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DL-methionyl-DL-methionine as an efficient methionine source for promoting zootechnical performance and methionine-related pathways in the whiteleg shrimp (*Penaeus vannamei*)

Marina Machado[1]*, Sergio Fernández-Boo[1], Carla Teixeira[1,2], Michael Viegas[3], Renata Serradeiro[4], Jorge Dias[3], Benjamín Costas Refojos[1,2] and Karthik Masagounder[5]

[1]*Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), Universidade do Porto, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos s/n, 4450-208, Matosinhos, Portugal*
[2]*Instituto de Ciências Biomédicas Abel Salazar (ICBAS-UP), Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-123, Porto, Portugal*
[3]*SPAROS Lda., Área Empresarial de Marim, Lote C, Olhão, Portugal*
[4]*RIASEARCH Lda, Cais da Ribeira de Pardelhas 21, Murtosa, Portugal*
[5]*Evonik Operations GmbH, Rodenbacher Chaussee 4, 63457 Hanau-Wolfgang, Germany*

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Abstract

Methionine (MET) supplementation is a current strategy to achieve shrimp requirement. Notwithstanding, the efficiency of the precisely formulated feeds can be diminished since shrimps are slow eaters and masticate feed externally that results in nutrient leaching. In this regard, a methionine dipeptide (DL-methionyl DL-methionine) benefits the feed industry by reducing MET water solubility while increasing its bioavailability. Therefore, the effects of feeding whiteleg shrimp (*Penaeus vannamei*) with increasing levels of methionine dipeptide were evaluated on zootechnical performance and methionine-, immune- and antioxidant-related pathways. A 74 d growth trial was conducted by feeding a control diet and four diets supplemented with AQUAVI® Met-Met at 0·08, 0·12, 0·24 and 0·32% of DM. Diet digestibility, body amino acids (AA) composition and nitrogen metabolites, metabolic enzymes, oxidative status and gene expression were evaluated. It can be concluded that graded dietary increase of methionine dipeptide up to 0·24 % for 74 d translated in significant gains on the growth performance, feed efficiency, nutrient and nitrogen gain and shrimp survival. Moreover, it was showed that Met-Met dietary spare leads to an improvement of free-AA pool and nitrogen metabolites concentration and reduces the signs of oxidative stress. Finally, in a closer look to the MET-related pathways passive to be altered by Met-Met spare, a clear modulation of the described antioxidant and cell proliferation routes was detected.

Key words: **Immunonutrition: Antioxidant response: Immune system: Growth**

Ten amino acids (AA) have been defined as essential for aquatic species[1] since their dietary deficiency may lead to a reduction of growth and feed efficiency. However, the need for the introduction of increasing amounts of plant protein sources in aquafeeds[2], together with the need of high dietary protein levels in of some species[3], raised up the scenario of dietary deficiency of specific AA[4]. Even though the relatively high protein content of some plant ingredients, such as soyabean, wheat gluten, corn gluten and others, usually present low content of methionine. Methionine is in fact one of the first-limiting essential AA in plant protein-based diets[4], and its supplementation is a current strategy to achieve shrimp methionine requirement (i.e. about 0·7 to 0·9 % of the diet)[1]. An additional problematic is raised by the fact that the requirements of some AA appear to increase in plant-based diets, since feed intake and protein utilisation may be reduced[5]. In fact, Xie, Lemme[6] showed the ability of DL-methionine dietary supplementation (0·1 to 0·3 % of the diet) in low-fish meal diets to improve growth performance (final body weight, weight gain and specific growth rate) of whiteleg shrimp (*Penaeus vannamei*).

Additionally, most AA requirements were established by means of optimal growth which overlooks the animal needs of certain functional AA associated with immune responses, oxidative stress response and health, leading to an

Abbreviations: AA, amino acids; ADC, apparent digestibility coefficients; ALT, aminotransferase; AST, aspartate aminotransferase; BG, biomass gain; FBW, final mean body weight; FCV, feed conversion ratio; FI, feed intake; HCys, homocysteine; IBW, initial body weight; PER, protein efficiency ratio; SAHH, S-adenosyl homocystainase; SAM, S-adenosylmethionine; SGR, specific growth rate.

* **Corresponding author:** Marina Machado, email mcasimiro@ciimar.up.pt

underestimation of the true AA requirement[7]. Indeed, methionine is also a key player in the biosynthesis of vital molecules for immune-related cellular functions and works as a precursor of key hormones and enzymes[8–16]. Briefly, as a precursor of S-adenosylmethionine (SAM), the universal methyl donor, methionine participates in the regulation of cell functioning (e.g. polyamine biosynthesis, bioactive amines, DNA methylation and apoptosis)[12,17–19] and antioxidant system (e.g. precursor of GSH and inactivation of reactive oxygen species by methionine residues)[11,20,21]. The immune system is in fact highly dependent of the proper supply of specific AA[22]. Dietary methionine surplus has showed to improve the immune status of poultry[23,24] and fish[25–29]. Moreover, Machado, Engrola[28] suggested that methionine requirements could be increased in the context of an extreme diet formulation (100 % plant ingredients) compared with a practical fish meal diet (32 % plant ingredients incorporation), since a clear impact on the immune status of a European seabass (*Dicentrarchus labrax*) was observed. These works put into evidence that methionine dietary surplus above the established requirements may be key for the development of a stronger immune response in a practical feed formulation scenario and confirms the presuppose that the requirement of specific AA increases in the event of infection or even inflammation[8]. However, there is a current lack of studies reporting the effects of dietary methionine surplus on shrimp immune and antioxidant responses.

In addition to the need of balancing dietary methionine levels, shrimps are slow eaters and masticate feed externally, which results in nutrient loss by leaching due to the prolonged time of feed under water. Also, Richard, Blanc[30] hypothesised that the supplemented AA may be partially excreted through shrimp gills. Such events reduce the efficiency of the precisely formulated feeds, by reducing the effective use of the supplemented AA[31]. This problematic is recognised for some time and has been effectively surpassed by the use of a dipeptide DL-methionyl-DL-methionine (Met-Met) developed by Evonik (commercialised under the name AQUAVI® Met-Met). This dipeptide presents reduced water solubility, and both fish and crustaceans were able digest Met-Met to free D- and L-methionine *in vitro* (data from Evonik's aqua R&D group). Also, Ji, Wang[32] pointed to Met-Met ability to improve the negative outcome of methionine dietary deficiency reducing its impact on growth in a low-fish meal scenario.

In whiteleg shrimp, Met-Met dietary incorporation and supplementation as a strategy to surpass the lower methionine content of plant ingredients resulted in a higher bioavailability, based on growth and feed efficiency, when compared with its common alternative, the feed grade DL-methionine[33]. With that results in mind, the present study intended to evaluate the effects of Met-Met supplementation on growth performance, whole-body composition, nutrient retention, apparent digestibility and methionine immune and antioxidant-related pathways.

Material and methods

Experimental diets

To evaluate the effects of Met-Met dietary supplementation and dietary Met levels, five diets were formulated. The negative control diet (NC) was formulated to contain 0·56 % Met (DM basis) using high levels of soyabean meal (26 %) and wheat (36%), and moderate levels of fishmeal (7 %) and poultry meal (2·8 %). Krill meal was used at a low level (2%) to guarantee feed palatability. NC was also supplemented with essential amino acids (i.e. lysine, threonine and tryptophan) and monocalcium phosphate to avoid any nutritional deficiencies. This basal NC formulation served as basis for the remaining four diets, which were supplemented with the AQUAVI® Met-Met (Met-Met) at 0·08, 0·12, 0·24 and 0·32 %. Feed formulation is presented in Table 1. All diets were formulated to be isonitrogenous (crude protein: 36 % DM), isolipidic (crude lipid: 8·7 % DM) and isoenergetic (gross energy: 19·8 MJ/kg DM). Diets NC and Met-Met 0·32% were also supplemented with 1 % chromic oxide as an inert marker for digestibility measurements.

