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Effect of dietary methionine and energy on growth performance and feed utilization of juvenile *Penaeus vannamei* reared under hyperosmotic salinity

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ABSTRACT - This study assessed the impact of varying dietary methionine (Met) and digestible energy (DE) levels on the growth performance, feed utilization, and glycemia in hemolymph of juvenile *Penaeus vannamei* reared under hyperosmotic salinity (43-48 g L^{-1}). A 2 × 3 factorial design was used to evaluate six diets, varying in two DE levels (15.7 and 16.6 MJ kg^{-1}, DM basis), achieved through soybean oil addition, and three Met levels (7.6, 8.5, and 9.9 g kg^{-1}, DM basis), adjusted with crystalline DL-Met-Met supplementation. Dietary crude protein was maintained at 371 ± 3.0 g kg^{-1} (DM basis). Shrimp of 2.9 ± 0.04 g were reared at 100 animals m^{-2} in forty 0.5-m^3 tanks for 60 days. No differences were found in shrimp survival, specific growth rate, weight gain, and feed conversion ratio (FCR) across the different DE and Met levels (P>0.05). However, dietary DE levels significantly influenced daily gross energy intake (P<0.01) and daily DE intake (P<0.01). Dietary Met levels significantly affected daily Met intake (P<0.01) and total sulfur amino acid intake (P<0.01). Glucose concentration in shrimp hemolymph was significantly influenced by dietary Met levels (P = 0.02), with higher levels leading to increased glucose concentrations. Results suggested that while Met levels affect certain metabolic responses, they did not translate into enhanced growth performance under hyperosmotic conditions. Experimental diets met the shrimp's dietary requirements for energy and Met, with no additional benefits observed from increased levels.

Keywords: aquaculture, digestible energy, hemolymph glucose, nutrient optimization, shrimp metabolism

1. Introduction

The whiteleg shrimp, *Penaeus vannamei*, is one of the most important species in global aquaculture due to its ability to adapt to a wide range of salinities, making it suitable for diverse farming environments (Briggs et al., 2004). This euryhaline species is capable of efficient osmoregulation in both low and high salinity conditions (Chong-Robles et al., 2014). However, extreme salinities, particularly hyperosmotic conditions (i.e., > 35 g L^{-1}) impose substantial metabolic demands on this species, primarily by increasing the energy required for osmoregulation (Villarreal et al., 1994; Pequeux, 1995). At salinities between 20 and 25 g L^{-1}, *P. vannamei* maintains hemolymph isosmotic with its environment, minimizing the energy required for osmoregulation (Castille and Lawrence, 1981; Jaffer et al., 2020).

In coastal aquaculture systems, especially those located in arid and semi-arid regions, hypersaline conditions frequently occur due to the disparity between evaporation rates and freshwater inputs (Zampatti, 2010; Gillanders et al., 2011; Valentim et al., 2018). Water salinities of more than 40 g L^{-1} have been reported in shrimp farming regions across Saudi Arabia, Egypt, Iran (Rosenberry, 1999; Sadek et al., 2002), Mexico (Moreno-Figueroa et al., 2018), and Brazil (Castro et al., 2018). Hypersaline culture environments have been shown to hinder shrimp growth and decrease feed efficiency (Saoud and Davis, 2005; Castro et al., 2018; Ponce-Palafox et al., 2019; Rufino et al., 2021). As a result, there is a growing interest in developing dietary strategies that can alleviate the negative impacts of high salinity on shrimp performance.

To address these challenges, various dietary strategies have been explored, including the manipulation of carbohydrates, proteins, lipids, and essential amino acids (EAA) (Wang et al., 2014; Sui et al., 2015; González-Félix et al., 2009; Gong et al., 2004). Methionine (Met) is a key amino acid (AA) that plays a vital role in protein synthesis, antioxidant response, and immune function (Belghit et al., 2014; Wu et al., 2024). Despite its importance, the specific Met and digestible energy (DE) requirements for shrimp under hyperosmotic conditions remain underexplored.

Given the increased metabolic demands and stress associated with hyperosmotic environments, higher dietary DE and Met levels may be necessary to maintain optimal growth performance in shrimp under these conditions. Although previous studies have established Met requirements for *P. vannamei* in various conditions, the focus has primarily been on more isosmotic environments (Façanha et al., 2016; Façanha et al., 2018; Façanha et al., 2019). However, there remains a critical gap in understanding how these dietary requirements might change when shrimp are reared in hyperosmotic conditions.

This study aimed to assess the impact of varying dietary Met and DE levels, achieved through crystalline Met and soybean oil supplementation, on the growth performance and metabolic responses of shrimp reared in hyperosmotic salinity conditions. The research specifically seeks to determine whether increased dietary DE and Met levels are necessary to support the elevated energy demands of shrimp under these stressful environmental conditions. By addressing this critical knowledge gap, the study intends to contribute to the development of more effective feed formulations, ultimately enhancing the sustainability and production of shrimp aquaculture in high-salinity regions.

2. Material and methods

The study was conducted in the aquaculture research facilities of the Laboratory of Aquatic Animal Nutrition (3°50'01.55"S and 38°25'22.74" W) of the Instituto de Ciências do Mar (LABOMAR) of the Universidade Federal do Ceará (UFC), Eusébio, CE, Brazil.

The study followed a 2 × 3 factorial design, evaluating six dietary treatments that combined two independent variables: total dietary DE content (15.7 and 16.6 MJ kg^{-1} of the diet, dry matter (DM) basis) and total dietary Met content (7.6, 8.5, and 9.9 g kg^{-1} of the diet, DM basis). The experiment was conducted under hyperosmotic conditions (43-48 g L^{-1}) for *P. vannamei*. Each of the six diets was evaluated in six to seven tanks, resulting in 40 rearing tanks. The experimental replicates were assigned to treatments using a completely randomized design. 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.

