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Effects of water temperature on digestive protease activity and apparent nutrient digestibility in Pacific whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931)

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ABSTRACT

Water temperature strongly influences metabolic rate, digestive physiology, and nutrient utilization in poikilothermic aquatic species such as shrimp. This study evaluated the effects of two culture temperatures (27 and 31 °C) on growth performance, apparent nutrient digestibility, hepatopancreatic protease production, and protease activity in Pacific white shrimp (*Litopenaeus vannamei*). Adult shrimp (≈16 g) were maintained at the experimental temperatures for 21 days, following a gradual acclimation period. Growth performance and apparent digestibility coefficients (AD) of dry matter, protein, methionine, and lysine were not significantly affected by temperature. However, lipid digestibility was significantly higher at 27 °C. Shrimp reared at 27 °C also exhibited a significantly higher hepatosomatic index and greater total quantity of stored protease throughout the circadian cycle, indicating enhanced digestive enzyme synthesis. Although protease catalytic activity was higher at 31 °C, shrimp maintained digestive efficiency at 27 °C through compensatory increases in enzyme production and lipid utilization. These findings demonstrate that *L. vannamei* exhibits substantial digestive plasticity, allowing maintenance of growth and protein utilization at moderately sub-optimal temperatures through coordinated metabolic and enzymatic adjustments.

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most widely farmed crustaceans worldwide, contributing significantly to global aquaculture production (FAO, 2024). *L. vannamei* is characterized by rapid growth, high environmental adaptability, and considerable economic importance in aquaculture. As an ectothermic organism, shrimp lacks the capacity to regulate its internal body temperature, making it highly sensitive to environmental temperature fluctuations (Ren et al., 2021).

Although *L. vannamei* exhibits a relatively wide thermal tolerance, optimal physiological performance is generally observed between 25 and 30 °C (Pérez-Rostro et al., 2022), while the general culture temperature range reported for the species spans approximately 23–33 °C (Ponce-Palafox et al., 1997; Wyban et al., 1995). This temperature variability is also commonly observed across shrimp farming regions in Ecuador, including estuarine and mangrove systems, where seasonal fluctuations typically range between approximately 25 and 28 °C in inner estuaries and may extend from ~20–32 °C across coastal mangrove environments (Marín Jarrín et al., 2022). Within this range, moderate deviations from the thermal optimum may induce measurable physiological adjustments in metabolism and digestion without necessarily causing severe thermal stress. Temperature strongly influences metabolic processes, feeding activity, and physiological performance in

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crustaceans (Dayalan et al., 2022; Ren et al., 2021). Shrimp can maintain normal behavioral patterns during moderate increases in temperature, tolerating rapid rises to approximately 34 °C; however, prolonged exposure to higher temperatures may compromise survival and physiological homeostasis, indicating an upper limit to the species' thermal tolerance (Dayalan et al., 2022). On the other hand, for sub-optimal water temperature (<25°C), feed consumption and feed efficiency are reduced, metabolism slows down, and slow growth is observed (Cheng et al., 2005; Ren et al., 2021).

At temperatures below the thermal optimum, shrimp typically exhibit reduced feed intake, slower metabolism, and reduced growth performance (Cheng et al., 2005; Ren et al., 2021). Growth suppression under these conditions is primarily associated with decreased feed consumption (James et al., 1995), which has prompted the aquaculture feed industry to develop strategies aimed at improving feed palatability through specialized formulations containing specific additives and raw materials (He et al., 2022).

In addition to changes in feeding behavior, low temperatures can induce physiological alterations affecting oxygen consumption, nutrient digestibility, and digestive enzyme activity (Zhou et al., 2010; Millard et al., 2021). Among digestive enzymes, proteases play a central role in dietary protein hydrolysis, and changes in their activity may directly influence protein digestibility and nutrient utilization efficiency. These biochemical and physiological responses reflect adaptive mechanisms that allow aquatic ectotherms to cope with environmental temperature fluctuations through phenotypic plasticity and physiological acclimation (Buckley et al., 2001; Fox et al., 2019).

