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Effects of dietary protein and methionine levels on the growth and feed utilization of juvenile *Penaeus vannamei* raised in hypo- and hyperosmotic conditions

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ABSTRACT - This study evaluated the effects of dietary crude protein (CP) and methionine (Met) levels on growth performance, feed utilization, and daily nutrient intake of juvenile *Penaeus vannamei* reared under 16 and 40 psu. A 2 × 3 × 2 factorial design tested six diets with CP levels (33.3 and 37.4%, as-is basis) adjusted via soy protein concentrate, and Met levels (0.6, 0.7, and 0.9%, as-is basis) supplemented with DL-methionine. Shrimp of 1.49 ± 0.11 g were fed for 91 days in 92 tanks of 0.5 m^3 at 140 animals/m^2. Salinity was the dominant factor affecting shrimp performance, significantly influencing survival, with higher rates observed at 40 psu (95.5 ± 4.5%) compared with 16 psu (90.7 ± 5.7%). However, a higher salinity negatively impacted final body weight (13.6 ± 1.2 vs. 11.4 ± 0.7 g), yield (13.8 ± 0.9 vs. 16.9 ± 1.6 MT/ha), weight gain (665.5 ± 44.5 vs. 816.1 ± 84.0%), specific growth rate (2.24 ± 0.06 vs. 2.43 ± 0.10%/day), and feed conversion ratio (1.46 ± 0.10 vs. 1.32 ± 0.08). Except for daily feed intake, shrimp reared at 16 psu consistently outperformed those at 40 psu ($P<0.001$). Dietary CP and Met levels did not significantly affect survival, yield, or other growth parameters ($P>0.05$), though raising CP and Met levels significantly increased daily CP and Met intake ($P<0.001$). Although shrimp at 40 psu showed increased intake of CP, Met, total sulfur amino acids, and total amino acids, this did not improve growth performance or feed utilization under hyperosmotic conditions. These findings suggest that elevated energy demands for osmoregulation in hyperosmotic environments limited nutrient conversion into growth, showing that dietary interventions were insufficient to fully overcome the challenges posed by high salinity.

Keywords: feed utilization, methionine supplementation, *Penaeus vannamei*, suboptimal salinity

1. Introduction

Salinity is a critical environmental factor that significantly influences the physiological and metabolic functions of aquatic species, including *Penaeus vannamei* (Ponce-Palafox et al., 1997; Tseng and Hwang, 2008; Ern et al., 2014). As a euryhaline species, *P. vannamei* exhibits remarkable adaptability to salinities ranging from freshwater to hypersaline conditions, enhancing its suitability for aquaculture across diverse environments (Roy et al., 2010). This broad adaptability allows for flexibility in farming conditions, but it also requires careful consideration of the salinity impact on shrimp performance.

At salinities between 20 and 25 practical salinity unit (psu), *P. vannamei* maintains hemolymph isosmotic with its environment, minimizing the energy required for osmoregulation (Castille and Lawrence, 1981; Jaffer et al., 2020). However, when salinity levels fall outside this optimal range, energy is increasingly redirected from growth-related processes, such as digestion, to support osmoregulation (Wang et al., 2013; Méndez-Martínez et al., 2021).

To address the increased energy demands caused by salinity stress, dietary interventions, including higher protein and essential nutrient levels, may support the shrimps' osmoregulatory needs (Li et al., 2015). Protein is not only important for tissue growth but also plays a significant role in osmoregulation. Amino acids derived from dietary protein act as osmolytes, molecules that help cells maintain homeostasis under osmotic stress (Li et al., 2015). Free amino acids such as glycine, alanine, and proline accumulate in shrimp muscle tissue, aiding in cell volume regulation (Roy et al., 2007).

Essential amino acids, like methionine (Met), contribute to both protein metabolism and the synthesis of osmolytes such as taurine. Methionine also supports antioxidant defense mechanisms through the synthesis of compounds like glutathione, which mitigates oxidative stress (Wu et al., 2023). Therefore, increased dietary protein and Met could enhance shrimp resilience in hyperosmotic conditions by supporting both osmoregulation and cellular protection (Brosnan and Brosnan, 2006).

Based on these considerations, we hypothesized that higher levels of dietary protein and Met are necessary to meet the increased nutrient demands of shrimp reared in hyperosmotic environments. Therefore, this study aimed to assess the impact of varying dietary crude protein (CP) and methionine (Met) levels on shrimp growth performance and feed utilization, while exploring whether these dietary adjustments can enhance overall shrimp performance under hyperosmotic environments.

2. Material and methods

The study was conducted in the aquaculture research facilities of the Laboratório de Nutrição de Organismos Aquáticos (3°50'01.55" S, 38°25'22.74" W) of the Instituto de Ciências do Mar (Institute of Marine Sciences; LABOMAR) at the Universidade Federal do Ceará, Eusébio, Ceará, Brazil.

The experimental design followed a 2 × 3 × 2 factorial arrangement, combining three independent variables: CP content (33.3 and 37.4%, as-is basis), total dietary Met levels (0.6, 0.7, and 0.9%, as-is basis), and salinity (16 and 40 psu). The study involved six dietary treatments, each under both salinity conditions, resulting in 12 different combinations. Each combination was replicated across seven to eight tanks in a completely randomized design, using 92 tanks. This factorial design facilitated the evaluation of the main effects and interactions among CP levels, Met levels, and salinity on shrimp growth performance, feed utilization, and daily nutrient intake. All rearing procedures were performed in compliance with relevant laws and institutional guidelines, including those related to animal welfare. Ethical review and approval were waived for this study, since shrimp do not fall under phylum Chordata and subphylum Vertebrata which require ethical approval for research activities according to the Brazilian Federal Laws.

Six experimental diets (A–F) were formulated to ensure consistent energy levels across all treatments, by varying the CP and Met content to evaluate their impact on shrimp performance under different salinity conditions. The CP and Met levels were adjusted by altering the dietary inclusion of soy protein concentrate and crystalline DL-methionine (Table 1).