In a previous study, Nunes et al. (2019) demonstrated that the crude protein (CP) content of shrimp diets could be reduced from 436 to 371 g kg^{-1} (DM basis) without negatively affecting the growth performance of *P. vannamei*, if total dietary Met (Met + Cys) content was maintained at a minimum of 7.8 g kg^{-1} (13.2 g kg^{-1} DM basis), in diets containing 20 MJ kg^{-1}, tested under salinity conditions of 34 ± 3.1 g L^{-1}. Building on these findings, all diets in the current study were formulated to be isonitrogenous, ensuring consistent CP levels across all treatments. The average CP level across diets

was 371.2 ± 3.0 g kg^{-1}, (DM basis), enabling the isolation of effects of dietary Met and DE variations without confounding influences from differing CP levels (Table 1).

Table 1 - Raw material and proximate composition of experimental diets

Raw material	Dietary inclusion (g kg^{-1})/Diet (Met g kg^{-1}/DE MJ kg^{-1})					
	8/16	9/16	10/16	8/17	9/17	10/17
Soybean meal[1]	377.9	376.3	374.7	337.0	338.4	339.9
Wheat flour[2]	263.5	264.0	264.5	270.0	270.0	270.0
Soy protein concentrate[3]	80.2	79.9	79.7	108.5	106.2	103.8
Salmon meal[4]	60.0	60.0	60.0	60.0	60.0	60.0
Cassava starch	30.0	30.0	30.0	30.0	30.0	30.0
Kaolin	30.0	30.0	30.0	30.0	29.3	28.6
Salmon oil	20.0	20.0	20.0	20.0	20.0	20.0
Krill meal[5]	20.0	20.0	20.0	20.0	20.0	20.0
Soy lecithin	19.3	19.3	19.3	19.3	19.3	19.3
Soybean oil	-	-	-	17.7	17.8	18.0
Calcium carbonate	15.9	15.9	15.9	15.9	15.9	16.0
Sodium monophosphate	11.5	11.6	11.6	11.0	11.1	11.1
Dextrin	10.0	10.0	10.0	10.0	10.0	10.0
Potassium chloride	11.1	11.1	11.1	9.9	10.0	10.1
Magnesium sulfate	18.0	18.0	18.0	8.0	8.0	8.0
EAA mixture[6]	19.2	19.3	19.6	19.3	19.4	19.7
DL-Met-Met[7]	2.6	3.6	4.7	2.6	3.6	4.7
Synthetic binder[8]	4.5	4.5	4.5	4.5	4.5	4.5
Vitamin-mineral premix[9]	3.0	3.0	3.0	3.0	3.0	3.0
Coarse salt	1.9	1.9	1.9	1.8	1.8	1.9
Vitamin C[10]	0.6	0.6	0.6	0.6	0.6	0.6
Cholesterol[11]	0.4	0.4	0.4	0.4	0.4	0.4
Proximate composition (g kg^{-1}, dry matter)						
Dry matter	920.8	918.3	916.7	922.2	918.7	909.2
Crude protein	371.6	368.4	377.0	370.1	369.3	371.0
Ether extract	64.9	65.8	63.7	82.7	81.7	80.3
Crude fiber	30.2	29.4	30.3	28.1	28.5	29.7
Ash	113.6	120.4	121.4	112.1	113.3	113.1
NFE[12]	370.2	389.4	401.3	364.6	383.7	395.1
Gross energy (MJ kg^{-1})	18.4	19.1	18.9	19.1	19.6	19.8
Digestible energy (MJ kg^{-1})[13]	15.6	15.7	15.7	16.4	16.4	16.6

[1] Bunge Alimentos S.A. (Luiz Eduardo Magalhães, Brazil): 109.4 g kg^{-1} moisture, 452.6 g kg^{-1} CP, 22.8 g kg^{-1} ether extract (EE), 68.0 g kg^{-1} crude fiber (CF), 61.0 g kg^{-1} ash, 5.8 g kg^{-1} Met, 27.5 g kg^{-1} Lys, 12.2 g kg^{-1} Met + Cys.
[2] Dona Benta (J. Macêdo, Fortaleza, Brazil): 130.5 g kg^{-1} moisture, 119.2 g kg^{-1} CP, 12.0 g kg^{-1} EE, 5.6 g kg^{-1} CF, 7.5 g kg^{-1} ash, 1.7 g kg^{-1} Met, 2.4 g kg^{-1} Lys, 4.0 g kg^{-1} Met + Cys.
[3] XSoy600 (Sementes Selecta S.A., Araguari, Brazil): 69.6 g kg^{-1} moisture, 622.4 g kg^{-1} CP, 12.0 g kg^{-1} EE, 46.0 g kg^{-1} CF, 66.0 g kg^{-1} ash, 8.1 g kg^{-1} Met, 37.6 g kg^{-1} Lys, 16.8 g kg^{-1} Met + Cys.
[4] Pesquera Pacific-Star (Puerto Montt, Chile): 105.8 g kg^{-1} moisture, 638.6 g kg^{-1} CP, 93.0 g kg^{-1} EE, 1.4 g kg^{-1} CF, 162.0 g kg^{-1} ash, 16.5 g kg^{-1} Met, 41.9 g kg^{-1} Lys, 23.5 g kg^{-1} Met + Cys.
[5] Krill meal from the Antarctic. Qrill™ (Aker BioMarine Feed Ingredients AS, Lysaker, Norway): 90.5 g kg^{-1} moisture, 502.0 g kg^{-1} CP, 231.7 g kg^{-1} EE, 31.4 g kg^{-1} CF, 97.1 g kg^{-1} ash, 14.3 g kg^{-1} Met, 34.5 g kg^{-1} Lys, 22.2 g kg^{-1} Met + Cys.
[6] Essential amino acid (EAA) mixture; average dietary inclusion of 7.0 g kg^{-1} L-Lysine, 3.5 g kg^{-1} L-Threonine, 4.5 g kg^{-1} L-Arginine HCl, 2.9 g kg^{-1} L-Phenylalanine and 1.5 g kg^{-1} L-Histidine.
[7] AQUAVI® Met-Met with a minimum of 950 g kg^{-1} DL-Methionyl-DL-Methionine; Evonik Operations GmbH (Hanau, Germany).
[8] Nutri-Bind Aqua Veg Dry, Nutri-Ad International NV (Dendermonde, Belgium). Synthetic binder consisting of calcium lignosulfonate (940 g kg^{-1}) and guar gum (60 g kg^{-1}).
[9] Rovimix® 2050 BR0738A010. DSM Produtos Nutricionais Brasil Ltda. (São Paulo, São Paulo, Brazil). Guaranteed levels: vit. A, 3,500,000 IU; vit. D3, 1,500,000 IU; vit. E, 75,000 mg kg^{-1}; vit. B1, 12,500 mg kg^{-1}; vit. B2, 10,000 mg kg^{-1}; vit. B6, 12,500 mg kg^{-1}; vit. B12, 10,000 mg kg^{-1}; nicotinic acid, 50,000 mg kg^{-1}; pantothenic acid, 40,500 mg kg^{-1}; biotin, 500 mg kg^{-1}; folic acid, 5000 mg kg^{-1}; vit. C, 100,000 mg kg^{-1}; Fe 15,000 mg kg^{-1}; Cu, 12,500 mg kg^{-1}; Zn, 50,000 mg kg^{-1}; Mn, 15,000 mg kg^{-1}; Se, 175 mg kg^{-1}; I, 500 mg kg^{-1}; Co, 100 mg kg^{-1}, 10 mg kg^{-1} moisture.
[10] Rovimix® Stay C® 35. Minimum of 350 g kg^{-1} phosphorylated vitamin C activity; DSM Nutritional Products AG (Schweiz, Switzerland).
[11] Cholesterol SF (Dishman Netherlands B.V., Veenendaal, Netherlands). Minimum of 910 g kg^{-1} active cholesterol.
[12] Nitrogen-free extract calculated by difference [dry matter − (CP + EE + CF + ash)].
[13] Digestible energy calculated based on the apparent digestibility coefficients of raw material provided in the literature. See Material and methods section for details.