Diets were manufactured by extrusion at Sparos Lda (Olhão) facilities. All powder ingredients, including AQUAVI® Met-Met, were mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion) and ground (below 400 μm) in a micropulveriser hammer mill (model SH1, Hosokawa-Alpine). Diets (pellet size: 1·2 and 2·0 mm) were manufactured with a twin-screw extruder (model BC45, Clextral) with a screw diameter of 55·5 mm. Extrusion conditions: feeder rate (90 kg/h), screw speed (255 rpm), water addition in barrel 1 (340 ml/min), temperature barrel 1 (36°C) and temperature barrel 3 (116–118°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen). Coating conditions were pressure (700 mbar), spraying time under vacuum (approximately 90 s) and return to atmospheric pressure (120 s). After coating, diets were packed in sealed plastic buckets stored at room temperature.

Trials design

Two trials were conducted at the experimental facilities of Riasearch (Murtosa) under the full responsibility of Sparos Lda. Experiments were conducted by FELASA certified scientists and technical staff, in full compliance with the European (Directive 2010/63/EU) and Portuguese (Decreto-Lei nº. 113/2013, 7 August) legislation on the protection of animals for scientific purposes.

Whiteleg shrimp were obtained from Shrimp Improvement Systems, LLC, and a stock of post-larvae shrimp (±30 000 individuals) were transferred to the experimental facilities by a duly authorised carrier and kept on sanitary quarantine for 2 weeks. No pathological signs were observed in association to transport. Prior to start of the trial, the shrimp stock was kept in four 500 l tanks supplied with recirculated seawater (salinity 20 ‰, water flow 2·3 l/min, temperature 26 ± 1°C, dissolved oxygen kept above 6 mg/l) for approximately 2 months. During this period, shrimp were fed a commercial diet for marine fish larvae (WinFlat, Sparos Lda). Shrimp post-larvae were fed continuously (hourly meals) with automatic feeders at approximately 12 % biomass/d. Prior to the start of the trials, shrimp were manually sorted to constitute a sub-stock with a homogenous weight range.

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Table 1. Formulation of the experimental diets

Ingredients, %	NC	Met-Met 0·08 %	Met-Met 0·12 %	Met-Met 0·24 %	Met-Met 0·32 %
Fishmeal*	7·00	7·00	7·00	7·00	7·00
Krill meal†	2·00	2·00	2·00	2·00	2·00
Poultry meal‡	2·76	2·76	2·76	2·76	2·76
Soya protein concentrate§	12·73	12·73	12·73	12·73	12·73
Soybean meal 44‖	26·10	26·10	26·10	26·10	26·10
Wheat meal¶	36·11	36·11	36·11	36·11	36·11
Wheat bran**	4·50	4·50	4·50	4·50	4·50
Fish oil††	1·00	1·00	1·00	1·00	1·00
Soybean oil‡‡	3·20	3·20	3·20	3·20	3·20
Soy lecithin§§	1·39	1·39	1·39	1·39	1·39
MCP‖‖	1·25	1·25	1·25	1·25	1·25
Guar gum¶¶	0·20	0·20	0·20	0·20	0·20
Biolys®***	0·53	0·53	0·53	0·53	0·53
ThreAMINO®†††	0·21	0·21	0·21	0·21	0·21
TrypAmino®‡‡‡	0·02	0·02	0·02	0·02	0·02
Vitamin mineral premix§§§	1·00	1·00	1·00	1·00	1·00
AQUAVI® Met-Met‖‖‖		0·08	0·12	0·24	0·32

* Fishmeal: 59·6 % crude protein (CP), 8·9% crude fat (CF), CONRESA, Spain.
† Krill meal: 65% CP, 14% CF, Aker Biomarine, Norway.
‡ Poutry meal 65:64·6% CP, 11·6% CF, SAVINOR UTS, Portugal.
§ Soycomil P: 64·5% CP, 0·7% CF, ADM, The Netherlands.
‖ Solvent extracted soybean meal: 44·6% CP, 3·3% CF, Cargill, Spain.
¶ Wheat: 10·2 % CP, 1·2 % CF, Casa Lanchinha, Portugal.
** Wheat bran: 14·9% CP, 4·2% CF, Casa Lanchinha, Portugal.
†† Sopropêche, France.
‡‡ Henry Lamotte Oils GmbH, Germany.
§§ Lecico P700IPM, LECICO GmbH, Germany.
‖‖ MCP (monocalcium phosphate): 22% P, 18% Ca, Fosfitalia, Italy.
¶¶ Seah International, France.
*** Biolys: 54·6% Lysine, Evonik Nutrition & Care GmbH, Germany.
††† ThreAMINO: 98·5% Threonine, Evonik Nutrition & Care GmbH, Germany.
‡‡‡ TrypAMINO: 98% Tryptophan, Evonik Nutrition & Care GmbH, Germany.
§§§ PREMIX Lda, Portugal: Vitamins (µg or mg/kg diet): DL-α tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20 000 µg; DL-cholecalciferol, 2000 µg; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0·1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0·5 mg; manganese oxide, 9·6 mg; sodium selenite, 0·01 mg; zinc sulphate,7·5 mg; sodium chloride, 400 mg; excipient wheat middling's.
‖‖‖ AQUAVI® Met-Met, Evonik's aqua R&D, Evonik Industries AG.

Growth trial. After the quarantine period, quintuplicate groups of 150 shrimp with a mean initial body weight of 1·72 ± 0·11 g (mean ± SD) were fed one of the five experimental diets during 74 d. Shrimp were grown in plastic circular tanks (volume: 500 l) supplied with recirculated seawater. Temperature was regulated at 27·9 ± 0·5°C (mean ± SD), salinity and pH maintained at ~ 20 ‰ and ~7·7, respectively. Dissolved oxygen levels were kept above 5·9 mg/l and total ammonia levels below 0·25 mg/l. During the trial, shrimp were subjected to a photoperiod regime of 16 h light and 8 h dark. Shrimp were fed manually during the day, in four meals (08.00, 10.00, 14.00 and 18.00 h) and by automated feeders during the night, in two meals (22·00, 04.00 h). Daily feed ration was based on a commercial feeding table for shrimp. However, to avoid feed wastage, the feed ration per tank was adjusted on a daily basis, taking into account any feed residues present in the tank at the first morning meal. For this purpose, if all pellets were eaten, the feed ration was increased by 0·2 %; if < 5 pellets remained in the tank, the ration was maintained; if > 5 pellets remained in the tank, the feed ration was decreased by 0·2 %. Shrimp were group weighed after 30, 57 and 74 d of feeding.