Soy protein concentrate was incorporated at 8% in diets A, B, and C, and increased to 15.6–15.7% in diets D, E, and F to elevate CP levels. Proximate composition analysis revealed a CP content of 33.3 ± 0.3% for diets A, B, and C, and 37.4 ± 0.5% for diets D, E, and F (as-is basis). Within each protein group, the diets were isonitrogenous, with nitrogen content maintained at 6.1 ± 0.1% for diets A, B, and C, and 6.8 ± 0.1% for diets D, E, and F (dry matter [DM] basis).

Table 1 - Formulation and proximate composition of experimental diets

Item	Diet/composition (%, as-is)					
	A	B	C	D	E	F
Raw material (% of diet)						
Soybean meal	36.00	36.00	36.00	36.00	36.00	36.00
Wheat flour	27.44	26.61	25.59	25.58	25.56	25.54
Soy protein concentrate	8.00	8.00	8.00	15.70	15.56	15.38
Salmon meal	6.00	6.00	6.00	6.00	6.00	6.00
Cassava starch	4.51	5.18	6.00	0.00	0.00	0.00
Salmon oil	3.30	3.31	3.32	3.23	3.23	3.23
Wheat gluten	3.00	3.00	3.00	3.00	3.00	3.00
Krill meal	3.00	3.00	3.00	3.00	3.00	3.00
Calcium carbonate	1.51	1.51	1.51	1.44	1.44	1.45
Soy lecithin, oil	1.39	1.39	1.39	1.39	1.39	1.39
Potassium chloride	1.19	1.20	1.21	0.89	0.90	0.91
Monosodium phosphate	1.12	1.12	1.12	1.00	1.00	1.00
Magnesium sulphate	1.00	1.00	1.00	1.00	1.00	1.00
L-Lysine	0.66	0.67	0.67	0.14	0.15	0.16
Salt	0.54	0.54	0.54	0.53	0.53	0.53
Vitamin-mineral premix	0.45	0.45	0.45	0.45	0.45	0.45
Synthetic binder	0.30	0.30	0.30	0.30	0.30	0.30
L-Threonine	0.28	0.28	0.28	0.10	0.10	0.11
DL-Methionine	0.20	0.34	0.52	0.14	0.28	0.46
Vitamin C	0.10	0.10	0.10	0.10	0.10	0.10
Proximate composition (% of diet, as-is)						
Dry matter	87.4	87.2	87.2	88.8	87.3	88.5
Moisture	12.6	12.8	12.9	11.2	12.7	11.5
Crude protein	33.7	33.2	33.0	37.9	37.2	37.0
Crude fat	7.1	6.8	7.0	6.9	6.7	7.0
Crude fiber	2.3	2.0	1.9	1.9	3.2	2.7
Ash	8.5	8.4	8.4	8.5	8.3	8.4

DL-methionine was supplemented to achieve the following total Met levels (from both intact and supplemental sources): 0.63% in diet A, 0.74% in diet B, 0.85% in diet C, 0.60% in diet D, 0.71% in diet E, and 0.86% in diet F (as-is basis) (Table 2).

The diet formulation included practical ingredients such as soybean meal, wheat flour, and salmon meal. To maintain a uniform crude fat content and meet shrimps' requirements for omega-3 fatty acid and phospholipid, all diets were supplemented with salmon oil (3.30–3.32%) and soy lecithin (1.39%). Cassava starch levels were adjusted to balance the energy contribution from varying protein and Met levels, ensuring all diets met recommended energy ranges. Krill meal was included at 3% to enhance feed attractability, while wheat gluten, crystalline amino acids (e.g., L-Lysine, L-Threonine), mineral salts, a vitamin-mineral premix, and a synthetic binder were consistently added to ensure balanced nutrition and optimal pellet stability.

All dried ingredients were ground to a particle size of 300 µm for feed manufacturing, weighed, and thoroughly mixed with liquid raw materials and feed additives in a planetary mixer until a uniform dough was achieved. The dough was then manually pressed through a rigid net to break it into small particles, which were processed using a laboratory extruder set at 95 °C. After extrusion, the pellets with 2.0 mm in diameter and 4.8 mm in length, were steamed for 10 min. and then dried in a convection oven (model MA-035/3, Marconi Equipamentos para Laboratório Ltda., Piracicaba, Brazil) to an average moisture content of 12.3 ± 0.7%. All experimental diets were stored at −20 °C until use.

Table 2 - Amino acid composition of experimental diets (%, as-is)

