet, 2023). Male individuals were selected based on the presence of the gonopore in each coxopodite of the fifth pair of periopods and the appendix masculina on the second pleopod. The selected male prawns (n = 72) were 11.54 ± 0.45 g total wet weight, had complete cephalothoracic appendages, and showed no evidence of lacerations on the body or chelipeds.

2.4. Experimental system, stocked and feeding

The experimental cultivation system consisted of 12 aquariums (0.60 ×0.30 ×0.35 m, 55 L). Each aquarium included six circular containers (19 cm diameter, and 8 cm high, 284 cm^2) arranged in two groups of three levels. The prawns were cultured individually in each cultivation container (6 prawns/aquarium = 32 prawns/m^2) and distributed randomly. Each dietary treatment was carried out in

Table 2
Supplementation and proximal composition of diets with different levels of chitosan.

	Chitosan (g/kg of diet)			
	0 (Control)	1	2	4
Dietary supplementation				
Diet basal (g)	1000.00	999.00	998.00	996.00
Chitosan (g)	0.00	1.00	2.00	4.00
Proximate composition	33.02 ± 0.15[a]	35.18 ± 0.15[b]	37.63 ± 0.15[c]	37.21 ± 0.15[c]
Crude protein (%)	6.88 ± 0.30[a]	7.12 ± 0.30[ab]	7.86 ± 0.30[c]	7.54 ± 0.30[ab]
Crude fat (%)	2.61 ± 0.15[a]	2.40 ± 0.15[a]	2.17 ± 0.15[a]	2.34 ± 0.15[a]
Crude fiber (%)	10.29 ± 0.10[c]	9.94 ± 0.10[b]	9.15 ± 0.10[a]	9.28 ± 0.10[a]
Ash (%)	47.20 ± 0.71[c]	45.35 ± 0.71[bc]	43.20 ± 0.70[a]	43.64 ± 0.70[ab]
Carbohydrates (%)	6.87 ± 0.41[b]	6.37 ± 0.37[ab]	5.51 ± 0.30[a]	5.79 ± 0.32[a]
Carbohydrates/Fats				

Data are expressed as mean ± standard deviation (n = 2). Data with different superscript letters in the same row indicate significant differences ($P < 0.05$).

triplicate. The initial feeding level was 4% wet weight per day, followed by a decrease of 1% each month. The food was distributed twice a day (40% at 08:00 h and 60% at 18:00 h) during the 90-day experiment.

2.5. Growth and survival

The total wet weight (g) of each prawn was obtained on Adam® AQT600 digital balance (± 0.1 g), and weight gained (WG), absolute growth rate (AGR), specific growth rate (SGR), survival (S), and protein efficiency rate (PER) were calculated with the following formulas:

$$WG\ (\%) = [(\text{Final weight} - \text{Initial weight}) / \text{Initial weight})] \times 100$$

$$AGR\ (g/day) = [(\text{Final weight} - \text{Initial weight}) / \text{Day cultivation})]$$

$$SGR\ (\%\ \text{weight} / \text{day}) = [(\ln \text{Final weight} - \ln \text{Initial weight}) / \text{Day cultivation})] \times 100$$

$$PER = \text{Weigh gain} / \text{Protein intake}$$

$$S\ (\%) = (\text{Final number prawn} / \text{Initial number prawn}) \times 100$$

Dead or dying prawns were evaluated by visual observation and classified according to substates of incomplete ecdysis syndrome (Reyes, 2018).

2.6. Prophenoloxidase (proPO)

At the end of the diet trial experiment, two prawns in molting state C or Do (Silva et al., 2019) were selected from each treatment and kept fasting for 12 h. Hemolymph was extracted from the fifth pair of periopods with a 1-mL syringe (Insulin Syringe 26 G ½, previously rinsed with 10% sodium citrate) and added to a 1.5 mL Eppendorf tube. Then 20 μL of hemolymph was added to another 1.5 mL Eppendorf tube containing 80 μL of 10% sodium citrate. The proPO of the hemocytes was analyzed in duplicate according to the protocol of Hose et al. (1987) with some modifications: one drop (20 μL) of hemolymph was placed on a slide for a smear, then fixed with 2.5% glutaraldehyde diluted in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4 °C. The cells received three 15-min rinses in phosphate buffer, incubated in 0.1% L-DOPA in phosphate buffer for 18 h at room temperature, and examined by light microscopy. Black-stained granular cells were counted and the percentage of proPO-positive hemocytes was calculated (Kumar et al., 2015).