The experimental diets were named based on their Met and DE content. The Met treatments were designated as 8, 9, and 10, corresponding to methionine levels of 7.6, 8.5, and 9.9 g kg^{-1}, respectively. The DE treatments were labeled as 16 and 17, reflecting DE levels of 15.7 and 16.6 MJ kg^{-1}, respectively. The factorial nomenclature combined these designations; for example, treatment 8/16 represented a diet with 7.6 g kg^{-1} Met and 15.7 MJ kg^{-1} DE. To achieve increased dietary Met levels, a dipeptide, DL-Met-Met (AQUAVI® Met-Met, Evonik Operations GmbH, Hanau, Germany), was supplemented at 2.6, 3.6, and 4.7 g kg^{-1} of the diet.

The dietary lipid content was increased to 82 g kg^{-1}, primarily through the addition of soybean oil, aimed at evaluating the impact of increased energy density on nutrient utilization in shrimp under varying Met levels. This modification resulted in a variation of DE content from 15.7 MJ kg^{-1} in diets containing 65 g kg^{-1} crude lipid to 16.5 MJ kg^{-1} in diets with 82 g kg^{-1} crude lipid (DM basis), corresponding to a difference of 0.8 MJ kg^{-1}.

The digestible protein and DE of the raw material were estimated by applying apparent digestibility coefficients (%) reported in the literature for penaeid shrimp: wheat flour (WF), 68.2 and 62.9% (Glencross et al., 2018); soy protein concentrate (SPC), 92.4 and 78.2% (Carvalho et al., 2016); soybean meal (SBM), 85.6 and 92.9% (Siccardi et al., 2006); salmon meal, 84.1 and 85.8% (Guo et al., 2020); and krill meal, 85.0 and 83.9% (Siccardi et al., 2006), respectively.

Aside from slight variations in the inclusion levels of SBM, WF, and SPC between the two sets of diets, the inclusion levels of other macro-ingredients remained consistent across all formulations (Table 1). Diets were designed based on the ideal protein concept, with lysine (Lys) serving as the reference AA. To ensure balanced AA profiles, all diets were supplemented with L-Lysine (Biolys®, Evonik Operations GmbH, Hanau, Germany), L-Threonine (ThreAMINO®, Evonik Operations GmbH, Hanau, Germany), L-Arginine HCl (Sigma-Aldrich do Brasil Ltda., São Paulo, Brazil), L-Phenylalanine, and L-Histidine (Ajinomoto do Brasil Ltda., Laranjal Paulista, Brazil). The following Lys to EAA ratios were achieved: 100 Lys: 73 threonine, 120 arginine, 46 histidine, and 42 phenylalanine. Except for Met, the coefficient of variation (CV) for the dietary content of all other AA was maintained below 3% (Table 2). The experimental diets were produced following the methodology described by Nunes et al. (2011).

Whiteleg shrimp (*P. vannamei*) were obtained as PL10 from a commercial hatchery (Samaria Unidade de Pós-Larvas Ltda., Touros, Brazil) and transported to the laboratory in seawater. Initially, the shrimp were cultured in five nursery tanks, each with a volume of 23 m^3, at a density of 2,000 PL m^{-3}, until they reached juvenile stage. During this phase, shrimp were gradually adapted to the target salinity concentration of 45 g L^{-1}. Hypersalinity was achieved by dissolving crude sea salt (996.4 g kg^{-1} sodium chloride) into seawater at 35 g L^{-1}.

For stocking in the experimental units, shrimp were size-graded to achieve a homogeneous BW of 2.9 ± 0.04 g (n = 2,280; CV = 1.4%). They were then reared in indoor 0.5-m^3 tanks (bottom area of 0.57 m^2 × 0.56 m height) under an artificial 12-h light cycle starting at 05:30 h. The stocking density was set at 100 animals m^{-2} (57 shrimp per tank). Shrimp were fed a commercial diet for four days before transitioning to the experimental diets.