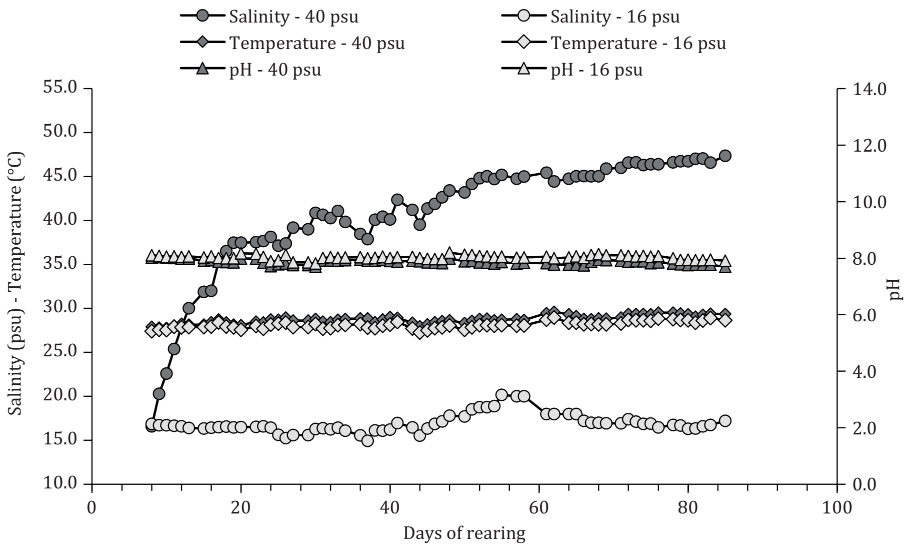
Amino acid	Diet/composition (%, as-is)					
	A	B	C	D	E	F
Essential amino acids						
Arginine	1.96	1.95	1.93	2.25	2.25	2.26
Histidine	0.82	0.82	0.80	0.92	0.91	0.94
Isoleucine	1.39	1.40	1.38	1.59	1.61	1.62
Leucine	2.33	2.33	2.29	2.66	2.64	2.67
Lysine	2.08	2.08	2.08	2.07	2.09	2.09
Methionine	0.63	0.74	0.85	0.60	0.71	0.86
Phenylalanine	1.57	1.56	1.54	1.79	1.78	1.79
Threonine	1.47	1.44	1.41	1.46	1.45	1.49
Tyrosine	1.04	1.03	1.04	1.18	1.17	1.18
Valine	1.46	1.46	1.44	1.65	1.67	1.69
Non-essential amino acids						
Alanine	1.41	1.40	1.36	1.59	1.58	1.60
Aspartic acid	2.96	2.98	2.85	3.42	3.40	3.38
Cysteine	0.42	0.42	0.42	0.48	0.46	0.44
Glutamic acid	6.29	6.26	6.07	7.00	6.95	7.00
Glycine	1.54	1.50	1.46	1.67	1.65	1.71
Proline	2.03	1.99	1.94	2.18	2.17	2.23
Serine	1.54	1.50	1.45	1.75	1.69	1.74
Taurine	0.09	0.09	0.08	0.09	0.08	0.09
Sum of amino acids	31.04	30.94	30.40	34.35	34.26	34.76

Shrimp were reared in circular indoor tanks with a volume of 0.5 m^3 (0.57 m^2 bottom area × 0.56 m height) under a roof and subjected to a 12-h artificial light cycle starting at 05:30 h. Except during the salinity adjustment phase, the system operated under a zero-exchange regime for the first five weeks, after which a 10% weekly water exchange was implemented. The tanks were organized into two independent systems, each comprising 50 tanks. Each system featured its own filtration units and had a total water capacity of 40 m^3, including a 20 m^3 header tank and the experimental tanks. This arrangement enabled precise control of water salinity, maintained at either 16 or 40 psu throughout the experiment.

Non-specific pathogen-free post-larvae (PL) of *P. vannamei* were obtained at PL10 stage from a commercial hatchery (Aquatec Aquacultura Ltda., Canguaretama, Brazil) and transported to the laboratory. At arrival, shrimp were acclimated to temperature, pH, and salinity and stocked in five nursery tanks (23 m^3 each) until they reached an average body weight (BW) of 1.5 g. Prior to stocking in the experimental tanks, shrimp were size-graded to ensure uniform BW across treatments. The average initial BW was 1.49 ± 0.11 g (n = 7,360; min-max: 1.3–1.9 g), with a coefficient of variation of 7.6%. Shrimp were stocked under 140 animals/m^2 (80 animals per tank) at an initial salinity of 16.7 ± 0.3 psu and acclimated to the target salinities over a 10-day period, following the method described by Castro et al. (2017). To increase salinity to 40 psu, crude sea salt (996.4 g/kg sodium chloride) was dissolved into seawater, mixed in a 20-m^3 header tank, and filtered through sand for one week. Salinity adjustments were made gradually at a rate of 1.5–2.0 psu/day to minimize stress.

Continuous aeration was provided using a 2.0 hp blower (model CR-5, Ibram Industria Brasileira de Maquinas Ltda., São Paulo, Brazil), with air distributed via h 0.5-m long microporous flexible hoses (Aero-Tube™, Tekni-Plex Aeration, Austin, Texas, USA) laid at the bottom of each tank. Water quality parameters were measured daily. The pH and temperature were recorded using a portable pH meter (H98107 pHEP, Hanna Instruments Brasil, Barueri, Brazil), while salinity was measured using a refractometer (RTS-101ATC, Instrutherm Instrumentos de Medição Ltda, São Paulo, Brazil) at 13:00 h. Measurements were taken in all tanks. The average recorded water quality parameters were:

temperature, 28.4 ± 0.59 °C (n = 6,417); pH, 7.95 ± 0.14 (n = 6,417); salinity, 16.9 ± 1.17 psu (n = 3,186) for the 16 psu condition, and 40.5 ± 6.73 psu (n = 3,231) for the 40 psu condition (Figure 1).



Each data point represents the daily average value of all rearing tanks within each salinity treatment.

Figure 1 - Daily fluctuations in salinity (psu), temperature (°C), and pH during the experimental period for *Penaeus vannamei* reared under low and high salinity conditions.

Shrimp were fed experimental diets for 91 days using feeding trays. The feed was delivered four times daily at 07:00, 10:00, 13:00, and 16:00 h. Feeding trays, measuring 14.3 × 3.5 cm (diameter × height), were centrally placed in each tank at one unit per tank. The daily feed ration was divided into four meals. The first feeding was 25%, the second and third feedings were each 15%, and the fourth feeding was 45% of the total daily ration. Feeding rates were calculated using the equation $FR = 0.0931 \times BW^{0.6200}$, in which FR represents the maximum daily feed intake of a shrimp with a specific BW (Nunes and Parsons, 2000). A 30% feed restriction was applied to the calculated FR to prevent an excessive feed conversion ratio (FCR). Daily feed amounts were adjusted assuming a fixed daily decrease in shrimp survival and a BW gain of 100 mg shrimp per day. From the 22nd day of culture onwards, five shrimp per tank were sampled bi-weekly to monitor BW gain. Feed adjustments for the following period were made based on the average daily BW gain recorded during the previous sampling for each specific tank, while maintaining a fixed daily survival decrease of 0.2%. The feeding trays were inspected daily before each meal to check for dead animals and leftovers. Any uneaten feed was collected from each tray and pooled by tank. The DM content of the uneaten feed was then determined by drying the samples to a constant weight in a convection oven.