Most studies investigating temperature stress in shrimp have focused on acute cold shock experiments, where animals experience rapid decreases in temperature that trigger strong physiological and molecular responses (Donaldson et al., 2008; Zhu et al., 2024a). However, comparatively less attention has been given to physiological adjustments occurring under moderate but sustained sub-optimal temperatures, conditions that may induce compensatory responses in metabolism and digestion without necessarily causing severe thermal stress. Consequently, the effects of sub-optimal temperatures on digestive protease activity and protein digestibility in juvenile *L. vannamei* remain insufficiently characterized.

The present study aimed to evaluate the effect of water temperature on protein digestibility and digestive protease activity in juvenile *L. vannamei*.

2. Materials and methods

2.1. Rearing facilities

The facilities used for the experiments were located at Vitapro Aquaculture Research Center (CEA) in Lima, Peru. The nursery is a 6 m^3 cubic tank connected to a water recirculation aquaculture system (RAS, composed of a water pump, heat pump, biofilter, mechanical filter, and UV sterilizer). The experimental tanks were 2 m^3 cylindrical tanks connected in groups of five to RAS similar to the nursery system. The water in these systems was maintained at a temperature of 29°C prior to experiments.

2.2. Experimental shrimp

Post-larval shrimp were carefully transported by air from commercial hatcheries in Tumbes, Peru, and maintained in the nursery tank containing natural seawater with salinity of 35 ppt and temperature of 29°C for 4 weeks until they reached a mean body weight of 1.2 g. Shrimp were fed commercial starter diets of 0.5 and 0.8 mm particle size. The daily feeding rate was scaled from 35% to 6% of total body weight (BW) over the 4-week experimental period.

Subsequently, shrimp were transferred to grow-out tanks at a density of 50 shrimp per tank (25 shrimp/m^2) for an 8-week rearing period until reaching an average weight of 16 g (adults). During this period, they were fed a 35% crude protein commercial diet at a rate of 4% of the total stocked biomass, split into two daily doses at 09:00 and 15:00 h. Feed particle size was 1.2 mm until shrimp reached 4 g, after which a 2 mm pellet was used until the end of the trial. All grow-out diets were commercial formulations produced in the VITAPRO pilot plant and contained 0.5% chromium oxide as an inert marker for subsequent apparent digestibility (AD) calculations. The ingredient composition and proximate analysis of the standard diets are provided in Table 1.

2.3. Growth performance

To evaluate shrimp growth performance, the following parameters were recorded: initial body weight, final body weight, total feed intake, and survival. The corresponding performance metrics were calculated as follows:

$$Weekly\ growth = \frac{final\ body\ weight\ (g) - initial\ body\ weight\ (g)}{culture\ period\ (weeks)}$$

$$Feed\ conversion\ ratio = \frac{total\ feed\ intake\ (g)}{total\ shrimp\ weight\ gain}$$

$$Total\ feed\ intake\ (g) = feed\ supplied\ (g) - uneaten\ feed\ (g)$$

(*) feed weight on a dry basis

$$Survival\ rate\ (\%) = \left(\frac{Number\ of\ shrimp\ at\ harvest}{number\ of\ shrimp\ at\ stocking}\right) * 100$$

2.4. In vivo digestibility assay

When shrimp reached 16 g, a RAS with its five replicates was brought to a temperature of 27°C and another RAS with the same number of replicates to 31°C, both with a temperature change rate of 0.1°C/4 h (48 in total). Shrimp were maintained at their experimental temperatures for two weeks. Water parameters were monitored daily and kept within the recommended ranges: dissolved oxygen (> 4.0 mg/L), pH (8.0 ± 0.1), alkalinity (> 120 mg/L), total ammonia nitrogen (NH_3 < 0.2 mg/L; Lin and Chen, 2001) and nitrite (NO_2 < 3.17 mg/L; Lin and Chen, 2003). Water temperature was monitored twice daily.