A pool of fifty whole shrimp from the initial stock, at the start of the trial, and a pool of ten whole shrimp per tank at the end of the trial (74 d) were sampled and stored at –20°C for subsequent analysis of whole-body moisture, ash, protein, fat, energy and amino acids content. Also, at the end of the trial, a pool of three shrimps per tank (n 5 pools per dietary treatment) were used to collect samples of haemolymph, hepatopancreas and muscle. All shrimp sampled were at the molting stage D1. Haemolymph was withdrawn from the ventral sinus into a 1 ml disposable syringe containing 1:1 proportion of an anticoagulant buffer (27 mM sodium citrate, 385 mM NaCl, 115 mM glucose; pH 7·5). Samples were put into tubes, snap-frozen in liquid nitrogen and stored at −80°C until subsequent analysis of several additional humoral parameters. Samples of hepatopancreas were also preserved in RNA Later, snap-frozen in liquid nitrogen and stored at −80°C for subsequent gene expression analysis.

Digestibility trial. In parallel to the growth performance trial, triplicate groups of twenty-five shrimp, with a mean initial body weight of 10·2 ± 1·1 g were used to measure the apparent digestibility of protein, lipid, energy and amino acids, by the indirect method with diets containing 1 % chromic oxide as an inert marker. The apparent digestibility measurements were only performed on dietary treatments NC and Met-Met 0·32 %. Prior to initiation of faeces collection, shrimp were fed the chromic oxide marked diets for 12 d, under identical rearing conditions

as those adopted for the growth performance trial. Approximately 2 h following the first morning meal and after the removal of all uneaten feed pellets, excreted faeces were collected by siphoning. After decanting and removal of excess water, faeces per replicate tank were stored frozen at −20°C. The collection of faeces per replicate tank lasted for 15 d, and daily collection of faeces from the same replicate was pooled and stored frozen at −20°C until subsequent analysis.

Analytic methods

Biochemical composition of feeds, whole-fish and faeces. Representative samples of each diet were taken for proximate and AA profile analyses (Table 2). Proximate analysis of diets, whole-fish and faeces followed the methodology described by AOAC[34]. Dry matter was measured after drying at 105°C for 24 h; total ash evaluated by combustion (550°C during 6 h) in a muffle furnace (Nabertherm L9/11/B170); crude lipid by petroleum ether extraction (40–60°C) using a Soxtec™ 2055 Fat Extraction System (Foss, Denmark), with prior acid hydrolysis with 8·3 M HCl; gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA); chromium was determined by spectrometry (SpectrAA 220 FS, Varian) according to Bolin, King[35], after perchloric acid digestion. All analysis of protein and amino acids were performed by EVONIK by HPLC methodology.

Growth performance. During the growth trial shrimp were group weighed after 30, 57 and 74 d of feeding with the experimental diets and growth performance was evaluated.

IBW (g): Initial mean body weight.

Final mean body weight (FBW) (g): Final mean body weight.

Table 2. Proximate composition and amino acids content of the experimental diets

		Met-Met			
Nutrients, % DM	NC	0·08	0·12	0·24	0·32
Dry matter	93·22	91·62	93·28	92·79	92·93
Ash	7·15	7·03	7·09	7·03	7·12
Protein	36·16	35·93	35·95	36·12	36·23
Lipid	8·70	8·76	8·59	8·74	8·65
Energy, kJ/g DM	19·89	19·95	19·79	19·89	19·80
Chromic oxide	0·91	–	–	–	0·95
Arginine	2·40	2·33	2·31	2·32	2·31
Histidine	0·90	0·88	0·87	0·87	0·87
Isoleucine	1·46	1·47	1·47	1·47	1·47
Leucine	2·52	2·53	2·52	2·52	2·53
Lysine	2·33	2·22	2·23	2·20	2·19
Threonine	1·52	1·53	1·53	1·51	1·51
Valine	1·61	1·61	1·61	1·61	1·61
Methionine	0·56	0·66	0·70	0·82	0·90
Cysteine	0·47	0·47	0·47	0·46	0·47
Methionine + Cysteine (Met + Cys)	1·03	1·13	1·17	1·28	1·37
Phenylalanine	1·62	1·63	1·63	1·62	1·63
Aspartic acid + Asparagine	3·39	3·41	3·39	3·39	3·39
Glutamic acid + Glutamine	6·43	6·45	6·42	6·42	6·43
Alanine	1·60	1·61	1·60	1·60	1·60
Glycine	1·74	1·74	1·73	1·73	1·73
Proline	2·06	1·96	2·00	2·00	2·00
Serine	1·66	1·66	1·65	1·65	1·65
Tryptophan	0·45	0·44	0·45	0·44	0·04
Met-Met	<0·01	0·09	0·12	0·25	0·30

Specific growth rate, SGR (%/d): (Ln FBW–Ln IBW) × 100/d.

Weight gain, WG (g): final mean biomass–initial mean + (dead body weight–(number of dead × IBW))

Feed conversion ratio, FCR: crude feed intake/weight gain.

Feed intake, FI (%BW/d): (crude feed intake/(IBW + FBW)/2/d) × 100.

Protein efficiency ratio, PER: wet weight gain/crude protein intake.

$$\text{Retention (\%)} = 100 \times \frac{(\text{FBW} \times \text{NFS}) - (\text{IBW} \times \text{NIS})}{\text{Nutrient intake}}$$

NFS: Nutrient content of final shrimp

NIS: Nutrient content of initial shrimp

Apparent digestibility coefficients. The apparent digestibility of protein, lipid, energy and amino acids was evaluated in the digestibility trial by the indirect method with diets containing 1 % of chromic oxide as an inert marker in NC and Met-Met 0·32% dietary treatments. Apparent digestibility coefficients (ADC) of dietary nutrients and energy in the experimental diets were calculated according to NRC (2011) (1):

$$\text{ADC, \%} = 100 \times \frac{\text{\% marker diet}}{\text{\% marker faeces}} \times \frac{\text{\% nutrient faeces}}{\text{\% nutrient diet}}$$

Daily N gain: (final body N content–initial body N content)/(IBW + FBW)/2)/d.

Daily N intake: N intake/(IBW + FBW)/2)/d.

Daily faecal N losses: daily N intake × (100–ADC N)/100.

Daily metabolic N losses: daily N intake–(daily N gain + daily faecal N losses).

Free amino acids and nitrogen-related metabolites. Hepatopancreas samples (three shrimps per pool, five pools per treatment) were firstly homogenised (0·1 M HCl, on ice) and then the supernatant (obtained after centrifugation at 1500 × ***g***, 15 min, 4°C) was deproteinised by centrifugal ultrafiltration (10 kDa cut-off, 2500 × ***g***, 20 min, 4°C). Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters). Analyses were done by ultra-high-performance liquid chromatography in a Waters reversed-phase amino acid analysis system, using norvaline as an internal standard. The resultant peaks were analysed with EMPOWER software (Waters).

Enzyme activities. The activity of several enzymes was measured in samples (three shrimp per pool, five pools per treatment) of haemolymph, muscle and hepatopancreas by enzymatic colorimetric and fluorometric methods using commercially available kits.

Tissue samples were placed into a sterile 2 ml round-bottom tube containing 0·1M phosphate buffer and an appropriate size and number of stainless-steel beads. Samples were mechanically disrupted using a Tissue Lyser II (Qiagen) for 3 × 15 s at 30 Hz, followed by centrifugation at 14 000 × ***g*** for 10 min. The supernatant was removed and placed in a sterile 1·5 mL Eppendorf

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tube for subsequent enzyme analysis. Soluble protein concentration of tissue samples was determined using the Micro BCA™ Protein Assay Kit (ThermoScientific Ref. 23235), with bovine serum albumin used as a standard. Amount of protein is expressed as mg of soluble protein.