2.7. Water quality

The water temperature was determined with a digital thermometer (± 0.1 °C), dissolved oxygen with a Hanna HI 9146 Oximeter (± 0.01 mg/L), and the pH with a PH-222 digital pH meter pH-222 (± 0.02 units). Total ammoniacal nitrogen (TAN = NH_3-N + NH_4-N), ammonia nitrogen (NH_3-N), ammonium nitrogen (NH_4-N), nitrite nitrogen (NO_2-N), and nitrates (NO_3-N) were measured with a commercial kit (Hanna) whose reading was made with the Hanna multiparameter photometer (HI 83399) (± 0.01 mg/L).

2.8. Statistical analysis

The data were tested for normality and homoscedasticity using the Shapiro-Wilk and the Levene test, respectively. Differences among treatment means were determined by one-way analysis of variance (ANOVA) and Duncan's post hoc test, with significance set at $P < 0.05$. Data were expressed at the mean with standard deviation. Statistical analyses were performed on SPSS software version 26 for Windows.

3. Results

3.1. Growth

Overall, prawn growth parameters were similar ($P > 0.05$) among the dietary treatments, although the greatest growth (mainly in weight gain) was observed in prawns fed on 2 g of chitosan/kg diet. The PER was similar among treatments (Table 3).

3.2. Survival

Our results showed that prawn survival was similar ($P > 0.05$) among the three experimental diets, although lower survival rates were observed in prawns from the 1 g chitosan/kg treatment group (Table 3). The highest prawn mortality occurred within experimental diets and was due to incomplete ecdysis syndrome (substages E1 and E2).

3.3. proPO in hemocytes

The hemocytes of *C. (C.) caementarius* with the highest proPO content (66.28%) ($P < 0.05$) were obtained with the diet containing 1 g of chitosan/kg. On the other hand, proPO content was low and similar ($P > 0.05$) among the 4 g of chitosan/kg diet (19.36%), 2 g of chitosan/kg diet (12.52%) and followed by the control diet (1.85%) (Fig. 1).

3.4. Water quality

Water quality parameters in the culture system were similar ($P > 0.05$) during the experimental period. Except for the TAN which was higher ($P < 0.05$) in the control diet and lower with 4 g of chitosan/kg diet. Nitrites in the water from control diet and with 2 and 4 g chitosan/kg diet treatments were elevated compared to those from the 1 g of chitosan/kg treatment, but there were no significant differences among dietary treatments, due to the variability of the data (Table 4). An increase in nitrogenous products in all treatments was observed during the last two weeks of experimental culture.

4. Discussion

The effect of chitosan on growth and immune response in aquatic animals is still controversial (Niu et al., 2013a; Niu et al., 2013b). Several studies reported that chitosan improves proteases activity, produces greater growth, and contributes to healthy immune function in crustaceans, although this depends on the species and the concentration

Table 3
Growth and survival parameters of *C. (C.) caementarius* fed on different levels of chitosan in the diet.

Parameters	Chitosan (g/kg of diet)			
	0 (Control)	1	2	4
Initial diet (g)	11.53 ±	11.72 ±	11.82 ±	11.10 ±
Final weight (g)	0.13	0.76	0.58	0.50
Weight gained (%)	13.40 ±	13.68 ±	14.24 ±	13.24 ±
Absolute growth rate (g/	0.48	1.52	1.30	0.59
día)	16.21 ±	16.58 ±	21.03 ±	19.48 ±
Specific growth rate	5.06	7.70	16.90	8.75
(%/día)	0.021 ±	0.022 ±	0.027 ±	0.024 ±
Protein efficiency ratio	0.006	0.011	0.021	0.010
Survival (%)	0.50 ± 0.15	0.45 ±	0.52 ±	0.52 ±
Σ Deaths by incomplete	0.166 ±	0.21	0.40	0.23
ecdysis syndrome	0.049	0.169 ±	0.205 ±	0.196 ±
Σ Deaths from unknown	72.22 ±	0.073	0.154	0.083
causes	9.62	55.56 ±	61.11 ±	72.22 ±
	2	9.62	9.62	9.62
	3	5	6	4
		3	1	1

Data are expressed as mean ± standard deviation (n = 3).

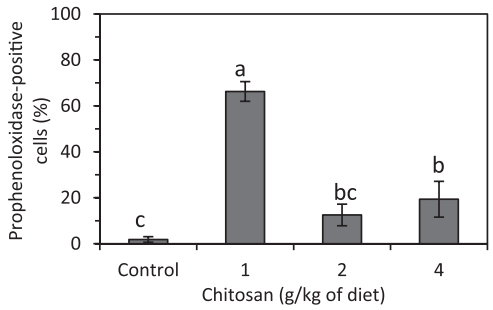

Fig. 1. Prophenoloxidase-positive cells from the hemocytes of *C. (C.) caementarius* fed on dietary chitosan. Columns with different letters indicate significant differences ($P < 0.05$) (n = 2).