Shrimp were fed the experimental diets for 56 days, following a four-day acclimation period. Feeding was exclusively done in feeding trays (14.3 × 3.5 cm, diameter × height), which were centrally placed in each tank at one unit per tank. The daily feed ration was divided into four meals, corresponding to 25, 15, 15, and 45% of the total daily ration delivered at 07:00, 10:00, 13:00, and 16:00 h, respectively. Feeding rates were calculated using the equation $MM = 0.0931 \times BW^{0.62}$, in which MM represents the maximum daily feed intake for a shrimp of a given body weight (Nunes and Parsons, 2000). To optimize feed efficiency and prevent an excessive feed conversion ratio (FCR), a 30% reduction was applied to the calculated MM. This adjustment is not a feed restriction but rather a correction to provide feed at an optimal rate, considering variations in shrimp species, historical growth performance, and feed utilization data from previous studies. The daily feed ration was also adjusted based on an assumed daily decrease in shrimp survival and a body weight gain of 100 mg shrimp day^{-1}. From the 19th day

of culture onwards, five shrimp per tank were sampled weekly to monitor BW gain. Feed adjustments for the following week were made based on the average daily BW gain recorded during the previous week for each specific tank, while maintaining a fixed daily survival decrease of 0.2%. Feeding trays were inspected daily before each meal to check for dead animals and feed leftovers. All feed leftovers were 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 culture system operated with its own filtration system with a total water storage capacity of 40 m^3. The system initially operated under static conditions; however, from the fifth week onward, culture water was drained from the tank bottom once a week from at 14% of the total tank volume.

Continuous aeration was provided to all tanks using a 2.5-hp blower, with air distribution achieved through a micro-perforated hose laid at the bottom of each tank. Water quality parameters were measured daily, including pH and temperature, which were determined using a portable pH meter with an attached thermometer (H98107 pHEP, Hanna Instruments Brasil, Barueri, Brazil). Salinity was measured using a refractometer (Model RTS01ATC, Instrutherm Instrumentos de Medição Ltda, São Paulo, Brazil) at 13:00 h in all tanks. Total ammonia nitrogen (TAN) analysis was measured using a visible spectrophotometer (DR 2800 Spectrophotometer, Hach Company, Loveland, USA) following Castro et al. (2021). The recorded water quality parameters were as follows: temperature was 26.1 ± 0.8 °C (mean ± SD), salinity was maintained at approximately 45.1 ± 0.9 g L^{-1}, pH was 8.0 ± 0.2, and TAN was 0.08 ± 0.01 mg L^{-1}.

Table 2 - Amino acid composition of experimental diets (g kg^{-1}, dry matter)

	Diet (Met g kg^{-1}/DE MJ kg^{-1})					
	8/16	9/16	10/16	8/17	9/17	10/17
Essential amino acids (EAA)						
Arginine	26.7	27.0	27.2	26.8	26.3	26.5
Histidine	7.7	7.7	7.7	7.7	7.5	7.6
Isoleucine	14.9	15.0	14.9	14.9	14.8	14.7
Leucine	24.4	24.6	24.7	24.4	24.3	24.3
Lysine	22.5	22.5	22.7	22.4	22.1	22.1
Methionine	7.6	8.5	9.9	7.6	8.6	9.8
Met + Cys	12.4	13.3	14.7	12.4	13.3	14.4
Phenylalanine	19.0	19.4	19.4	19.1	19.3	19.4
Threonine	16.0	16.1	16.1	15.9	15.8	15.8
Valine	15.5	15.7	15.7	15.6	15.5	15.5
Total EAA	154.3	156.6	158.4	154.4	154.2	155.6
Non-essential amino acids (NEAA)						
Alanine	14.9	15.0	15.2	15.0	14.8	14.8
Aspartic acid	34.2	34.5	34.5	34.0	33.7	33.8
Cysteine	4.8	4.8	4.8	4.8	4.7	4.7
Glycine	16.2	16.3	16.4	16.2	16.1	16.2
Glutamic acid	60.7	61.2	61.3	60.7	60.3	60.5
Proline	18.7	18.7	18.8	18.4	18.0	18.5
Serine	15.9	16.1	16.1	15.9	15.8	15.7
Total NEAA	165.4	166.7	167.0	164.9	163.3	164.1
Total (EAA + NEAA)	319.6	323.3	325.4	319.3	317.5	319.7
Free amino acids						
Methionine	0.1	0.1	0.2	0.1	0.2	0.2
Lysine	3.8	3.8	3.9	3.9	3.8	3.7
Threonine	3.7	3.6	3.7	3.7	3.6	3.6
Valine	0.2	0.2	0.2	0.2	0.2	0.2
Alanine	0.5	0.4	0.5	0.4	0.4	0.4
Arginine	5.4	5.3	5.6	5.4	5.4	5.4
Phenylalanine	2.9	2.8	2.9	3.0	3.0	3.1

Glucose concentration in shrimp hemolymph was determined following the method described by Lorenzon et al. (2000). Ten shrimp per dietary treatment were sampled one day prior to harvest. Shrimp were captured 1 h after feeding. Immediately following capture, hemolymph was collected using a sterile 1-mL syringe with a 21G needle. About 25 to 40 µL of hemolymph was drawn from each animal, and glucose levels were immediately determined using the OneTouch® Select Plus Flex meter (LifeScan Europe GmbH, Switzerland). Given the short measurement time, no anticoagulant was mixed with the hemolymph. This measurement was conducted to assess the shrimp's physiological response to the dietary treatments, as glucose concentration is an indicator of stress and energy metabolism.

The proximate dietary composition was determined according to AOAC methods (AOAC, 2023). 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 ether extract 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 dietary gross energy (GE) content was determined using the traditional bomb calorimetry method with an IKA Model 5000 calorimeter. The AA composition was determined using high-performance liquid chromatography following the method described by Figueiredo-Silva et al. (2015).