For muscle, hepatopancreas and haemolymph, alanine aminotransferase (ALT) activity was determined using the GPT/ALT Kit (Spinreact, Ref. 41280). ALT catalyses the reversible transfer of an amino group from alanine to α-ketoglutarate forming glutamate and pyruvate. The pyruvate produced is reduced to lactate-by-lactate dehydrogenase and NADH. The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ALT present in the sample. Enzyme activity is expressed as U/L.mg protein. One unit (U) is the amount of enzyme that transforms 1 µmol of substrate per minute, in standard conditions.

For all three tissues, aspartate aminotransferase (AST) activity was determined using the GOT/AST Kit (Spinreact, Ref. 41273). AST catalyses the reversible transfer of aspartate to α-ketoglutarate forming glutamate and oxalacetate. The oxalacetate produced is reduced to malate-by-malate dehydrogenase and NADH. The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of AST present in the sample. Enzyme activity is expressed as U/L.mg protein. One unit (U) is the amount of enzyme that transforms 1 µmol of substrate per minute, in standard conditions.

Superoxide dismutase (EC 1·15·1·1) activity in homogenised hepatopancreas and muscle samples was measured by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction was monitored at 550 nm according to McCord and Fridovich (1969). Reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7·8), 0·1 mM EDTA, 0·1 mM xanthine, 0·012 mM cytochrome C and 0·025 µg/ml xanthine oxidase. Activity is reported in units of superoxide dismutase per mg of protein. One unit of activity was defined as the amount of enzyme necessary to produce a 50 % inhibition of the ferricytochrome C reduction rate.

Catalase (EC 1·11·1·6) activity in homogenised muscle and hepatopancreas samples was determined by measuring the decrease of hydrogen peroxide concentration at 240 nm according to Aebi (1984). Reaction mixture contained 50 mM potassium phosphate buffer (pH 7·0) and 10 mM H_2O_2 freshly added. Enzyme activity is expressed as units per mg of soluble protein (U/mg). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate per min under the above assay conditions. Soluble protein concentration of tissue samples was determined using the Micro BCA™ Protein Assay Kit (ThermoScientific Ref. 23235), with bovine serum albumin used as a standard. Amount of protein is expressed as mg of soluble protein.

Oxidation status. The protein carbonyl content of homogenised muscle and hepatopancreas samples (three shrimp per pool, five pools per treatment) was determined using the Protein Carbonyl Content Assay Kit (Sigma-Aldrich, Ref. MAK094).

Carbonyl content was determined by the derivatisation of protein carbonyl groups with 2,4-dinitrophenylhydrazine leading to the formation of stable dinitrophenyl hydrazone adducts, which can be detected spectrophotometrically at 375 nm, proportional to the carbonyls present. Protein carbonyl content is expressed as nmole carbonyl/mg protein.

Lipid peroxidation in muscle and hepatopancreas samples was determined by assessing the concentration of thiobarbituric acid reacting substances, according to the method of Buege and Aust (1978). An aliquot of the supernatant from the homogenate (100 µl) was mixed with 500 µl of a previously prepared solution containing 15 % (w/v) TCA, 0·375 % (w/v) thiobarbituric acid, 80 % (v/v) hydrochloric acid 0·25 N and 0·01 % (w/v) butylated hydroxytoluene. The mixture was heated to 100°C for 15 min and after cooling at room temperature and centrifuged at 1500 × ***g*** for 10 min. Absorbance in the supernatant was measured at 535 nm and compared with blank. Concentration was expressed as nanomole malondialdehyde/g of hepatopancreas, calculated from a calibration curve. Total glutathione (GSH and GSSG) was measured in hepatopancreas following the method described by Griffith (1980) and Vandeputte *et al.* (1994) with modifications.

Total glutathione analyses were carried out at 37°C, and changes in absorbance due to reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (Sigma) were monitored at 412 nm in a Multiskan GO microplate reader. The molar extinction coefficient used for 5,5′-dithiobis (2-nitrobenzoic acid) was 13 600 $M^{-1} \times cm^{-1}$. Total GSH was determined using a reaction mixture containing 133 mM-phosphate buffer with 5·8 mM-EDTA at pH 7·4 (Sigma), 0·71 mM 5,5′-dithiobis (2-nitrobenzoic acid), 0·24 mM-NADPH (Sigma) and 1·2 µg/ml GR (Sigma). Total GSH is expressed as uM glutathione/mg protein.

Gene expression analysis. Total RNA isolation from hepatopancreas (three shrimp per pool, five pools per treatment) was conducted with NZY Total RNA Isolation kit (NZYTech, Portugal) following manufacturer's specifications and resuspended in free nuclease water (Nzytech, Portugal). RNA quantity and purity were then verified using the D-11 Spectrophotometer (DeNovix) and first-strand cDNA was synthesised with NZY First-Strand cDNA Synthesis Kit (NZYTech). cDNA amplification was carried out with specific primers (Table 3) for genes that have been selected for their involvement in immune and antioxidant responses and methionine metabolism (Table 3). Primers were designed with NCBI Primer Blast Tool according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content and self-dimer or cross-dimer formation). Serial fivefold dilutions of cDNA were used to analyse the efficiency of the primer pairs by calculating the slope of the regression line of the cycle thresholds (Ct) *v.* the relative concentration of cDNA. Accession number, efficiency values, annealing temperature, product length and primers sequences are presented in Table 3.

Primer efficiency and quantitative PCR assays were performed with CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using 4·4 µl of cDNA (23 ng cDNA per well) mixed with 5 µl of iTaq Universal SYBR Green Supermix (Bio-Rad) and 0·3 µl of 10 µM of each specific primer in a final

Table 3. Gene panel analysed in hepatopancreas

Function	Gene	Acronym	Gene Bank ID	Forward and reverse primer sequences	Eff*	Product lenght‡
House-keeping	Cytoplasmic-type actin 4	–	MF627841·1	F- CACGAGACCACCTACAACTCCATC R- TCCTGCTTGCTGATCCACATCTG	87·60	260
	Ribosomal protein L8	–	DQ316258·1	F- AGCCAAGCAAGATGGGTCG R- TGTAACGATAAGGGTCACGGAAG	110·68	219
Nutrient trafficking	Rab GTPase	*rab*	JX073679·2	F- AGGTTCCGCAGCCTTATTCC R- CGCTCTGTTCGCACATCATCTA	131·81	125
Antimicrobial peptide	PvHm117 crustin P	*crustin*	AY488497·1	F- GAAACCACCACCAACACCTACTCC R- TCTGTGCGGCCTCTTTACGG	104·44	109
	Penaeidin-3a	*pen3*	Y14926·1	F- ATACCCAGGCCACCACCCTT R- TGACAGCAACGCCCTAACC	100·12	137
Immunity	Hemocyanin	*hemocy*	KY695246·1	F- GTCTTAGTGGTTCTTGGGCTTGTC R- GGTCTCCGTCCTGAATGTCTCC	117·69	124
	Lysozyme C-like	*lys-like*	XM_027352857	F- CGGGAAAGGCTATTCTGCCT R- CCAGCACTCTGCCATGTACT	98·03	82
	C-type lectin 2-like	*lectin*	DQ858899·2	F- GCTTCTGTTGGTGCTGTTGGC R- GTTCCCTTCCCGTATGTGGC	115·44	138
Oxidative status	Thioredoxin 1	*thdox*	EU499301·1	F- TTAACGAGGCTGGAAACA R- AACGACATCGCTCATAGA	118·44	116
	Glutathione transferase	*gst*	AY573381	F- AAGATAACGCAGAGCAAGG R- TCGTAGGTGACGGTAAAGA	94·84	146
	Glutathione peroxidase	*gpx*	XM_027372127·1	F- AGGGACTTCCACCAGATG R- CAACAACTCCCCTTCGGTA	88·90	117
Methionine metabolism	S-adenosylmethionine synthase-like	*sam-synth*	XM_027354783·1	F- TCTCTTTTCCAACTCGCGCT R- GCTACCTTAGCATCCGGGTC	98·44	214
	Spermine synthase	*sms*	XM_027377948·1	F- TGCATGCTACTAACGGCTCC R- CAGGCACCTCACACACAGAT	101·78	241
	Ornithine decarboxylase antizyme	*odc-antizyme*	XM_027351356·1	F- ATTCCTCCTAATGGAACTGGGAT R- TTGTCTGCTCCGCCAGTTG	92·71	300
	Adenosylhomocysteine hydrolase	*sahh*	MH603330·1	F- GCAAGACCTGTGTTGTGGCT R- GTAACCCTCACAAGCAGCCTG	105·21	134
Apoptosis	Caspase 3	*casp3*	KC660103·1	F- ACATTTCTGGGCGGAACACC R- GTGACACCCGTGCTTGTACA	103·98	182