During the experiment, feces from three randomly chosen tanks of each treatment were collected twice a day using 200 mL pipettes with a suction cup, rinsed in Petri dishes with distilled water to remove excess salt, and stored at −20°C, according to the methodology described by Méndez-Martínez et al. (2021). Once 40 g of wet feces per tank were obtained, they were sent to the Vitapro Central Laboratory in Trujillo (Peru) for freeze-drying for 24 h or until a moisture content of 5% was reached (Méndez-Martínez et al., 2021). Subsequently, crude protein levels were quantified using the Dumas method (AOAC 990.03), methionine and lysine using the Waters AccQ Tag Amino Acid Analysis

Table 1
Chemical profile (%, as-is) of commercial diets used in this study.

Nutrient (%)	Starter	Starter	Pre-grower	Grower
Pellet diameter (mm)	0.5	0.8	1.2	2.0
Moisture	10.4	11.9	10.5	9.5
Total lipids	14.6	8.0	7.5	7.3
Ash	11.9	10.0	9.4	8.7
Crude protein	53.5	36.3	43.0	35.5
Carbohydrate	9.4	31.8	26.8	34
Fiber	0.2	2.0	2.8	2.0
Methionine	1.6	0.8	1.0	0.6
Lysine	4.0	2.0	2.7	2.2

Ingredients: Fish and other marine meals, fish oil, oilseed meal, cereals and by-products, soy lecithin, sodium/potassium phosphate, calcium carbonate, sodium chloride, preservative, mineral and vitamin premix, synthetic amino acids (methionine and lysine).

Method, and total lipid content by ether extraction (AOAC Official Method 2003.05). The apparent digestibility coefficient (AD) of protein, essential amino acids and total lipids was calculated following the methods described by Montaño-Vargas et al. (2002):

$$CDA\ nutrient\ (\%) = 1 - \left(\frac{\%\ \ Cr2O3\ \ in\ feed}{\%\ \ Cr2O3\ \ in\ feces} * \frac{\%\ \ nutrient\ in\ feces}{\%\ \ nutriente\ in\ feed}\right)$$

2.5. Analysis of enzyme production and activity

After the feces collection period, shrimp were checked for intermolt status (using uropod characteristics according to methodology of De Oliveira et al., 2006) and subjected to a 24-hour fasting. Upon completion of the fasting period, shrimp were sampled at three-hour intervals. For each sampling point, three individuals per tank were euthanized by immersion in 5°C water (Gamboa-delgado et al., 2003), followed by dissection and collection of the hepatopancreas. Samples were weighted using an Ohaus SPX 621 electronic balance with a precision of 0.01 g and stored at −20°C. The hepatosomatic index (HSI) was calculated using the following formula:

$$HSI = \frac{hepatopancreas\ weigth\ (g)}{shrimp\ weight\ (g)} * 100$$

The hepatopancreases samples were placed in 15 mL centrifuge tubes and homogenized in 50 mM Tris-HCl solution with pH 8.0 (1:2 w/v) using a glass rod (Casillas-Hernández et al., 2006). This homogenate was centrifuged at 3305 xg for 40 min at 5°C. The supernatant, considered as enzyme extract, was recovered and stored at −20 C.

We estimated both, the total quantity of the stored protease in the hepatopancreas (TQSP) and total protease activity (TPA) at culture temperatures. The latter was assessed by performing enzymatic reactions at temperatures simulating shrimp culture conditions.

To estimate the TQSP we used the methodology described by Casilla-Hernández (2006) for determining total specific protease activity to reaction temperature of 35°C. For this purpose, 20 μL of enzyme extract was incubated with 230 μL of 50 mM Tris-HCl buffer (pH 8) and 500 μl of 0.5% azocasein chromogenic substrate. The reaction was carried out at 35°C for 30 min. To terminate the reaction, 500 μL of 20% (w/v) trichloroacetic acid (ATC) was added followed by 15 min incubation at room temperature. The mixture was then centrifuged at 6866 xg for 10 min at room temperature. The supernatant was collected in 1.5 mL microcentrifuge tubes for absorbance measurement.