At harvest, shrimp were counted and weighed individually on a precision scale with 0.01-g accuracy. Growth performance and feed utilization calculations were as follows:

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

$$\text{Growth (g week}^{-1}\text{)} = \frac{\text{BIOg}}{60 \text{ days}} \times 7 \quad (2)$$

$$\text{Specific growth rate (SGR, \% day}^{-1}\text{)} = \frac{\ln \text{FBW} - \ln \text{IBW}}{60 \text{ days}} \times 100 \quad (3)$$

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

$$\text{Yield (MT ha}^{-1}\text{)} = \frac{\text{BIOg (MT)}}{\text{Tank bottom area (ha)}} \quad (5)$$

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

$$\text{Daily feed intake (g in DM of feed per kg BW per day)} = \frac{\text{Total feed intake}}{\text{Average BW} \times \text{average } N \times 60 \text{ days}} \quad (7)$$

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

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

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

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

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

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

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

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 two-way analysis of variance (ANOVA) was performed to assess the effects of dietary DE and Met levels, as well as their interaction on shrimp growth performance, feed utilization, and glucose concentration in shrimp hemolymph. The following mathematical model was adopted:

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

in which Yij is the *j*-th observation of the *i*-th dietary DE and Met levels; μ is the general mean response; τi is the non-random effect of dietary DE and Met levels, in which $\sum_{i=1}^{k} \tau i = 0$; and ϵij is the random dietary DE and Met levels error. When significant differences were detected, they were compared two-by-two with Tukey's Honest Significant Difference (HSD) post-hoc test. The significance level was set at $\alpha = 0.05$, with results considered significant when P-values were less than 0.05. Homogeneity of variances was verified using Levene's test, and normality of residuals was checked via the Shapiro-Wilk test. All statistical analyses were conducted using R software.

The assumptions of the ANOVA were tested to ensure the validity of the results. Levene's test for homogeneity of variances indicated that the assumption of equal variances was met for all parameters. The Shapiro-Wilk test for normality of residuals also confirmed that the residuals were normally distributed. These results suggest that the assumptions underlying ANOVA were not violated, supporting the robustness of the findings.

3. Results

No significant differences were observed in shrimp growth performance parameters, including weekly growth rate, weight gain, SGR, and yield, across the different dietary Met and DE levels (Table 3). The P-values for these parameters indicated that neither the individual effects of dietary DE and Met nor their interaction significantly influenced growth performance (P>0.05).

On the other hand, the analysis of feed utilization parameters revealed several significant findings. Dietary DE levels significantly influenced both daily GE (not shown) intake (P<0.01) and daily DE intake (P<0.01). Higher dietary DE levels resulted in an increased energy intake, demonstrating a clear impact of dietary DE content on the energy intake of shrimp. However, dietary Met levels and the interaction between DE and Met did not show significant effects on energy intake (P>0.05). As expected, dietary Met levels had a significant effect on daily Met intake (P<0.01) and total sulfur AA (TSAA) intake (P<0.01). Higher Met levels in the diet led to increased intake of these AA. Dietary DE levels and the interaction between dietary DE and Met did not significantly influence daily Met or TSAA intake (P>0.05). Total feed intake, daily feed intake, daily CP intake, FCR, FE, and PER did not exhibit significant differences across the different DE and Met levels (P>0.05).

The two-way ANOVA showed that dietary Met significantly increased glucose levels at medium and high supplementation levels compared with the lowest one (P = 0.022, Figure 1). Although dietary DE showed a trend toward increasing glucose levels, this effect was not statistically significant (P = 0.064). The interaction between Met and DE was not significant (P = 0.934).

Table 3 - Growth performance and feed utilization of the juvenile *P. vannamei* fed different dietary digestible energy (DE) and methionine (Met) levels under hyperosmotic conditions (data represent mean ± SD of six to seven replicates)

DE (MJ kg^{-1}, DM)	16			17			P Two-Way ANOVA		
Met (g kg^{-1}, DM)	8	9	10	8	9	10	Met	DE	Met × DE
Initial body weight (g)	2.90±0.04	2.89±0.04	2.91±0.05	2.92±0.03	2.89±0.04	2.90±0.07	0.67	0.90	0.67
Final body weight (g)	10.93±0.84	11.13±0.61	10.96±0.71	11.05±0.49	11.38±0.40	11.06±0.49	0.54	0.44	0.95
Survival (%)	99.3±1.57	98.5±2.75	100±0.00	100±0.00	97.93±2.06	99.3±1.57	0.10	0.72	0.62
Growth performance									
Weekly growth (g week^{-1})	0.94±0.09	0.96±0.07	0.94±0.09	0.95±0.06	0.99±0.05	0.95±0.05	0.53	0.47	0.94
Weight gain (%)	377.2±27.1	384.5±19.4	376.8±28.5	378.3±18.4	393.4±15.3	382.3±19.5	0.43	0.47	0.91
Specific growth rate (% day^{-1})	2.21±0.12	2.24±0.08	2.21±0.13	2.22±0.08	2.28±0.06	2.23±0.09	0.43	0.45	0.92
Yield (MT ha^{-1})	7.96±0.84	8.06±0.45	8.05±0.74	8.13±0.5	8.25±0.32	8.08±0.41	0.90	0.48	0.94
Feed utilization									
Total feed intake (g, DM)	728.3±45.5	732.9±41.6	727.9±32.1	741.7±23.3	761.8±16.7	731.6±37.2	0.51	0.18	0.68
Daily feed intake[1]	30.9±0.58	30.8±1.28	30.7±0.35	31.0±0.16	31.5±0.43	30.8±0.80	0.43	0.19	0.51
Daily DE intake[2]	482.6±9.1	483.6±20.1	481.9±5.5	509.0±2.62	517.2±6.9	510.6±13.2	0.76	<0.01	0.76
Daily crude protein intake[3]	11.48±0.22	11.35±0.47	11.57±0.13	11.48±0.06	11.64±0.15	11.41±0.29	1.00	0.49	0.15
Daily Met intake	0.24±0.01a	0.26±0.01b	0.30±0.01c	0.24±0.00a	0.27±0.00b	0.30±0.01c	<0.01	0.36	0.15
Daily TSAA intake[4]	0.39±0.01a	0.41±0.02b	0.45±0.00c	0.38±0.00a	0.42±0.01b	0.44±0.01c	<0.01	0.81	0.20
Feed conversion ratio	1.61±0.09	1.60±0.08	1.59±0.08	1.60±0.05	1.62±0.05	1.59±0.03	0.78	0.78	0.82
Feed efficiency	0.63±0.03	0.64±0.03	0.63±0.03	0.62±0.02	0.63±0.02	0.63±0.01	0.69	0.90	0.83
Protein efficiency ratio	1.69±0.08	1.73±0.07	1.67±0.09	1.69±0.05	1.71±0.05	1.71±0.02	0.44	0.85	0.47