The proximate composition of the diets was determined according to AOAC methods (AOAC, 2002). Dry matter was determined by drying samples in an air-circulating oven for 24 h at 105 °C. The Dumas combustion method was applied for CP analysis (AOAC 968.06), while crude fat was determined by acid hydrolysis (AOAC 954.02). Ash content was determined by burning samples in a muffle furnace at 600 °C for 2 h (AOAC 942.05), while crude fiber was determined by the enzymatic-gravimetric method (AOAC 992.16). The amino acid composition was determined using high-performance liquid chromatography following the method described by Figueiredo-Silva et al. (2015).

At shrimp harvest, animals were counted and weighed individually on a precision scale with 0.01-g accuracy (Ohaus Adventurer, model ARA520, Toledo do Brasil Indústria de Balanças Ltda., São Bernardo do Campo, Brazil). Growth performance, feed utilization, and daily nutrient intake calculations were as follows:

$$\text{Survival rate (\%)} = \frac{\text{Final } N}{\text{Initial } N} \times 100 \quad (1)$$

$$\text{Weekly growth (g/week)} = \frac{(\text{FBW} - \text{IBW})}{91 \text{ days}} \times 7 \quad (2)$$

$$\text{Specific growth rate (SGR, \%/day)} = \frac{\ln \text{FBW} - \ln \text{IBW}}{91 \text{ days}} \times 100 \quad (3)$$

$$\text{Weight gain (\%)} = \frac{(\text{FBW} - \text{IBW})}{\text{IBW}} \times 100 \quad (4)$$

$$\text{Yield (MT/ha)} = \frac{\text{BIOg (MT)}}{\text{Tank bottom area (ha)}} \quad (5)$$

$$\text{Total feed intake (g, DM)} = \text{amount of feed distributed} - \text{amount of uneaten feed} \quad (6)$$

$$\text{Daily feed intake (g, DM/kg BW per day)} = \frac{\text{Total feed intake}}{\text{Average BW} \times \text{average } N \times 91 \text{ days}} \quad (7)$$

$$\text{Daily nutrient intake (g, DM/kg BW per day)} = \text{Daily feed intake} \times \text{\% Nutrient content} \quad (8)$$

$$\text{Average BW (kg)} = \frac{(\text{IBW} + \text{FBW})}{(2 \times 1000)} \quad (9)$$

$$\text{Average } N = \frac{(\text{Initial } N + \text{Final } N)}{2} \quad (10)$$

$$\text{FCR} = \frac{\text{Total feed intake}}{(\text{BIOg})} \quad (11)$$

$$\text{Feed efficiency (FE)} = \frac{\text{BIOg} + \text{dBIO}}{\text{Total feed intake}} \quad (12)$$

$$\text{dBIO} = (\text{FBW} - \text{IBW}) \times (\text{Initial } N - \text{Final } N) \quad (13)$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{BIOg}}{\text{Protein intake (g, DM)}} \quad (14)$$

in which Initial N = number of shrimp at stocking, Final N = number of shrimp at harvest, IBW = initial body weight (g), FBW = final body weight (g), BIOi (g) = initial biomass, BIOf (g) = final biomass, BIOg (g, wet weight) = gained biomass (BIOf – BIOi), and dBIO (g) = dead biomass.

A three-way ANOVA was conducted to assess the main effects and interactions of the three experimental factors: dietary CP content, Met content, and salinity on shrimp performance. This analysis was used to determine whether these factors, individually or in combination, significantly influenced shrimp growth performance, feed efficiency, and daily nutrient intake. The model accounted for all main effects and all possible interactions between the factors, providing a detailed understanding of their influence on the parameters evaluated. The following mathematical model was adopted:

$$Yij = \mu + \tau i + \epsilon ij, \quad (15)$$

in which Yij is the j-th observation of dietary CP content, Met content, and salinity; μ is the general mean response; τi is the non-random effect of dietary CP content, Met content, and salinity, wherein $\sum_{i=1}^{k} \tau i = 0$; and ϵij is the random dietary CP content, Met content, and salinity error.

Where significant effects were detected in the ANOVA, Tukey's Honest Significant Difference (HSD) test was employed for post-hoc comparisons. The normality of residuals was assessed using the Shapiro-Wilk test, which evaluated whether the data followed a normal distribution. The homogeneity of variances across different groups was tested using Levene's test. In cases where the homogeneity of variances assumption was violated, Welch's ANOVA and a robust three-way ANOVA approach were used. All statistical analyses were performed using R software, with a significant level set at 5%.

3. Results

The three-way ANOVA revealed that salinity had a highly significant effect on survival (P = 0.001), with higher survival rates were observed at 40 psu (95.5 ± 4.5%) than at 16 psu (90.7 ± 5.7%). Crude protein showed a marginal effect on survival (P = 0.059), indicating a potential trend towards higher survival at increased CP levels, though not statistically significant. Methionine did not significantly affect survival (P = 0.68). None of the interaction effects were significant: CP × Met (P = 0.793), CP × salinity (P = 0.921), and Met × salinity (P = 0.813). However, the three-way interaction CP × Met × salinity approached significance (P = 0.063). These results emphasize the significant impact of salinity on survival rates, while CP and Met, along with their interactions, appear to have less effect (Table 3).