* Efficiency of PCR reactions were calculated from serial dilutions of tissue RT reactions in the validation procedure.
† Annealing temperature (°C).
‡ Amplicon (nt).

volume of 10 µl. Melting curve analysis was also performed to verify that no primer dimers were amplified. The standard cycling conditions were 10 min at 95°C for initial denaturation, 40 cycles of 2 steps (95°C for 15 s and primer annealing temperature for each different gene for 1 min), 1 min at 95°C followed by 35 s at the annealing temperature (60°C for all used primers) and ending with 95°C for 0·5 s. All reactions were carried out as technical duplicates. The expression of the target genes was normalised using the average expression of whiteleg shrimp cytoplasmic-type actin 4 and ribosomal protein L8.

Statistical analysis

All results are expressed as mean of five replicates ± standard deviation (mean ± SD). Data were analysed for normality and homogeneity of variance, and, prior to ANOVA, values expressed as percentage were subjected to arcsin square root transformation[36]. Data were analysed by a one-way ANOVA, with methionine supplementation level as an independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. All statistical tests were performed using the IBM SPSS Statistics software (version 21).

In addition, exponential (Ex), liner broken-line and quadratic broken-line (QBL) models were used to estimate the optimal level of total Met and Met + Cys inclusion to maximise shrimp responses including final body weight, biomass gain (BG) and FCR. Linear broken-line model was expressed as $Y = \beta0 + \beta1 \times (\beta2X)$, where $(\beta2X) = 0$ for $X > \beta2$, Y is the dependent variable, X is the dietary Met or Met + Cys level, $\beta0$ is the value at the plateau, $\beta1$ is the slope and $\beta2$ is the Met or Met + Cys concentration at the break point. Quadratic broken-line model is $Y = \beta0 + \beta1 \times (\beta2X)2$, where $(\beta2X) = 0$ for $X > \beta2$, Y is the dependent variable, X is the dietary Met or Met + Cys concentration, $\beta0$ is the value at the plateau, $\beta1$ is the slope and $\beta2$ is the Met or Met + Cys concentration at the break point. Exponential model was expressed as $Y = \beta0 + \beta1 \times (1–EXP(–\beta2 \times (X–\beta3)$, where Y is the dependent variable, X is the dietary Met or Met + Cys concentration, $\beta0$ is the response for the dependent variable estimated for the basal diet concentration of Met or Met + Cys, $\beta1$ is the difference estimated between the minimum and maximum response obtained by the increasing Met or Met + Cys, $\beta2$ is the slope of the exponential curve, $\beta3$ is the Met or Met + Cys level in the basal diet; Met or Met + Cys requirement was estimated by calculating $(\ln(0{\cdot}05)/ \pm \beta2) + \beta3$ for 95 % of the max response.

Results

Growth performance

Growth performance was evaluated at 30 and 57 d of feeding and at the end of the trial (74 d) and consisted in the evaluation of shrimp survival, FBW, SGR, BG, FCR, feed intake (FI) and PER (Table 4).

Thirty days of feeding. At 30 d of feeding, methionine supplementation had a significant effect on the overall zootechnical performance criteria. Shrimp fed all diets supplemented with Met-Met showed a significantly higher FBW, SGR, BG, PER and a significantly lower FCR and FI than those fed the NC diet. Moreover, shrimp fed Met-Met 0·32% showed higher BG than those fed Met-Met 0·08 %, while individuals fed Met-Met 0·24% and Met-Met 32% showed a significantly lower FCR than those fed the Met-Met 0·08 diet. Shrimp fed diets Met-Met 0·24% and Met-Met 0·32% showed a significantly lower feed intake and significantly higher PER than those fed the Met-Met 0·08% and Met-Met 0·12% diets.

Fifty-seven days of feeding. After 57 d of experimental feeding, the overall performance data were within the expected range for the species. Although some mortality occurred, after 57 d of experimental feeding, the average survival rate was 93 % and was not significantly affected by dietary treatments. Shrimp FBW at this time ranged from 6·24 g to 8·08 g, with shrimp from the best performing treatment showing a 4·7-fold increase of their IBW. At 57 d of feeding, methionine supplementation had a significant effect on the overall zootechnical performance criteria. Shrimp fed all diets supplemented with Met-Met showed a significantly higher FBW, SGR, BG, PER and a significantly lower FCR and FI than those fed the NC diet. Shrimp fed diets supplemented with Met-Met at levels equal or higher than 0·12 %, showed a higher FBW, SGR and BG than those fed the Met-Met 0·08 % diet. Additionally, shrimp fed with the Met-Met 0·24 % and Met-Met 0·32 % presented a higher BG than those fed the remaining diets. Met-Met 0·32 % diet showed a higher FBW and SGR than those fed the Met-Met 0·12 % diet. Shrimp fed diet Met-Met 0·8 % showed a significantly higher FCR than those fed diets with higher Met-Met supplementation doses (0·12, 0·24 and 0·32 %). Shrimp fed with the Met-Met 0·24 % diet showed a significantly higher PER than those fed with the Met-Met 0·08 diet.

Seventy-four days of feeding. At the end of the trial, the average survival was 85·2 %, which can be considered as acceptable for the species, but was significantly affected by dietary treatments. Survival was significantly lower in shrimp fed the NC diet compared with all supplemented diets. Moreover, shrimp fed Met-Met 0·12 % dietary treatment showed a significantly higher survival than those fed the Met-Met 0·8 % diet. Shrimp FBW ranged from 9·54 g to 15·29 g, with shrimp in the best-performing treatments showing an 8·9-fold increase of their initial body weight. After 74 d of experimental feeding, methionine supplementation had a significant effect on the overall zootechnical performance criteria. Shrimp fed all diets supplemented with Met-Met showed a significantly higher FBW, SGR, BG, PER and a significantly lower FCR and feed intake than those fed the NC diet. Shrimp fed diets supplemented with Met-Met at levels equal or higher than 0·12 %, showed a higher FBW, SGR and BG than those fed the Met-Met 0·8 diet. Shrimp fed diets with the two higher Met-Met supplementation doses (0·24 and 0·32 %) showed a significant reduction of FCR and feed intake, when compared with those fed the lower Met-Met supplementation doses (0·08 and 0·12 %). Moreover, shrimp fed with the Met-Met 0·12 diet showed a significantly lower FCR and FI and lower PER than those fed the Met-Met 0·08 diet. Shrimp fed diets with the two higher Met-Met supplementation doses (0·24 and 0·32 %) showed a significantly higher PER than those fed diets supplemented with the lower Met-Met doses (0·08 and 0·12 %).