[1] Daily intake reported in g of feed ingested per kg of shrimp BW per day.
[2] Daily intake reported in kJ of DE ingested per kg of shrimp BW per day.
[3] Daily intake reported in g of CP ingested per kg of shrimp BW per day.
[4] TSAA - total sulfur amino acids. Daily intake reported in g of TSAA ingested per kg of shrimp BW per day.
Values in the same row with different letters are significantly different (P<0.05).

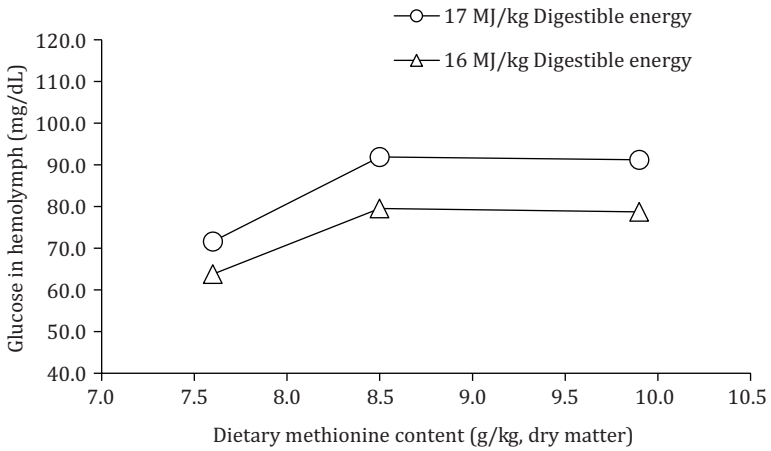

Figure 1 - Effect of dietary methionine and digestible energy levels on glucose concentration in shrimp hemolymph.

To further explore the significant main effect of dietary Met levels, post-hoc comparisons were conducted using Tukey's HSD test. The results showed that glucose concentrations were significantly higher in the 9 g kg^{-1} Met group compared with the 8 g kg^{-1} Met group (mean difference = 18.22 mg dL^{-1}, P = 0.043) and in the 10 g kg^{-1} Met group compared with the 8 g kg^{-1} Met group (mean difference = 17.48 mg dL^{-1}, P = 0.040). However, there was no significant difference in glucose levels between the 9 g kg^{-1} Met and 10 g kg^{-1} Met groups (mean difference = −0.74 mg dL^{-1}, P = 0.994). The comparison of dietary DE contents revealed a non-significant effect, with glucose concentration tending to be lower in shrimp fed diets containing 16 MJ kg^{-1} DE compared with those fed diets with 17 MJ kg^{-1} DE (mean difference = 0.97 mg dL^{-1}, P = 0.064). The post-hoc comparisons for the interaction between Met and DE levels showed no significant differences between the groups (P>0.05). The average glucose concentrations for the 16 MJ kg^{-1} DE group were 63.78 ± 20.86 mg dL^{-1} for the 8 g kg^{-1} Met level, 79.5 ± 14.55 mg dL^{-1} for the 9 g kg^{-1} Met level, and 78.7 ± 22.13 mg dL^{-1} for the 10 g kg^{-1} Met level. The 17 MJ kg^{-1} DE group exhibited average glucose concentrations of 71.62 ± 17.7 mg dL^{-1} for the 8 g kg^{-1} Met level, 91.88 ± 8.25 mg dL^{-1} for the 9 g kg^{-1} Met level, and 91.2 ± 31.25 mg dL^{-1} for the 10 g kg^{-1} Met level.

4. Discussion

This study explored the effects of varying dietary Met and DE levels on the growth performance of juvenile *P. vannamei* under hyperosmotic salinity conditions. It has been reported that hyperosmotic environments place additional energy demands on shrimp due to the increased effort required for osmoregulation (Hurtado et al., 2006). Previous research by Villarreal et al. (1994) demonstrated that *P. vannamei* exhibited elevated oxygen consumption at higher salinities, indicating a significant increase in metabolic demand. This raised the question of whether enhancing dietary energy levels could better meet these demands and whether this effect might be amplified by increasing Met, an EAA known for its benefits in stress response and cellular protection. Methionine not only supports protein synthesis but also plays a critical role in enhancing antioxidative defenses and immune functions in aquatic organisms (Belghit et al., 2014; Wu et al., 2024). It is crucial for the synthesis of glutathione, a key antioxidant that protects cells from oxidative stress, and taurine, which aids in osmoregulation and immune modulation (Brosnan and Brosnan, 2006; Forman et al., 2009). Given the heightened metabolic demands and stress associated with hyperosmotic environments, higher dietary DE and Met levels may be necessary to sustain optimal growth performance in shrimp under these conditions. However,

the results of the present study indicated no significant improvement in key growth parameters, such as weekly growth rate, weight gain, SGR, and yield, across the different dietary treatments.

The absence of enhanced growth performance with increased DE and Met levels likely indicates that the diets used in this study already met or exceeded the nutritional requirements of shrimp. Under the experimental conditions of this study, shrimp may have effectively allocated sufficient energy for maintenance (i.e., additional energy needed for osmoregulation under hyperosmotic environment) and growth, even when fed diets with the lowest DE and Met content (15.7 MJ DE kg^{-1} feed; 7.6 g Met kg^{-1} feed). This suggests that the shrimp's growth potential had already been maximized, and any additional dietary energy or Met (TSAA) provided beyond this point did not translate into further improvements in growth performance.