Yield, an important indicator of overall treatment efficacy, is reported separately because it includes both survival and growth metrics. The three-way ANOVA revealed a highly significant effect of salinity on yield (P<0.001). In contrast, neither CP (P = 0.690) nor Met (P = 0.206) demonstrated significant main effects. Additionally, no significant interaction effects were observed between CP × Met, CP × salinity, Met × Salinity, or CP × Met × salinity (P>0.05). Post-hoc analysis with Tukey's HSD test indicated that yield was significantly lower at 40 psu (13.8 ± 0.9 MT/ha) compared with 16 psu (16.9 ± 1.6 MT/ha; difference = −3.0 MT/ha, P<0.001), suggesting that lower salinity (16 psu) is more beneficial to achieving higher yields (Table 3).

Salinity had a highly significant effect on all growth parameters — FBW, weekly growth, weight gain (WG), and SGR — with all showing P<0.001. Crude protein and Met did not exhibit significant main effects (P>0.05). No significant interactions were found between CP × Met, CP × salinity, Met × salinity, or CP × Met × salinity (P>0.05). Post-hoc Tukey HSD tests showed that shrimp reared at 16 psu consistently outperformed those at 40 psu across all parameters: FBW was higher by 2.2 g (13.6 ± 1.2 vs. 11.4 ± 0.7 g), weekly growth by 0.18 g (0.94 ± 0.10 vs. 0.76 ± 0.05 g), WG by 150.6% (816.1 ± 84.0 vs. 665.5 ± 44.5%), and SGR by 0.19%/day (2.43 ± 0.10 vs. 2.24 ± 0.06%/day, respectively; all P<0.001). These results indicate that lower salinity (16 psu) significantly promoted better growth performance in shrimp (Table 3).

Salinity had a highly significant effect on daily feed intake (P<0.001), as did Met (P = 0.03). Crude protein did not significantly affect feed intake (P>0.05). The interaction Met × salinity was significant (P = 0.03), while other interactions were not. Tukey's HSD test showed that daily feed intake was higher at 40 psu compared with 16 psu (24.05 ± 0.90 vs. 22.00 ± 0.89 g/kg BW/day, respectively; P<0.001). Shrimp fed 0.6% Met had significantly higher feed intake compared with 0.7% Met (P = 0.021), with no significant differences between other Met levels. Additionally, at 40 psu, feed intake varied significantly with Met levels, indicating that salinity influences the effect of Met on feed intake (Table 4).

Salinity also significantly affected feed efficiency (P = 0.001), with no significant effects for CP (P = 0.36) or Met (P = 0.64). Tukey's HSD test revealed that feed efficiency was lower at 40 psu compared with 16 psu (difference = −0.11 or −11%, P<0.001). This suggests that 16 psu promotes better feed efficiency (Table 4).

Crude protein had a significant main effect on the protein efficiency ratio (PER) (P<0.001), as did salinity (P = 0.001), with no significant effect of Met (P = 0.44). Tukey's HSD test showed that PER

Table 3 - Survival, yield, and growth performance of the juvenile *P. vannamei* fed different dietary crude protein (CP) and methionine (Met) levels at different salinities

CP	Met	Salinity	Survival (%)	Initial body weight (g)	Final body weight (g)	Yield (MT/ha)	Weekly growth rate (g/week)	Weight gain (%)	Specific growth rate (%/day)
33	0.6	16	92.1 ± 5.0	1.49 ± 0.01	13.7 ± 1.6	16.9 ± 2.1	0.94 ± 0.12	819.0 ± 108	2.43 ± 0.13
33	0.7	16	88.5 ± 6.0	1.49 ± 0.01	13.8 ± 1.2	17.0 ± 1.6	0.94 ± 0.09	825.8 ± 85.9	2.44 ± 0.10
33	0.9	16	91.1 ± 3.5	1.50 ± 0.02	13.5 ± 0.8	16.7 ± 1.1	0.93 ± 0.06	804.9 ± 56.9	2.42 ± 0.07
37	0.6	16	91.5 ± 5.5	1.49 ± 0.01	13.2 ± 0.9	16.4 ± 1.2	0.91 ± 0.07	789.3 ± 61.7	2.40 ± 0.08
37	0.7	16	93.0 ± 5.4	1.49 ± 0.02	13.2 ± 0.8	16.3 ± 1.0	0.90 ± 0.06	783.9 ± 57.4	2.39 ± 0.07
37	0.9	16	92.5 ± 5.4	1.48 ± 0.02	13.7 ± 1.4	17.1 ± 1.9	0.94 ± 0.11	827.1 ± 93.3	2.44 ± 0.11
33	0.6	40	93.0 ± 7.3	1.49 ± 0.03	11.2 ± 0.6	13.5 ± 0.8	0.75 ± 0.04	650.5 ± 40.0	2.21 ± 0.06
33	0.7	40	96.1 ± 3.2	1.48 ± 0.02	11.9 ± 0.7	14.5 ± 0.9	0.80 ± 0.05	701.4 ± 40.8	2.29 ± 0.06
33	0.9	40	95.0 ± 3.7	1.50 ± 0.01	11.3 ± 0.4	13.6 ± 0.5	0.75 ± 0.03	651.9 ± 24.8	2.22 ± 0.04
37	0.6	40	99.1 ± 2.2	1.50 ± 0.01	11.5 ± 0.9	14.0 ± 1.3	0.77 ± 0.07	665.6 ± 62.2	2.23 ± 0.09
37	0.7	40	93.6 ± 3.2	1.49 ± 0.02	11.6 ± 0.5	14.0 ± 0.7	0.78 ± 0.04	677.2 ± 29.7	2.25 ± 0.04
37	0.9	40	97.3 ± 3.2	1.50 ± 0.02	11.3 ± 0.7	13.7 ± 1.1	0.75 ± 0.06	654.2 ± 51.4	2.22 ± 0.07
Summary of the ANOVA (P-values)									
CP			0.06	0.84	0.55	0.63	0.55	0.55	0.57
Met			0.68	0.62	0.81	0.82	0.80	0.75	0.12
Salinity			***	0.68	**	**	**	**	***
CP × Met			0.79	0.24	0.61	0.55	0.61	0.52	0.39
CP × salinity			0.92	0.53	0.79	0.74	0.74	0.81	0.65
Met × salinity			0.81	0.47	0.32	0.30	0.29	0.26	0.31
CP × Met × salinity			0.06	0.76	0.61	0.55	0.63	0.62	0.70

Data represents the means (±standard deviation) of seven to eight replicate tanks.
** $P<0.05$; *** $P<0.001$.

was lower at 40 psu compared with 16 psu (difference = −0.27, P<0.001) and lower in the 37% CP treatment compared with the 33% CP treatment (difference = −0.23, P<0.001). These findings indicate that both higher salinity and CP levels negatively impacted PER (Table 4).