Table 4
Quality parameters of *C. (C.) caementarius* culture water fed on different levels of chitosan in the diet.

Parameters	Chitosan (g/kg of diet)			
	0 (Control)	1	2	4
Temperature (°C)	21.63 ± 1.31	21.89 ± 1.28	21.94 ± 1.25	21.86 ± 1.25
Dissolved oxygen (mg/L)	7.45 ± 0.40	7.82 ± 1.52	7.07 ± 1.29	7.64 ± 0.92
Total hardness (mg/L)	165.00 ± 1.41	185.50 ± 4.95	196.50 ± 16.26	190.00 ± 14.14
pH	8.11 ± 0.01	8.22 ± 0.09	8.15 ± 0.08	8.17 ± 0.08
Total ammoniacal nitrogen (mg/L)	1.09 ± 0.05[a]	0.97 ± 0.07[ab]	0.84 ± 0.15[ab]	0.75 ± 0.25[b]
Ammonia nitrogen (mg/L)	0.49 ± 0.03	0.47 ± 0.04	0.41 ± 0.08	0.37 ± 0.12
Ammonium nitrogen (mg/L)	0.33 ± 0.58	0.50 ± 0.04	0.44 ± 0.08	0.39 ± 0.13
Nitrite nitrogen (mg/L)	2.50 ± 1.75	0.17 ± 0.29	1.00 ± 0.50	0.50 ± 0.50
Nitrate nitrogen (mg/L)		4.20 ± 3.45	4.25 ± 0.82	5.27 ± 0.99

Data are expressed as mean ± standard deviation. (n = 3). Data with different superscript letters in the same row indicates significant differences ($P < 0.05$).

of chitosan in the diet (Niu et al., 2013a; Jongyotha et al., 2015; Tseng et al., 2021; Mohan et al., 2023). Contrarily, other crustacean studies showed that chitosan reduces growth and survival (Shiau and Yu, 1998), decreases proPO activity, but increases antioxidant enzymes (Cheng et al., 2021). In the present research, male prawns of *C. (C.) caementarius* fed on chitosan-containing diets did not show significant growth (Table 3) and survival was affected by the appearance of incomplete ecdysis syndrome, however, there was a significant increase in proPO activity in hemocytes (Fig. 1).

Our results showed that the growth of *C. (C.) caementarius* was greater with the 2 g chitosan/kg diet, while the opposite was observed with the 1 and 4 g chitosan/kg diets, although without significant differences among dietary treatments. In postlarvae of *P. vannamei*, the optimal level of chitosan was between 2.13 and 2.67 g/kg of diet, while lower or higher values than this range affect negatively growth and survival (Niu et al. (2011). In contrast, results from other studies on *P. vannamei* (Cheng et al., 2021) and *P. monodon* (Niu et al., 2013a) showed that greater growth is achieved with 4 g of chitosan/kg of diet. The response of *C. (C.) caementarius* to diets with chitosan related to the growth values observed in the present work could be explained by the interference of chitosan on the assimilation of proteins and lipids from diets, as it was reported in other crustacean species including *P. monodon* (Shiau and Yu, 1998). In the present research, all experimental diets contained higher levels of protein than the control diet (Table 2). However, relatively low PER values were observed in the prawns fed on these diets (Table 3). PER is considered an indicator of the efficiency by which the proteins present in the diet are used by the prawn (Goytortúa-Bores et al., 2006). Similarly, despite the high-fat content detected in experimental diets (Table 2), the growth parameters obtained herein might be a consequence of the presence of chitosan in those diets, since it is known that chitosan reduces the absorption of fats and cholesterol from the diets (Aranaz et al., 2009), because it limits micelle formation during lipid digestion by bonding directly with acids, bile salts, fatty acids, and lipids (Uyanga et al., 2023). Other studies using dietary chitosan and cholesterol have reported conflicting results regarding growth in crustaceans. For instance, Guo et al. (2022) and Tian et al. (2020) reported that diets with low ($\leq$ 0.25%) or no cholesterol retard growth in *Eriocheir sinensis* and *P. clarkii*, respectively. Su et al. (2023) observed poor growth in *P. vannamei* fed low cholesterol ($<$ 0.18%) and attributed this to abnormal energy metabolism caused by cholesterol deficiency. In contrast, Shiau and Yu (1998) showed that a high concentration of chitosan ($\geq$ 20 g/kg) lowers blood cholesterol and retards growth in *P. monodon*.