For the estimation of TPA, the same methodology was used, but the enzymatic extract was incubated with azocasein at 27 or 31°C, depending on the shrimp cultivation temperature from which each sample came.

The absorbance of the supernatants was read at a wavelength of 440 nm using a calibrated Spectronic® Genesys 20 spectrophotometer (Thermo Scientific Inc, Rochester, NY, USA). TQSP and TPAwere expressed as U/mg of protein, where the protease activity unit (U) was defined as the amount of enzyme required to increase the absorbance by 0.01 units at 440 nm per minute. Protein concentration of the enzyme extracts was quantified using the total protein Biuret method (Gornall et al., 1949).

2.6. Statistical analysis

All statistical analyses were performed using R software (version 3.1.0). Experimental results are presented as mean ± standard deviation (SD). Data normality was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated with Levene's test. Subsequently, comparisons of TQSP, TPA, HSI, and nutrient AD between treatments were conducted using Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* test. A significance level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Productive performance

Shrimp growth performance showed no significant differences during the 21 days of culture, with weekly growth rates of 1.96 ± 0.42 and 2.19 ± 0.26 g and feed conversion ratios of 1.62 ± 0.38 and 1.40 ± 0.16 for shrimp cultured at 27 and 31°C, respectively (Table 2). On the other hand, significant differences were observed in the hepatosomatic index, which was 2.93 ± 0.15 in shrimp cultured at 27°C and 2.77 ± 0.16 in those cultured at 31°C (Table 2).

3.2. In vivo digestibility assays

No significant differences were found in AD of dry matter, protein, methionine, and lysine between shrimp grown at 27 and 31°C. AD of dry matter averaged 72.3% and 71.2 for shrimp grown at 27 and 31°C, respectively ($p = 0.42$). AD of protein averaged 83.4 and 83.2% ($p = 0.78$); AD of methionine averaged 83.1 and 81.7% ($p = 0.15$); while AD of lysine averaged 85.1 and 85.1% ($p = 0.97$) for shrimp grown at 27 and 31 °C, respectively (Table 2). Lipid was the only nutrient for which apparent digestibility differed between treatments, with higher AD at 27°C (95.4%) than at 31°C (93.8%; $p = 0.04$; Table 3)."

3.2.1. Analysis of enzyme production and activity

Both TQSP and TPA varied significantly during the circadian cycle ($p < 0.05$; Figs. 1 and 2). In shrimp reared at 27 °C, TQSP reached its highest value at 03:00 h, while TPA showed its highest values at 12:00 and 03:00 h and the lowest value at 21:00 h. In shrimp reared at 31 °C, both TQSP and TPA peaked at 09:00 and 12:00 h and decreased to their lowest values between 18:00 and 21:00 h.

Water temperature had a significant effect on TQSP and TPA ($p < 0.05$). Across the circadian cycle, TQSP values were significantly higher in shrimp cultured at 27 °C than at 31 °C (Fig. 1). In contrast, TPA values were significantly higher in shrimp cultured at 31 °C than at 27 °C when considering all sampling times ($p < 0.05$; Fig. 2)

4. Discussion

Water temperature is a dominant driver of metabolic rate, ingestion, digestive physiology, and nutrient utilization in aquatic poikilothermic animals (Wyban et al., 1995; Spees et al., 2002; Croll and Watts, 2004; Spanopoulos-Hernández et al., 2005; Walker et al., 2011; Barbieri et al., 2016). Within the typical physiological range of L. vannamei, higher temperatures are often associated with increased metabolic rates and potentially higher growth. However, in the present study neither growth performance nor dietary protein and amino acid digestibility differed between shrimp maintained at 27 and 31°C. Similar patterns were reported by Wyban et al. (1995), who observed thermal effects on growth in small juveniles (3.8–10.8 g) but not in shrimp > 16 g, suggesting that

Table 2
Growth performance of *L. vanamei* cultured under 27 and 31°C for 21 days.