Salinity significantly affected FCR (P<0.001), while CP did not (P = 0.23). Methionine had a significant effect on FCR (P = 0.02). Tukey's HSD tests indicated that FCR was higher at 40 psu compared with 16 psu (difference = 0.18, P<0.001), and the 0.7% Met level resulted in a lower FCR compared with 0.6% Met (difference = −0.05, P = 0.012). These results suggest that higher salinity worsens FCR, while the 0.7% Met level improves it (Table 4).

Three-way ANOVA revealed significant effects of CP (P<0.001) and salinity (P<0.001) on daily CP intake, with no significant effect of Met (P = 0.12). Tukey's HSD tests showed higher CP intake in the 37% CP treatment compared with 33% CP (difference = 1.02 g/kg BW/day, P<0.001) and higher intake at 40 psu compared with 16 psu (difference = 0.75 g/kg BW/day, P<0.001) (Table 5).

For daily Met intake, significant main effects were found for CP (P = 0.042), Met (P<0.001), and salinity (P = 0.001), with a significant interaction between Met × Salinity (P = 0.036). Tukey's HSD tests indicated lower Met intake in the 37% CP group compared with the 33% CP group (difference = −6.1 mg Met/kg BW/day, P<0.001), with intake increasing significantly with higher Met levels. Methionine intake was also higher at 40 psu compared with 16 psu (difference = 16.7 mg/kg BW/day, P<0.001) (Table 5).

Daily total sulfur amino acid intake was significantly affected by Met (P<0.001) and salinity (P = 0.001), with a significant interaction between Met × salinity (P = 0.005). Tukey's HSD tests showed significant increases in sulfur amino acid intake with higher Met levels, and higher intake at 40 psu compared with 16 psu (difference = 25.2 mg Met+Cys/kg BW/day, P<0.001) (Table 5).

Table 4 - Feed utilization of the juvenile *P. vannamei* fed different dietary crude protein (CP) and methionine (Met) levels at different salinities

CP	Met	Salinity	Daily feed intake (g/kg BW per day)	Feed efficiency	Protein efficiency ratio	Feed conversion ratio
33	0.6	16	21.9 ± 1.2	0.84 ± 0.08	2.18 ± 0.21A	1.20 ± 0.12
33	0.7	16	22.2 ± 0.6	0.85 ± 0.06	2.22 ± 0.15A	1.19 ± 0.08
33	0.9	16	21.8 ± 0.6	0.85 ± 0.04	2.23 ± 0.10A	1.18 ± 0.05
37	0.6	16	22.4 ± 0.7	0.82 ± 0.05	1.92 ± 0.12B	1.22 ± 0.07
37	0.7	16	22.2 ± 0.5	0.82 ± 0.04	1.93 ± 0.08B	1.22 ± 0.06
37	0.9	16	22.0 ± 1.2	0.84 ± 0.07	2.01 ± 0.18B	1.20 ± 0.11
33	0.6	40	24.5 ± 0.9	0.72 ± 0.02	1.85 ± 0.04A	1.40 ± 0.03
33	0.7	40	23.1 ± 0.6	0.76 ± 0.03	1.99 ± 0.09A	1.32 ± 0.06
33	0.9	40	24.3 ± 0.7	0.71 ± 0.02	1.88 ± 0.06A	1.40 ± 0.04
37	0.6	40	24.1 ± 0.9	0.71 ± 0.04	1.67 ± 0.10B	1.41 ± 0.08
37	0.7	40	23.9 ± 0.6	0.74 ± 0.03	1.73 ± 0.07B	1.36 ± 0.05
37	0.9	40	24.1 ± 0.7	0.71 ± 0.04	1.71 ± 0.09B	1.41 ± 0.07
Summary of the ANOVA (P-values)						
CP			0.49	0.36	***	0.23
Met			**	0.64	0.44	**
Salinity			***	**	**	***
CP × Met			0.50	0.78	0.59	0.68
CP × salinity			0.64	0.89	0.55	0.71
Met × salinity			**	0.17	0.17	0.09
CP × Met × salinity			0.16	0.88	0.87	0.94

Data are presented as means (± standard deviation) based on seven to eight replicate tanks.
Different uppercase letters indicate significant differences between CP levels within the same Met × salinity treatment (ANOVA, P<0.05).
** P<0.05; *** P<0.001.

Lastly, daily total amino acid (TAA) intake was significantly influenced by CP (P<0.001) and salinity (P = 0.001), with no significant effect of Met (P = 0.87). Tukey's HSD tests revealed higher TAA intake in the 37% CP treatment compared with the 33% CP treatment (difference = 0.915 g/kg BW/day, P<0.001) and higher intake at 40 psu compared with 16 psu (difference = 0.690 g/kg BW/day, P<0.001) (Table 5).

The feeding rate, expressed as a percentage of shrimp BW, consistently declined throughout the experiment for both salinity levels. Initially, shrimp at both salinities were fed at rates around 4.5% of their BW, steadily decreasing as the shrimp grew. By the end of the trial, feeding rates had dropped to approximately 2% of shrimps' BW.