Shrimp whole-body composition

For proximate and AA whole-body composition, an initial pool of fifty shrimps was collected, while a pool of ten shrimps per tank (quintuplicate tanks) was collected at 74 d of feeding. No differences regarding moisture, protein, fat, ash or energy were observed between the dietary treatments (Table 5). Also, no differences were observed regarding AA body composition.

Nutrient retention

Dietary Met-Met supplementation had a significant effect on the retention of protein, fat and energy at whole-body level (Table 6). Protein retention ranged between 19·1 and 36·8%, with shrimp fed all diets supplemented with Met-Met showing a significantly higher protein retention than those fed the NC diet. The Met-Met supplementation dose had also a significant effect. Supplementation at 0·12% resulted in a significantly higher protein retention than the 0·08% dose, and the two highest doses (0·24 and 0·32%) led to further significantly increase of protein retention over the 0·12% dose. Fat retention ranged between 4·6 and 10·3%. Shrimp fed diets NC and Met-Met 0·08% showed a significantly lower fat retention than those fed diets supplemented with Met-Met at 0·12, 0·24 and 0·32%. Energy retention ranged between 9·1 and 17·5%. Shrimp fed all diets supplemented with Met-Met showed a significantly higher energy retention than those fed the NC diet. Moreover, shrimp fed diets supplemented with Met-Met at levels equal or higher than 0·12%, showed a higher energy retention than those fed the Met-Met 0·08 % diet. Shrimp fed all diets supplemented with Met-Met showed a significantly higher whole-body retention of all amino acids than those fed the NC diet (Table 8). At the exception of methionine, amino acids retention was highest with diets supplemented with Met-Met at 0·24 and 0·32%, intermediate with diets supplemented at 0·12% and lowest with diet supplemented at 0·08%. Methionine retention was highest at supplementation doses of 0·12 and 0·24%.

Apparent digestibility

The ADC for dry matter, protein and amino acids were measured only on diets NC and Met-Met 0·32 % (Table 8). The apparent digestibility of dry matter, protein and amino acids, at the

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Table 4. Growth performance after 30, 57 and 74 d of feeding (IBW: 1·72 ± 0·11 g). Final mean body weight (FBW), specific growth rate (SGR), biomass gain (BG), feed conversion rate (FCR), feed intake (FI) and protein efficiency ratio (PER)

	Survival %		FBW g		SGR %/d		BG g		FCR		FI %ABW/d		PER	
Diet	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
							30 d							
NC	95·07	1·46	4·56	0·12a	3·26	0·10a	439·58	12·51a	2·27	0·09c	7·34	0·14d	1·31	0·05a
Met-Met 0·08 %	95·60	2·43	4·88	0·14b	3·46	0·11b	477·02	17·01b	1·80	0·09b	5·95	0·20c	1·69	0·08b
Met-Met 0·12 %	95·07	1·53	4·95	0·10b	3·53	0·09b	495·60	15·52bc	1·69	0·09ab	5·80	0·23c	1·77	0·09b
Met-Met 0·24 %	94·80	1·97	4·99	0·04b	3·54	0·05b	500·22	8·24bc	1·56	0·05a	5·39	0·17a	1·91	0·06c
Met-Met 0·32 %	93·87	1·45	5·03	0·17b	3·57	0·11b	512·62	16·73c	1·56	0·11a	5·49	0·32b	1·92	0·14c
P value	ns		<0·001		<0·001		<0·001		<0·001		<0·001		<0·001	
							57 d							
NC	92·00	1·33	6·24	0·24a	2·26	0·06a	669·15	31·08a	2·15	0·12c	4·51	0·15b	1·38	0·08a
Met-Met 0·08 %	93·47	1·97	7·13	0·11b	2·49	0·04b	791·82	14·07b	1·67	0·05b	3·68	0·09a	1·82	0·05b
Met-Met 0·12 %	93·33	1·41	7·72	0·08c	2·64	0·03c	881·53	10·55c	1·55	0·10a	3·58	0·20a	1·93	0·12bc
Met-Met 0·24 %	92·53	1·10	7·92	0·14cd	2·68	0·04cd	905·70	16·66d	1·47	0·09a	3·43	0·21a	2·04	0·12c
Met-Met 0·32 %	92·53	1·45	8·08	0·18d	2·71	0·04d	934·79	24·53d	1·52	0·05a	3·61	0·11a	1·96	0·07bc
P value	ns		<0·001		<0·001		<0·001		<0·001		<0·001		<0·001	
							74 d							
NC	78·00	3·43a	9·54	0·91a	2·31	0·13a	1023·86	84·24a	2·50	0·18d	5·02	0·26d	1·19	0·09a
Met-Met 0·08 %	85·20	2·23b	12·53	0·28b	2·68	0·03b	1463·91	27·37b	2·00	0·12c	4·26	0·29c	1·52	0·09b
Met-Met 0·12 %	89·20	1·28c	14·75	0·69c	2·90	0·06c	1815·75	81·09c	1·67	0·15b	3·67	0·30b	1·80	0·17c
Met-Met 0·24 %	88·53	1·97bc	14·74	0·25c	2·90	0·03c	1803·35	33·06c	1·45	0·03a	3·18	0·10a	2·07	0·05d
Met-Met 0·32 %	88·67	2·36bc	15·29	0·89c	2·95	0·08c	1887·56	104·40c	1·42	0·12a	3·16	0·23a	2·10	0·18d
P value	<0·001		<0·001		<0·001		<0·001		<0·001		<0·001		<0·001	

Values are presented as means ± SD (*n* 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

Table 5. Proximate composition of whole shrimp (initial and 74 d after feeding the experimental diets)

	Moisture (%)		Protein (%)		Fat (%)		Ash (%)		Energy (kJ/g)	
Diet	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial	79·11		16·42		0·49		3·28		3·98	
NC	76·46	0·48	18·46	0·54	0·98	0·23	2·79	0·11	4·77	0·12
Met-Met 0·08 %	76·62	0·72	18·22	0·64	0·99	0·23	2·81	0·11	4·74	0·19
Met-Met 0·12 %	75·98	0·65	18·68	0·55	1·17	0·16	2·84	0·08	4·97	0·17
Met-Met 0·24 %	76·44	0·68	18·27	0·50	1·18	0·35	2·83	0·17	4·80	0·21
Met-Met 0·32 %	76·35	0·57	18·36	0·40	1·12	0·26	2·62	0·07	4·73	0·13
P value	ns		ns		ns		ns		ns	

Values are presented as means ± SD (*n* 5). Data were analysed by a one-way ANOVA, with methionine level as variable.