Although dietary manipulation resulted in significant increases in daily DE intake and daily Met intake, these increases appear to have surpassed the threshold necessary for optimal growth. Consequently, the surplus of dietary energy and AA did not contribute to further growth, likely due to the shrimp's ability to efficiently utilize the nutrients within the optimal range. This finding is consistent with the concept of nutrient saturation, in which nutrient intake beyond a certain level does not enhance growth but may instead be stored or excreted.

In this study, increasing dietary Met levels significantly influenced daily Met intake ($P<0.01$). As expected, higher Met levels in the diet led to increased intake of this AA. Shrimp in both DE groups, fed diets containing 7.6, 8.5, and 9.9 g kg^{-1} Met (on a DM basis), exhibited daily Met intakes of 0.24, 0.26, and 0.30 g per kg of BW per day, respectively. These values align with previous studies that reported optimal dietary Met levels ranging from 7.1 to 8.1 g kg^{-1} (Façanha et al., 2016; Nunes et al., 2019). Given that *P. vannamei* typically consumes around 4% of its body weight in feed per day during its juvenile stage, the estimated ideal daily Met intake is around 0.28-0.32 g per kg of BW per day. The daily Met intake observed across all treatments falls within this optimal range, reinforcing the adequacy of the dietary Met levels provided.

Similarly, dietary DE levels significantly influenced daily DE intake ($P<0.01$). Shrimp fed diets containing 15.7 and 16.5 MJ kg^{-1} DE presented average daily DE intakes of 482.7 and 512.3 kJ DE per kg of BW per day, respectively. These values exceed the daily DE requirement reported by Siccardi (2006), who found that an apparent daily DE intake of 334.7 kJ DE per kg of BW per day was sufficient for maximum growth in sub-adult *P. vannamei* (8.11–13.79 g) fed diets containing 350 g CP kg^{-1} of diet (as-is basis) under a 30.8 ± 0.4 g L^{-1} salinity. The higher DE intake observed in this study indicates that shrimp were ingesting sufficient energy to support growth, and the excess energy did not result in any additional growth improvements. Overall, these findings suggest that the nutritional needs of the shrimp were adequately met across all dietary treatments, with no further growth benefits observed from increased DE and Met intake beyond certain levels even under hypersalinity conditions.

Previous studies have explored the effects of dietary lipid on the growth performance and feed utilization of *P. vannamei* under salinity conditions outside the isosmotic range (20-25 g L^{-1}; Castille and Lawrence, 1981; Jaffer et al., 2020). Generally, these studies indicate that as salinity deviates from isosmotic conditions, both growth and feed efficiency tend to decline, with survival rates sometimes significantly impacted (Sui et al., 2015; Hurtado et al., 2006; Gao et al., 2016). The precise effects of increased dietary energy density, especially regarding lipid content, on shrimp growth performance and feed utilization under hypersalinity remain unclear. For instance, Chuphal et al. (2021) observed that diets with lower lipid content (46.5 – 51.4 g kg^{-1}) resulted in better growth performance in juvenile *P. vannamei* reared at low salinity (10 g L^{-1}). In contrast, Xu et al. (2018) found that juvenile *P. vannamei* (initial BW of 2.0 ± 0.08 g) reared for eight weeks at salinity of 3 g L^{-1} exhibited enhanced growth performance with higher dietary lipid levels, with the best growth rates observed in shrimp fed 90 g kg^{-1} of crude lipid, compared with those fed 60 or 120 g kg^{-1}. These mixed findings underscore the complexity of the relationship between dietary lipid content (as the main driver for DE changes) and growth performance, suggesting that other dietary components may need to be considered to support consistent growth under varying salinity conditions.

The ionic balance of culture water is another factor not addressed in the present study, which may have also influenced shrimp growth performance. In our study, Cl and Na were likely the most abundant ions due to the use of crude salt to achieve the desired hypersalinity conditions. Consequently, the balance of these ions would likely deviate from that of natural seawater, even if the hypersaline water originated from a natural source. Al-Sayegh et al. (2025) measured the concentrations of Ca, K, Mg, and Na, as well as their proportions, in seawater and natural hypersaline water at 44 g L^{-1} collected from the Arabian Gulf. They found significant discrepancies in the balance of these ions between the two water sources. These findings highlight the potential influence of ionic imbalance in culture water on shrimp performance, underscoring the need for further investigation into its effects under hypersaline conditions.

Studies on the EAA requirements of *P. vannamei* suggest that higher EAA levels may be necessary to maximize growth at elevated salinities. For instance, Huai et al. (2009) reported that juvenile *P. vannamei* required 13.6 g kg^{-1} of threonine at a salinity range of 0.50–1.50 g L^{-1}. In contrast, Zhou et al. (2013) observed that the requirement increased to 15.1 g kg^{-1} at a higher salinity range of 26–29 g L^{-1}. Interestingly, this trend does not appear to hold true for Met. In the present study, diets containing the lowest Met content (7.6 g kg^{-1}) among those tested were sufficient to promote maximum growth and feed utilization, comparable to the levels reported by Façanha et al. (2016) and Nunes et al. (2019), who found optimal Met levels ranging from 7.1 to 8.1 g kg^{-1} under more isosmotic conditions. These findings suggest that while certain AA may require adjustment in response to changes in environmental salinity, Met requirements remain relatively stable across a range of conditions. This stability in Met requirements may provide a useful baseline for feed formulation, allowing for more predictable growth outcomes even as other dietary and environmental factors vary.