Parameters	Treatment	Mean ± SD	*p*-value
Initial body weight (g)	27°C	16.1 ± 1.3	0.987
	31°C	16.1 ± 1.3	
Final body weight (g)	27°C	22.11 ± 1.9	0.380
	31°C	21.52 ± 0.4	
Weekly growth (g)	27°C	1.96 ± 0.42	0.275
	31°C	2.19 ± 0.26	
Hepatosomatic index (%)	27°C	2.93 + 0.15	< 0.001*
	31°C	2.77 + 0.16	
Feed conversion ratio	27°C	1.62 ± 0.38	0.228
	31°C	1.40 ± 0.16	

(*) Indicates significant difference (Student *t*-test)

Table 3
Results of apparent digestibility (AD) of dry matter (ADMS), protein (ADP), methionine (ADM), lysine (ADL) and lipids (ADL) of diets for shrimp cultured at 27 and 31 °C.

Parameter	Treatment	Mean ± SD	p-value
ADMS	27°C	72.3 + 2.4	0.42
	31°C	71.2 + 0.7	
ADP	27°C	83.4 + 1.1	0.78
	31°C	83.2 + 0.8	
ADM	27°C	83.1 + 1.6	0.15
	31°C	81.7 + 0.5	
ADL	27°C	85.1 + 1.2	0.97
	31°C	85.1 + 0.7	
ADL	27°C	95.4 + 1.0	0.04*
	31°C	93.8 + 0.4	

(*) Indicates significant differences (Student *t*-test)

larger individuals possess advanced physiological plasticity allowing them to buffer moderate thermal variation.

Our results suggest that *L. vannamei* activated compensatory digestive mechanisms at 27°C, allowing shrimp to maintain protein digestion and growth despite operating at the lower end of their optimal thermal window. Kır et al. (2023) showed that the species displays an optimal thermal range of 25–30°C, with standard metabolic rate increasing exponentially with temperature. Thus, although enzymatic catalytic efficiency may improve at 31°C, this occurs alongside substantially higher maintenance energy costs. Conversely, at 27°C shrimp likely compensate for reduced catalytic efficiency by increasing digestive enzyme synthesis, an energetically less costly strategy than sustaining elevated basal metabolism above the thermal optimum.

This interpretation is supported by classical work in Penaeus japonicus (Galgani, 1985), which demonstrated that protease activity is maintained within a narrow thermal compensation window (25–29 °C) but declines sharply below 21–25 °C. These findings highlight that

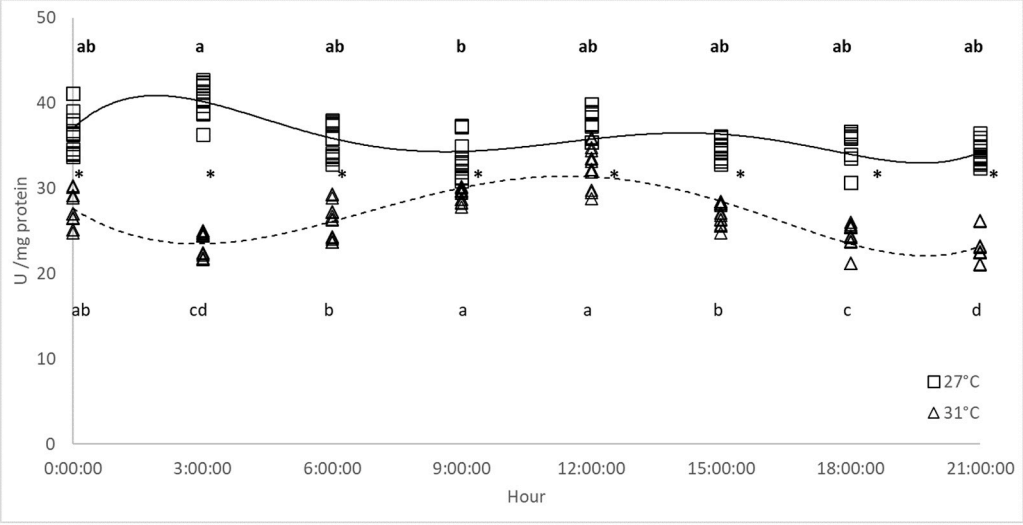

Fig. 1. Total quantity of stored protease (TQSP) in the hepatopancreas of shrimp cultured at 27 and 31 °C at different times of the day. Different letters indicate a statistical difference between hours within the same culture temperature (above 27 °C; below 31 °C) (ANOVA, Tukey's HSD test). Asterisks indicate significant differences between temperatures within the same hour (Student's *t*-test).