Table 6. Nutrient and energy retention in shrimp fed the diary treatments (expressed as percentage of intake)

	Protein (%)		Fat (%)		Energy (%)	
Diet	Mean	SD	Mean	SD	Mean	SD
NC	19·09	2·23a	4·64	1·24a	9·11	0·91a
Met-Met 0·08 %	25·84	1·71b	6·13	1·41a	12·25	0·67b
Met-Met 0·12 %	32·26	2·73c	8·98	1·10b	15·76	1·39c
Met-Met 0·24 %	36·07	1·44d	10·32	3·20b	17·38	0·75c
Met-Met 0·32 %	36·76	2·69d	10·07	2·66b	17·45	1·36c
P value	<0·001		0·001		<0·001	

Values are presented as means ± SD (*n* 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

Table 7. Retention of cysteine and essential amino acids in shrimp fed the diary treatments (expressed as percentage of intake)

Essential amino acids										
Diet	Arginine		Histidine		Isoleucine		Leucine		Lysine	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
NC	20·26	2·60a	15·62	1·88a	17·04	2·10a	17·20	2·12a	18·94	2·26a
Met-Met 0·08 %	28·72	2·16b	21·91	1·68b	23·40	1·55b	23·36	1·49b	26·87	1·84b
Met-Met 0·12 %	37·10	2·99c	27·79	3·32c	29·56	2·74c	29·52	2·64c	33·96	2·66c
Met-Met 0·24 %	40·89	1·50d	30·98	1·47d	33·26	1·23d	33·28	1·36d	38·27	1·96d
Met-Met 0·32 %	41·49	2·70d	31·44	2·11d	33·65	2·45d	33·52	2·42d	39·03	2·91d
P value	<0·001		<0·001		<0·001		<0·001		<0·001	

Diet	Threonine		Valine		Methionine		Cysteine (semi-essential)		Phenylalanine
NC	15·78	1·88a	18·06	2·15a	26·02	3·26a	16·23	1·93a	17·93a
Met-Met 0·08 %	21·34	1·40b	24·83	1·69b	29·81	1·89b	21·48	1·51b	24·69b
Met-Met 0·12 %	26·90	2·42c	31·28	2·76c	35·32	2·95d	27·59	1·72c	30·96c
Met-Met 0·24 %	30·37	1·12d	35·11	1·28d	34·02	1·74cd	31·09	1·92d	35·05d
Met-Met 0·32 %	30·47	2·01d	35·22	2·30d	31·09	2·49bc	31·67	2·16d	35·93d
P value	<0·001		<0·001		<0·001		<0·001		<0·001

Values are presented as means ± SD (*n* 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

exception of methionine, was not significantly affected by dietary treatments. Shrimp fed the Met-Met 0·32 diet showed a significantly higher digestibility of methionine than those fed the NC diet.

Nitrogen balance

Based on feed intake, weight gain, whole-body protein content and ADC of protein, a daily balance of nitrogen (N) was calculated and presented in Table 9.

Shrimp fed all diets supplemented with Met-Met showed a significantly higher daily nitrogen (N) gain and significantly lower faecal and metabolic N losses than those fed the NC diet. Among the supplemented diets, dose had also a significant effect on the daily N balance. Shrimp fed diets supplemented with Met-Met at levels equal or higher than 0·12 % showed a higher N gain than those fed the Met-Met 0·08 % diet. Both faecal and metabolic N losses were lowest with diets supplemented with Met-Met at 0·24 and 0·32 %, intermediate with diets supplemented at 0·12 % and highest with diet supplemented at 0·08 %.

Table 8. Apparent digestibility coefficients (ADC, %) of dry matter, protein, fat, energy and amino acids

	NC		Met-Met 0·32 %		
Diet	Mean	SD	Mean	SD	*P*-value
Dry matter	69·8	0·2	68·1	0·4	ns
Protein	86·0	0·6	85·9	0·8	ns
Fat	95·2	2·6	96·8	0·2	ns
Energy	81·4	0·3	80·5	0·7	ns
Arginine	92·1	0·7	92·1	0·4	ns
Histidine	87·6	0·5	87·3	0·7	ns
Isoleucine	87·4	0·9	87·4	0·5	ns
Leucine	87·2	0·8	87·3	0·3	ns
Lysine	91·2	0·6	90·9	0·2	ns
Threonine	85·9	0·5	85·7	0·1	ns
Valine	85·1	0·8	85·0	0·6	ns
Methionine	88·9	0·7a	91·9	0·3b	0·003
Cysteine	82·7	0·5	82·5	0·4	ns
Phenylalanine	80·1	1·5	80·0	2·0	ns
Aspartic acid + Asparagine	87·1	0·7	87·3	0·6	ns
Glutamic acid + Glutamine	90·9	0·7	91·1	0·7	ns
Alanine	84·6	0·7	84·3	0·6	ns
Glycine	84·4	0·5	83·8	0·5	ns
Proline	88·1	0·6	87·7	0·7	ns
Serine	86·3	0·6	86·4	0·4	ns

Values are presented as means ± SD (n 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

Table 9. Daily nitrogen balance

	Nitrogen gain (mg/kg/d)		Nitrogen faecal losses (mg/kg/d)		Metabolic nitrogen losses (mg/kg/d)	
Diet	Mean	SD	Mean	SD	Mean	SD
NC	515	35a	379	20d	1814	151d
Met-Met 0·08 %	579	23b	316	21c	1352	123c
Met-Met 0·12 %	632	21c	277	22b	1061	133b
Met-Met 0·24 %	615	18c	240	7a	851	46a
Met-Met 0·32 %	624	16c	240	17a	840	103a
P value	<0·001		<0·001		<0·001	

Values are presented as means ± SD (n 5). Data were analysed by a one-way ANOVA) with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

Free amino and nitrogen-related metabolites in hepatopancreas

Several free amino acids and nitrogenous metabolites were significantly affected by Met-Met supplementation (Table 10). In comparison with the non-supplemented diet (NC) and the lowest Met-Met dose (0·08 %), the higher levels of Met-Met supplementation led to a gradual and significant increase on the content of most amino acid and metabolites in shrimp hepatopancreas. Exceptions being taurine and beta-alanine contents, which tended to be significantly reduced with an increase of dietary Met-Met levels.

Metabolic enzymes and oxidation status

The effect of graded dietary supplementation levels of Met-Met on the general metabolic status of shrimp was assessed by measuring the activities of transaminases and oxidative enzymes (Table 11). In the various tissues, shrimp fed diets supplemented with Met-Met at levels equal or higher than 0·12 % showed a significant reduction on the activities of ALT and AST, than those found in shrimp fed with the NC and Met-Met 0·08 % diet. Supplementation with Met-Met at levels equal or higher than 0·12 % led to a significant increase on the activity of superoxide dismutase in the muscle, while without a significant effect on hepatopancreas. Highest doses of Met-Met (0·24 and 0·32 % in muscle and 0·32 % only in hepatopancreas) significantly enhanced the activity of catalase.

To further assess the oxidative status of shrimp fed the various diets, the levels of total glutathione, protein carbonyls and lipid peroxidation were measured in the muscle and hepatopancreas (Table 12). Dietary treatments had no significant effect on the levels of protein carbonyls. Lipid peroxidation in the muscle, but not in the hepatopancreas, was significantly reduced with the increase of supplemental Met-Met levels. Similarly, total GSH levels in the hepatopancreas were significantly enhanced with the graded increase of supplemental AQUAVI® Met-Met levels.

Hepatopancreas immune-related gene expression

Most genes associated to an immune and antimicrobial role were not affected by dietary treatments (Table 13). Genes involved in antioxidant response as GPX and GST, which codes glutathione peroxidase and glutathione transferase, respectively, were highest in shrimp fed the Met-Met 0·12 % diet. Moreover, the highest Met-Met dose (0·32 %) led to significant increase on the expression of S-adenosylmethionine synthase-like (*sam-synth*) and spermine synthase (*sms*). Finally, the mRNA expression of SAHH, which codes adenosyl-homocysteinase, was increased until the Met-Met 0·12 % diet.