Table 5 - Daily intake of crude protein (CP), methionine (Met), total sulfur amino acids (TSAA), and total amino acids (TAA) of the juvenile *P. vannamei* fed different dietary CP and Met levels at different salinities

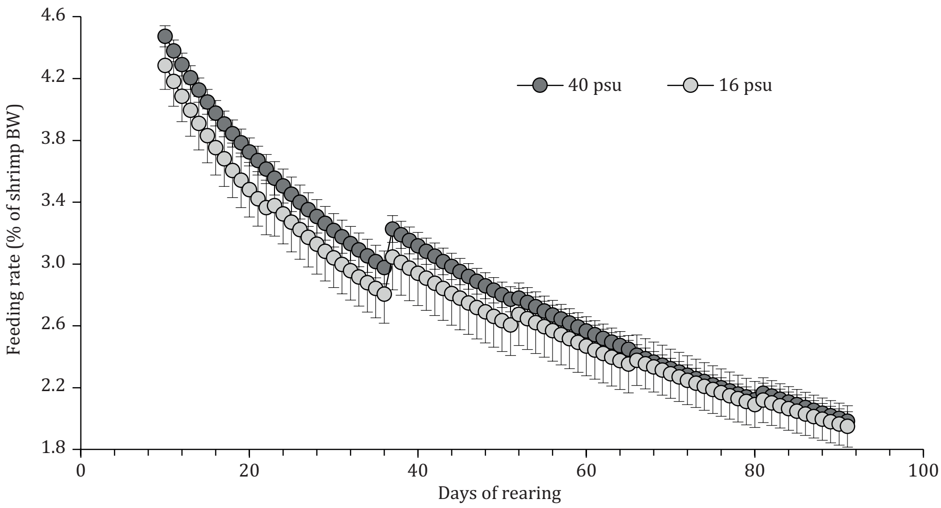
CP	Met	Salinity	Daily CP intake (g/kg BW per day)	Daily Met intake (g/kg BW per day)	Daily TSAA intake (g/kg BW per day)	Daily TAA Intake (g/kg BW per day)
33	0.6	16	8.5 ± 0.5A	0.16 ± 0.01aA	0.26 ± 0.02a	7.79 ± 0.42
33	0.7	16	8.4 ± 0.2A	0.19 ± 0.00bA	0.30 ± 0.01b	7.86 ± 0.20
33	0.9	16	8.2 ± 0.2A	0.21 ± 0.01cA	0.32 ± 0.01c	7.60 ± 0.23
37	0.6	16	9.5 ± 0.3B	0.15 ± 0.00aB	0.27 ± 0.01a	8.65 ± 0.25
37	0.7	16	9.4 ± 0.2B	0.18 ± 0.01bB	0.30 ± 0.01b	8.71 ± 0.21
37	0.9	16	9.2 ± 0.5B	0.21 ± 0.01cB	0.32 ± 0.02c	8.64 ± 0.46
33	0.6	40	9.4 ± 0.3A	0.18 ± 0.01aA	0.30 ± 0.01a	8.68 ± 0.31
33	0.7	40	8.8 ± 0.2A	0.20 ± 0.01bA	0.31 ± 0.01b	8.21 ± 0.23
33	0.9	40	9.2 ± 0.3A	0.24 ± 0.01cA	0.35 ± 0.01c	8.47 ± 0.25
37	0.6	40	10.3 ± 0.4B	0.16 ± 0.01aB	0.29 ± 0.01a	9.31 ± 0.34
37	0.7	40	10.2 ± 0.3B	0.20 ± 0.01bB	0.32 ± 0.01b	9.36 ± 0.25
37	0.9	40	10.1 ± 0.3B	0.24 ± 0.01cB	0.35 ± 0.01c	9.47 ± 0.30
Summary of the ANOVA (P-values)						
CP			***	**	0.09	***
Met			0.12	***	***	0.87
Salinity			***	***	***	***
CP × Met			0.47	0.17	0.99	0.50
CP × salinity			0.76	0.77	0.51	0.74
Met × salinity			0.06	**	**	0.06
CP × Met × salinity			0.35	0.20	0.13	0.35

Data are presented as means (± standard deviation) based on seven to eight replicate tanks.
Different lowercase letters indicate significant differences between Met levels within the same CP × salinity treatment (ANOVA, P<0.05)
Different uppercase letters indicate significant differences between CP levels within the same Met × salinity treatment (ANOVA, P<0.05).
** P<0.05; *** P<0.001.

Shrimp kept at a salinity of 40 psu exhibited slightly higher feeding rates compared with those at 16 psu, particularly between days 10 and 65 (Figure 2). During this period, the difference in feeding rate at 40 psu was 0.17% (ranging from 0.10 to 0.25%) higher than at 16 psu. By the end of the 91-day observation period, feeding rates for both salinities converged, indicating minimal differences between the two treatments.

4. Discussion

This study examined the interactive effects of dietary CP, Met levels, and salinity on the growth performance, feed utilization, and nutrient intake of juvenile *P. vannamei*. Given that the isosmotic point for *P. vannamei* ranges between 20 and 25 psu (Castille and Lawrence, 1981; Jaffer et al., 2020), we selected salinity levels of 16 and 40 psu to represent moderately hypo- and hyperosmotic conditions, respectively. Although 16 psu is below the isosmotic range, previous studies have reported it as optimal



Error bars indicate the standard deviation around the mean feeding rate, averaged across all dietary treatments (crude protein × methionine) for each salinity condition.

Figure 2 - Feeding rates (% of shrimp body weight [BW]) adopted during the experimental period under two salinity levels.

for the growth of juvenile shrimp of similar size (Bray et al., 1994), making it a valid reference point for the hyperosmotic condition.