Our findings also suggested that the deaths of prawns caused by incomplete ecdysis syndrome, which occurred mainly in the experimental diet groups, is the best evidence that chitosan affected the assimilation of lipids (mainly cholesterol). A soy lecithin deficiency in the diet of *C. (C.) caementarius* has been shown to cause deaths due to incomplete ecdysis syndrome (Acosta et al., 2018; Reyes, 2018). Interestingly, the observed prawn mortality in the experimental diet groups due to incomplete ecdysis occurred regardless of soy lecithin inclusion in the formulation of all diets used herein. In crustaceans, lecithin promotes greater assimilation of lipids and facilitates the transport of cholesterol (Baum et al., 1990; Liou et al., 2023) to be used in the synthesis of molting hormone (Bonilla-Gómez et al., 2012). Hence, an adequate amount of cholesterol activates ecdysone and improves molting in *E. sinensis* (Guo et al., 2022), while cholesterol deficiency decreases the frequency of molting in *P. clarkii* (Tian et al., 2020) and causes death by molting syndrome in *Portunus pelagicus* (Noordin et al., 2020). In addition, crustaceans do not synthesize cholesterol de novo, so supplementation is necessary to use dietary ingredients containing low cholesterol (Tian et al., 2020). However, the optimal level of dietary cholesterol needed to facilitate the molting process and enhance growth in different economically important crustacean species such as *P. clarkii* (Tian et al., 2020) and *C. (C.) caementarius* is still unknown.

We observed that the survival of *C. (C.) caementarius* was high in those fed with the control diet (72.22%) and lower but similar among dietary treatments (1 g chitosan/kg diet: 55.56%, 2 chitosan/kg diet: 61.11%, and 4 g chitosan/kg diet: 72.22%; Table 3). This variation in survival between the control and experimental groups reinforces the previous indication that chitosan in diets interferes with the assimilation of lipids affecting the process of ecdysis, since the highest frequency of deaths due to incomplete ecdysis syndrome occurred in substates E1 and E2, and incomplete ecdysis has been shown to cause imminent death in *C. (C.) caementarius* in those early stages (Reyes, 2018). It is known that chitosan can bind cholesterol and other sterols through hydrophobic interactions, thereby forming hydrophobic complexes, and in the small intestine, these complexes solidify, and are then excreted in the feces (Stanek et al., 2023). Therefore, the prawn deaths from this syndrome altered the average values of growth parameters, which would also explain the lack of significant differences among dietary treatments.

The proPO of *C. (C.) caementarius* hemocytes increased significantly (66.28%) with the administration of 1 g of chitosan/kg of diet (Fig. 1) compared to the higher concentrations of chitosan of the other experimental diets, indicating the ease by which low concentrations of chitosan stimulates proPO of the prawn hemocytes. It has been reported that in *P. clarkii*, a high proPO activity of the hemocytes is obtained with a 10 g chitosan/kg diet (Zhu et al., 2010), while in *P. vannamei* the phenoloxidase is increased with 4 g chitosan/kg diet (Cheng et al., 2021). In contrast, when *C. (C.) caementarius* was fed with the 2 and 4 g chitosan/kg diets, the proPO of hemocytes was low (12.52% and 19.36%,

respectively), and it was even lower (1.85%) with the control diet. These results could be explained by the high levels of nitrites present in the water of those experimental treatments (1.00 and 0.50 mg/L) and also of the control diet (0.33 mg/L), even though the values had high variation (Table 4), but at the same time indicating that chitosan contributed to attenuate the negative effect of nitrites on the activation of proPO of prawn hemocytes, although the toxicity of nitrites was more harmful. Nitrites in water are known to disrupt immunity in crustaceans, accumulate in hemocytes and produce morphological changes and cell damage (Liang et al., 2023). For example, a concentration of 0.314 mg/L of nitrites in water causes a significant decrease in phenoloxidase activity to less than half the control value in *Macrobrachium malcolmsonii* (Chand and Sahoo, 2006). However, there is still doubt as to whether the high concentrations of chitosan used in the diet of *C. (C.) caementarius* would favor a greater activity of the proPO of hemocytes when the nitrites of the culture water are $\leq$ 0.17 mg/L. At the laboratory level, it has been reported that water with nitrite concentrations of 0.14 mg/L do not affect the growth or survival of adult prawn of *C. (C.) caementarius* (Fuentes et al., 2021; Graciano et al., 2022).