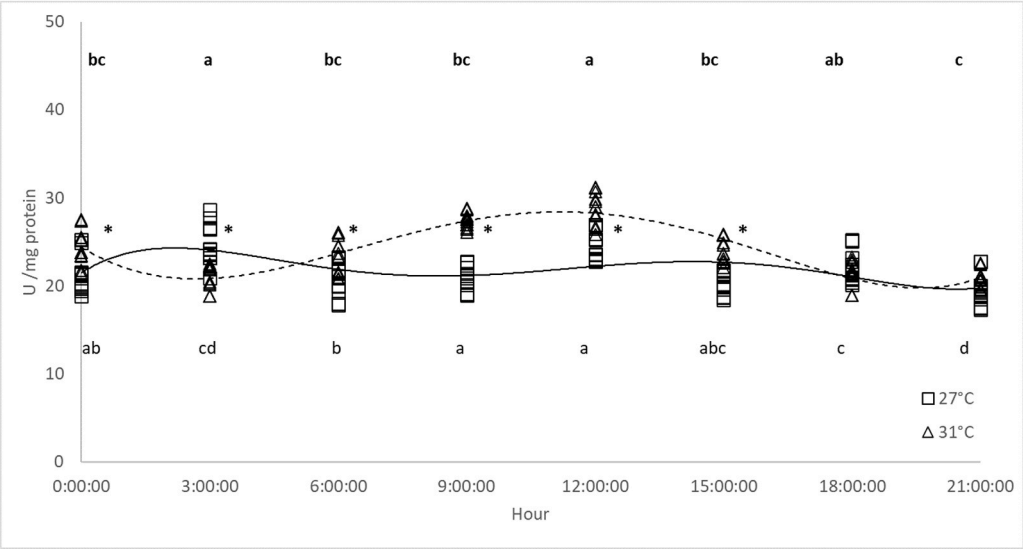

Fig. 2. Total protease activity (TPA) in shrimp cultured at 27 and 31 °C at different times of the day. Different letters indicate a statistical difference between hours within the same culture temperature (above 27 °C; below 31 °C) (ANOVA, Tukey's HSD test). Asterisks indicate significant differences between temperatures within the same hour (Student's *t*-test).

digestive plasticity operates under a physiologically bounded thermal range and that below this threshold compensatory capacity collapses. The present results suggest that 27°C lies within the lower, but still functional region of this window, allowing shrimp to maintain proteolysis via enzyme overproduction without compromising nutrient utilization.

One of the most notable findings in this study was the significantly higher hepatosomatic index (HSI) at 27°C. The hepatopancreas is the central organ for digestion, absorption, lipid deposition, glycogen storage, lipoprotein synthesis, and enzyme production in penaeids. Changes in its mass, therefore, reflect shifts in metabolic allocation. Cadena and Molina-Poveda (2000) reported elevated HSI during intermolt–premolt stages (B, C, D_0), when enzymatic activity, lipid storage, and nutrient assimilation intensify. This pattern suggests that the increased HSI observed at 27°C reflects enhanced hepatopancreatic workload, driven by increased lipid absorption and/or elevated digestive enzyme content.