Shrimp reared in high salinity environments, such as 40 psu, typically experience impaired growth due to increased energy demands for osmoregulation (Pequeux, 1995; Li et al., 2015). Energy that could otherwise support growth is redirected to maintain internal ionic balance (Ern et al., 2014). Based on this, we hypothesized that increasing dietary protein and Met could help compensate for the elevated metabolic demands by supplying essential nutrients to support both growth and homeostasis under hyperosmotic stress.

The rationale for increasing protein stems from its role in meeting the higher amino acid requirements associated with osmoregulatory processes. Shrimp exposed to high salinity accumulate higher levels of total ninhydrin-positive substances (TNPS), including free amino acids and small peptides critical for intracellular osmotic regulation (Roy et al., 2007). This diversion of amino acids for osmoregulation suggests a need for higher protein intake to maintain optimal growth and physiological function under salinity stress.

Methionine was also supplemented due to its multifunctional role in antioxidative defense, immune support, and osmoregulation, all of which become increasingly important in high-salinity environments. As a limiting amino acid, Met supports protein synthesis and serves as a precursor for both glutathione, a key antioxidant (Belghit et al., 2014; Wu et al., 2023), and taurine, a major osmolyte involved in osmoregulation and immune modulation (Brosnan and Brosnan, 2006; Forman et al., 2009). Therefore, Met supplementation was expected to enhance the shrimps' physiological resilience under high-salinity conditions.

Our results confirmed that salinity was the dominant factor influencing shrimp performance. Shrimp reared at 40 psu exhibited significantly lower growth, feed efficiency, and yield compared with those at 16 psu, consistent with previous reports on the negative effects of high salinity (Ponce-Palafox et al., 1997; Li et al., 2008; Gao et al., 2016). Interestingly, despite reduced growth, shrimp at 40 psu had higher survival rates (95.5%) than those at 16 psu (90.7%). Although this contrasts with Bray et al. (1994), who found no salinity effect on survival across a wide range (5–49 psu), it highlights the species' exceptional adaptability to varying salinities. These findings suggest that while growth is compromised under hyperosmotic stress, *P. vannamei* maintains robust survival capacity.

Shrimp reared at 40 psu also consumed significantly more nutrients, including CP (+0.75 g/kg BW/day), Met (+16.7 mg/kg BW/day), total sulfur amino acids (+25.2 mg Met+Cys/kg BW/day), and TAA (+0.69 g/kg BW/day) than those at 16 psu. However, this increased nutrient intake did not translate into improved growth or feed efficiency likely because the additional nutrients were redirected toward osmoregulation rather than somatic growth. This suggests that while dietary interventions provided some support, they were insufficient to fully offset the metabolic costs of high salinity. Moreover, the restricted feeding protocol used in this study may have influenced the results. Shrimp were fed biweekly based on BW, which meant those growing more slowly (i.e., at 40 psu) received proportionally more feed to maintain consistent nutrient delivery (Figure 2). Thus, the observed increase in nutrient intake at 40 psu may have been driven by the feeding protocol rather than a compensatory physiological response. In contrast, a more responsive feeding approach, such as *ad libitum* feeding, might allow shrimp to adjust intake in real time according to their metabolic needs, potentially improving growth and feed efficiency.

While the feeding regime used here reflects common commercial practices, in which feed inputs are based on estimated growth curves, it may not be optimal under environmentally stressful conditions. Under high salinity, in which metabolic demands are elevated, a static feeding strategy may fail to meet the shrimps' nutritional needs, especially for critical nutrients like methionine. This raises the possibility that a more flexible or dynamic feeding strategy could better support shrimp performance under suboptimal salinity conditions.

Future studies that utilize *ad libitum* or demand-based feeding protocols may offer clearer insights into how nutrient intake interacts with growth performance and osmoregulation under hyperosmotic conditions. Allowing shrimp to self-regulate feed intake could reveal whether they can compensate for increased energy expenditure by voluntarily increasing intake, leading to improved growth and feed efficiency. This approach could also better reveal the efficacy of dietary interventions, such as protein and methionine, in supporting shrimp health and productivity.

In conclusion, while the results of this study remain relevant for commercial applications, particularly in identifying the resilience of *P. vannamei* to hyperosmotic stress, the limitations observed suggest that future research could optimize shrimp growth and nutrient utilization through more flexible feeding regimes. This would not only enhance our understanding of shrimp physiology under challenging conditions but also offer practical solutions for improving productivity in commercial aquaculture systems, especially in environments with suboptimal salinity levels.

5. Conclusions

Our findings highlight salinity as the dominant factor affecting shrimp performance, with 16 psu resulting in superior growth, feed efficiency, and yield compared with the hyperosmotic condition of 40 psu. Although shrimp reared at 40 psu exhibited higher nutrient intake, this did not translate into improved growth, likely due to the increased energy demands of osmoregulation in hyperosmotic environments. Dietary protein and methionine levels have minimal effects on growth and survival, indicating that these nutrients alone may not be sufficient to counteract the stress imposed by high salinity. The study also suggests that restricted feeding protocols may limit the ability to fully capture the potential benefits of dietary interventions in high-salinity conditions.

Data availability

The entire dataset supporting the results of this study is available upon request to the corresponding author.

Author contributions

Conceptualization: Leite, J. S. and Nunes, A. J. P. **Data curation:** Leite, J. S. and Nunes, A. J. P. **Formal analysis:** Leite, J. S. and Nunes, A. J. P. **Funding acquisition:** Nunes, A. J. P. **Investigation:** Façanha,

F. N.; Leite, J. S. and Nunes, A. J. P. **Methodology:** Façanha, F. N.; Leite, J. S. and Nunes, A. J. P. **Project administration:** Leite, J. S. and Nunes, A. J. P. **Resources:** Nunes, A. J. P. **Supervision:** Nunes, A. J. P. **Writing – original draft:** Façanha, F. N. and Nunes, A. J. P. **Writing – review & editing:** Façanha, F. N.; Leite, J. S. and Nunes, A. J. P.

Conflict of interest

The authors declare no conflict of interest.

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