This study not only assessed growth performance and feed utilization but also examined the effects of dietary Met and DE levels on glucose concentration in the hemolymph of *P. vannamei*. The findings revealed that dietary Met levels significantly influenced glucose concentration, while the effects of DE levels approached but did not reach statistical significance. The interaction between Met and DE was not significant, indicating that the impact of Met on glucose levels was consistent regardless of dietary DE levels (Figure 1). Although no significant improvements in growth parameters and feed utilization were observed across the different dietary treatments, the significant increase in glucose levels with higher dietary Met levels suggests that Met plays a key role in the metabolic processes of *P. vannamei*.

Glucose is a crucial carbohydrate, serving as a primary energy source for cells in aquatic organisms and functioning as an indicator of physiological status (Long et al., 2021). Previous research by Shinji and Wilder (2012) demonstrated that shrimp respond to stressors, such as air exposure and low salinity, by increasing the levels of certain AA in their hemolymph, including glycine, arginine, and alanine. This is accompanied by an increase in ammonia-N, a product of AA catabolism, suggesting that AA are mobilized as energy sources under stressful conditions.

In this study, the elevated glucose concentrations in shrimp fed higher dietary Met levels could reflect a similar metabolic response, in which increased Met intake enhances the shrimp's ability to meet energy demands. Methionine plays an essential role in various metabolic functions, including protein synthesis, osmoregulation, and potentially gluconeogenesis (Chandler and White, 2019). Additionally, Met may influence glucose absorption at the intestinal level, further contributing to the observed increase in hemolymph glucose. It is also important to note that hemolymph sampling occurred one hour after feeding, meaning glucose levels could reflect postabsorptive glucose, which may partly explain the higher values compared with other studies. This dynamics may also explain the *plateau* in glucose levels observed between the 8.5 and 9.9 g kg^{-1} Met groups, suggesting that there could be a threshold beyond which the shrimp's capacity to utilize glucose becomes limited, potentially resulting in inefficiencies in energy allocation. These findings suggest that dietary Met may enhance the shrimp's metabolic capacity to generate or maintain glucose. As glucose is a critical energy source, its availability is essential for meeting the energy demands of vital physiological processes, particularly under stressful conditions such as hyperosmotic environments.

However, the results are not entirely clear. The lack of growth improvements despite increased glucose concentrations aligns with findings by Cuzon et al. (2000), who emphasized that shrimp, although capable of utilizing glucose, have a limited ability to regulate hemolymph glucose levels efficiently. This can make them prone to hyperglycemia, particularly when provided with diets high in carbohydrates. In this study, this could mean that excess glucose in the hemolymph was not effectively converted into growth, even with increased Met intake.

It is important to acknowledge methodological limitations when interpreting these results. The glucose concentration measurements were taken only on the final day of the culture period, offering a snapshot of the effects of dietary treatments at that specific moment. Glucose levels in shrimp can fluctuate throughout different life stages and in response to factors like stress, feeding schedules, or environmental changes. A single time-point measurement may not capture these dynamic changes in glucose metabolism. To gain a more comprehensive understanding, future research should involve longitudinal sampling across multiple time points to monitor how dietary Met and DE levels influence glucose levels over time.

Another limitation of this study is the absence of an isosmotic salinity group. Salinity is known to affect osmoregulation in shrimp, and including a comparison group under isosmotic conditions could have provided additional insights into how salinity interacts with dietary Met and DE levels. Previous studies, such as those by Shinji and Wilder (2012), highlight that shrimp subjected to salinity stress experience shifts in AA and carbohydrate metabolism, indicating that salinity is a key factor in metabolic regulation. Incorporating varying salinity levels in future research would help elucidate the interaction between Met, glucose metabolism, and osmotic stress in *P. vannamei*.

5. Conclusions

This study demonstrated that while dietary Met levels influence certain metabolic responses in juvenile *P. vannamei* under hyperosmotic conditions, such as increased glucose concentrations, these changes do not necessarily translate into enhanced growth performance. The results suggest that the shrimp's dietary requirements for energy and Met were already met by the experimental diets, and further increases did not yield additional growth benefits. Notably, diets containing 15.7 MJ DE kg^{-1} and 7.6 g Met kg^{-1} are sufficient to support optimal growth, indicating that higher levels of these nutrients are unnecessary under hypersaline conditions. From an industry perspective, this finding is particularly relevant, as it implies that investing in more energy-dense or Met-enriched diets may not be cost-effective. Optimizing feed formulations with these baseline levels could reduce production costs without compromising shrimp performance, enhancing the economic sustainability of shrimp farming in high-salinity environments. These findings emphasize the importance of fine-tuning nutrient levels in shrimp diets to meet specific metabolic demands without exceeding the threshold beyond which no further advantages are gained.

Data availability

The entire dataset supporting the results of this study is not publicly available due to confidentiality agreements and the sensitive nature of some of the data. However, access can be granted upon reasonable request to the corresponding author for research purposes.

Author contributions

Conceptualization: Rodriguez Mejia, E. R.; Masagounder, K. and Nunes, A. J. P. **Data curation:** Nunes, A. J. P. **Formal analysis:** Rodriguez Mejia, E. R.; Leite, J. S.; Façanha, F. N.; Masagounder, K. and Nunes, A. J. P. **Funding acquisition:** Masagounder, K. and Nunes, A. J. P. **Investigation:** Rodriguez Mejia, E. R.; Leite, J. S. and Nunes, A. J. P. **Methodology:** Masagounder, K. and Nunes, A. J. P. **Project administration:** Leite, J. S. and Nunes, A. J. P. **Resources:** Leite, J. S. and Nunes, A. J. P. **Software:**

Nunes, A. J. P. **Supervision:** Leite, J. S. and Nunes, A. J. P. **Validation:** Leite, J. S. and Nunes, A. J. P. **Visualization:** Rodriguez Mejia, E. R. and Nunes, A. J. P. **Writing – original draft:** Rodriguez Mejia, E. R. and Façanha, F. N. **Writing – review & editing:** Façanha, F. N.; Masagounder, K. and Nunes, A. J. P.

Conflict of interest

Karthik Masagounder is employed by Evonik Operations GmbH, Germany.

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