In this research, nitrites analysis from *C. (C.) caementarius* culture water showed how harmful this nitrogenous product can be when present above 0.33 mg/L, as at high concentrations it affects the activation of proPO of hemocytes and probably other immunological parameters, given its marked toxicity (Liang et al., 2023). In this regard, the nitrite content of the water of the rivers where *C. (C.) caementarius* occurs has only been recently reported for the Cañete River in Peru, which is one of the main locations from where this resource is extracted. In this river, no nitrites were detected in areas located at high altitudes (> 800 masl), but nitrite concentrations ranging from 0.15 to 1.70 mg/L were detected at low altitude (< 700 masl), which is where there are more population centers nearby (Wasiw and Yépez, 2017). Consequently, we may infer that in the lower part of the Cañete River and probably in other Pacific coast rivers with strong anthropogenic influence where *C. (C.) caementarius* is distributed, the high concentration of nitrites in the water would affect the immune response of this prawn species. Thus, subsequent studies are needed to evaluate the tolerance to nitrites of the water in the different ontogenetic states of *C. (C.) caementarius*. Likewise, relevant government agencies and environmental institutions should consider performing analyses for the determination of nitrite levels of the water in sampling sites during prawn population studies and river water quality monitoring programs, as it provides valuable information for the management of the river prawn resource.

The concentrations of NH_3-N (0.37–0.49 mg/L) and TAN (0.75–1.09 mg/L) of *C. (C.) caementarius* culture water from the present investigation were slightly higher than those reported in other studies that analyzed water used for prawn culture under controlled conditions (Reyes-Avalos et al., 2020; Fuentes et al., 2021; Graciano et al., 2022) and from the wild (Yépez and Bandín, 1998). However, it seems like *C. (C.) caementarius* can tolerate these relatively high NH_3-N and TAN levels, since the values were similar among the dietary treatments, except for TAN which was higher in the control diet. Previous studies on other prawn species revealed that NH_3-N concentrations ranging from 0.54 to 3.18 mg/L affect metabolism, growth, survival, and immune response in *M. rosenbergii* (Cheng and Chen, 2002; Dong et al., 2020). In the crab *P. trituberculatus* it was observed that low levels of ammonia (5 mg/L) stimulate molting, while high levels of ammonia (20 mg/L) suppress the molting process and cause molting death syndrome by over-activation of ecdysteroid signaling (Wang et al., 2023), while the LC50 at 96 h of TAN exposure is 40.42 mg/L in *M. nipponense* (Zhang et al., 2015).

5. Conclusions

The present study revealed for the first time that *C. (C.) caementarius* fed on 1 g of chitosan/kg of diet significantly increases the proPO of hemocytes. However, higher concentrations of dietary chitosan affect proPO likely due to the high concentration of nitrites in the culture water, as it was observed in the prawns from the 2 and 4 g of chitosan/kg diet treatments. Overall, prawn growth parameters were similar ($P > 0.05$) among the dietary treatments and the greatest growth was observed with the 2 g of chitosan/kg diet. However further studies are needed to overcome prawn mortalities due to incomplete ecdysis syndrome, which was likely influenced by a reduction of lipid assimilation caused by chitosan. The results presented herein contribute to a better understanding of the use of chitosan-containing diets in *C. (C.) caementarius* suggesting that it can be used at low concentrations to stimulate its immune response.

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Ethical statement

All experiments conducted in this study were performed in accordance with Peruvian Law on Animal Protection and Welfare (Law 30407), and every effort was made to minimize suffering during thermal stress.

CRediT authorship contribution statement

Walter Reyes-Avalos: Conceptualization, Validation, Acquisition of funds, Formal analysis, Writing - original draft. Writing: Proofreading and editing. **Carlos Azañero Díaz**: Conceptualization. Supervision, Validation, Formal analysis, Writing: Review and editing. **Gladis Melgarejo-Velásquez**: Conceptualization, Methodology, Research, Formal analysis, Data curation, Formal analysis. Review and editing. **Brian Alegre Calvo**: Methodology, Research. **Roberto Lezama Salazar**: Methodology, Research, Formal analysis. All authors read and approved the final manuscript. Data curation,

Declaration of Competing Interest

The authors declare that they have no financial interests or personal relationships that could have influenced this work.

Data availability

Data will be made available on request.

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Author agreement statement

We the undersigned declare that this manuscript "Effect of dietary chitosan on the growth, survival, and prophenoloxidase of male freshwater prawns *Cryphiops (Cryphiops) caementarius*" is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.

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