The higher apparent lipid digestibility observed at 27°C further supports this interpretation. Lipid metabolism plays a central role in thermal compensation in crustaceans. Previous studies have shown that shrimp can adjust energy reserves and exhibit compensatory responses under different thermal conditions, including changes in lipid and glycogen dynamics (Prates et al., 2022). Zhu et al. (2024b) demonstrated that L. vannamei exposed to decreasing temperatures strongly upregulates lipid metabolic pathways-including ELOVL7-mediated long-chain fatty acid synthesis, and accumulates unsaturated fatty acids to maintain membrane fluidity and cellular stability. Although our experimental temperatures were comparatively warm (27–31 °C), the underlying metabolic strategy appears analogous: shrimp at 27°C may rely more heavily on lipid assimilation and mobilization to compensate for thermal constraints on catalytic efficiency, while avoiding the higher ATP turnover required at 31°C. This suggests that 27°C may favor lipid utilization compared with 31 °C, although additional temperature levels would be required to determine whether this reflects a broader metabolic efficiency pattern.

Additional evidence supports the centrality of lipid metabolism in environmental compensation. Shirly-Lim et al. (2024) reported that shrimp predominantly mobilize glycogen and lipids under thermal and pH stress, highlighting these substrates as primary fuels supporting basal metabolism. This aligns closely with our findings, where higher lipid digestibility and increased HSI were observed concurrently at 27°C indicating that lipid pathways support both energetic demands and digestive compensation under moderate thermal deviation.

While temperature was the only variable manipulated in our study, responses to other environmental stressors provide valuable context. Yu et al. (2020) showed that chronic high-pH stress significantly increased HSI while reducing somatic growth in *L. vannamei*, demonstrating that hepatopancreas hypertrophy is a common physiological response to metabolic challenges. Their findings reinforce the interpretation that increased HSI at 27°C in our study represents a redistribution of assimilated nutrients toward hepatopancreatic reserves and metabolic maintenance rather than somatic accretion—consistent with the elevated lipid digestibility observed.

Enzymatic profiles further corroborate the compensatory response at 27°C. Protease content was higher at this temperature despite lower catalytic efficiency, indicating an upregulation of enzyme synthesis to maintain proteolytic flux. Positive associations between hepatopancreas mass and digestive enzyme output have been widely documented in penaeids (Comoglio et al., 2004; Aragón-Axomulco et al., 2012; Sriket et al., 2012). Moreover, shrimp are known to respond to both thermal and dietary stressors, such as soybean trypsin inhibitors via overexpression of proteases (Maytorena-Verdugo et al., 2017; Rojo-Arreola et al., 2019). Thus, the increase in protease levels observed at 27°C reflects a well-established physiological response that enables shrimp to maintain digestive performance under constrained catalytic conditions.

Although protease activity *per se* is reduced at 27°C, *L. vannamei* compensates through increased enzyme production and enhanced lipid assimilation. The coordinated increase in HSI, lipid digestibility, and protease synthesis illustrates a robust metabolic-digestive adjustment that stabilizes nutrient utilization and growth under moderate thermal variation. These findings demonstrate the remarkable digestive and metabolic plasticity of *L. vannamei*, supporting its capacity to maintain performance within a broad but physiologically structured thermal tolerance window.

5. Conclusion

Within the evaluated temperature range, *Litopenaeus vannamei* maintained growth and protein utilization despite temperature-dependent differences in digestive physiology. Higher lipid digestibility and increased hepatopancreatic protease reserves at 27°C suggest coordinated digestive adjustments that help sustain nutrient utilization under moderate thermal variation.

CRediT authorship contribution statement

Benjamín Castro Nole: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Writing – original draft. **Carlos Espinoza:** Formal analysis, Methodology, Resources, Writing – review & editing. **Zlatko Kovac:** Validation, Writing – review & editing. **Pablo Leyton:** Validation, Writing – review & editing. **Alberto Nunes:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that this study was funded by VITAPRO S.A. Benjamín Castro, Zlatko Kovac, Pablo Carlos, and Carlos Espinoza are affiliated with VITAPRO S.A. Alberto Nunes is not affiliated with the funding organization. The funder had no role in the design of the study, data collection, analysis, interpretation of results, or in the preparation of the manuscript.

Data availability

Data will be made available on request.

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