Optimal Met and Met + Cys requirements

Total Met and Met + Cys requirements estimates were estimated according to the Ex, liner broken-line and quadratic broken-line models (Table 14). All the three models fit the body weight, BG and FCR data very well with the R^2 values being ~90 %. The average Met requirement value, across the three different model estimates, was estimated at 0·80 % diet (DM basis) to optimise final body weight and BG and at 0·88 % diet (DM basis) to optimise FCR. Similar model estimate for Met + Cys was estimated to be 1·27 % diet (DM basis) to optimise final body weight and BG and at 1·35 % diet (DM basis) to optimise FCR.

Discussion

Taking into consideration that Met-Met dietary incorporation and supplementation resulted in a higher bioavailability, when compared with its common alternative (DL-methionine)[33], the present work was focused on the effects of Met-Met supplementation on growth performance, whole-body composition, nutrient retention, apparent digestibility and methionine immune and antioxidant-related pathways.

Table 10. Free amino acids and nitrogen-related metabolites in hepatopancreas

	NC		Met-Met 0·08 %		Met-Met 0·12 %		Met-Met 0·24 %		Met-Met 0·32 %		
mg/g (fresh weight)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	*P*-Value
Arginine	6·49	0·40ab	6·18	0·27a	6·50	0·41ab	6·98	0·34bc	7·33	0·25c	<0·001
Histidine	2·15	0·10a	2·08	0·05a	2·42	0·18b	2·62	0·17c	2·80	0·10d	<0·001
Isoleucine	7·04	0·17	7·09	0·16	7·33	9·14	7·21	0·30	7·12	0·17	ns
Leucine	9·21	0·17ab	9·06	0·23a	9·35	0·29ab	9·36	0·29ab	9·61	0·14b	0·018
Lysine	5·89	0·25a	6·03	0·20ab	6·13	0·34ab	6·40	0·36bc	6·74	0·17c	0·001
Threonine	3·16	0·10a	3·07	0·11a	3·43	0·14b	3·58	0·20bc	3·66	0·08c	<0·001
Valine	5·13	0·26	5·05	0·17	5·24	0·16	5·28	0·20	5·26	0·13	ns
Tryptophan	1·30	0·04	1·30	0·03	1·33	0·04	1·27	0·04	1·31	0·02	ns
Methionine	2·36	0·09a	2·46	0·08a	2·63	0·06b	3·02	0·13c	3·55	0·08d	<0·001
Cysteine	0·16	0·00a	0·19	0·01b	0·21	0·01c	0·21	0·01c	0·21	0·01c	<0·001
Phenylalanine	6·22	0·17b	5·90	0·20a	6·18	0·21b	6·35	0·26b	6·70	0·11c	<0·001
Tyrosine	3·89	0·08a	3·90	0·06a	4·11	0·14b	4·34	0·16c	4·52	0·05d	<0·001
Aspartic acid	3·67	0·09ab	3·68	0·06ab	3·59	0·21a	3·81	0·28ab	3·96	0·12b	0·023
Asparagine	1·29	0·04ab	1·24	0·04a	1·33	0·06b	1·34	0·04b	1·35	0·02b	0·004
Glutamic acid	5·03	0·23a	5·08	0·021ab	5·34	0·25ab	5·42	0·21b	5·83	0·20c	<0·001
Glutamine	6·01	0·19	6·23	0·12	6·26	0·33	6·30	0·22	6·32	0·15	ns
Alanine	4·69	0·10	4·67	0·10	4·85	0·08	4·68	0·16	4·80	0·11	ns
Glycine	6·85	0·20ab	6·73	0·12a	7·030	0·23ab	7·06	0·25ab	7·12	0·011b	0·022
Proline	11·46	0·37a	11·27	0·033a	11·75	0·34a	11·69	0·37a	12·22	0·13b	0·002
Serine	2·67	0·10a	2·97	0·16b	2·76	0·13a	3·02	0·15b	3·12	0·09b	<0·001
Taurine	1·92	0·12ab	1·96	0·11b	1·84	0·13ab	1·82	0·15ab	1·72	0·03a	0·041
Ornithine	3·65	0·13ab	3·52	0·19a	3·83	0·16b	4·31	0·17c	4·17	0·16c	<0·001
GABA	0·39	0·01	0·39	0·02	0·37	0·01	0·37	0·01	0·38	0·01	ns
HPro	0·19	0·01	0·18	0·01	0·17	0·01	0·19	0·00	0·18	0·01	ns
BAla	0·13	0·00b	0·11	0·00a	0·11	0·00a	0·11	0·01a	0·11	0·00a	<0·001
HCys	0·01	0·00a	0·01	0·00a	0·02	0·00b	0·02	0·00b	0·02	0·00b	<0·001
Cysta	0·03	0·00a	0·03	0·00b	0·04	0·00c	0·04	0·00c	0·04	0·00d	<0·001
TMG	0·02	0·00a	0·02	0·00b	0·02	0·00b	0·02	0·00b	0·02	0·00c	<0·001
SAM	1·07	0·05a	1·12	0·10a	1·14	0·01a	1·27	0·09b	1·29	0·07b	0·002
SAH	0·17	0·01	0·18	0·02	0·18	0·02	0·19	0·01	0·19	0·01	ns

GABA: γ-amino-n-butyric acid, Hpro: hydroxyproline, Bala: beta-alanine, HCys: homocysteine, Cysta: cystathionine, TMG: trimethylglycine, SAM: S – adenosyl methionine, SAH: S – adenosyl homocysteine. Values are presented as means ± SD (n 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

Shrimp growth performance, survival, whole-body composition and feed efficiency were improved by dietary AQUAVI® Met-Met supplementation after a feeding period of 74 d. The positive effect of the DL-methionyl-DL-methionine dipeptide dietary surplus on growth performance and feed efficiency was in fact early perceived after only 30 and 57 d of feeding. Though the observed improvement of shrimp final body weight and specific growth rate was not always parallel to the increment of Met-Met dietary surplus, since it reached a maximum growth performance in shrimp fed Met-Met 0·12 %. Nonetheless, the ratio of weight gain to the amount of protein consumed, given by the protein efficiency ratio, was found improved up to Met-Met 0·24 %, while the BG was gradually improved with the supplementation of Met-Met. In fact, at the end of the trial, the individuals fed Met-Met 0·24 and 0·32 % showed a significant higher survival rate despite the stabilisation of the growth performance indicators. The authors believe that this could be a reflection of the increase of the rearing density, due to the improved survival of these groups. This hypothesis is supported by the gradual increase of BG up to Met-Met 0·32 %, since it considers both survival and shrimp growth data. Thus, Met-Met efficacy to achieve shrimp robustness as an additive form of methionine, and the AA role in the support of shrimp development is where perceptible. The positive outcome of Met-Met dietary surplus on shrimp growth performance and survival is reinforced by the decrease of feed conversion ratio and feed intake in Met-Met 0·24 and 0·32 %. Model-estimate observations also provide similar conclusions projecting the 0·8% of methionine as the requirement for the optimal growth and body weight, which is equivalent to the 0·24% Met-Met dietary treatment (basal: 0·56% Met). On the other hand, the models estimate that the amount of methionine needed to optimise FCR is slightly higher, 0·88% DM, which corresponds to the Met-Met 0·32% diet. Same conclusions are withdraw by the Met + Cys model estimations. Altogether, these studies strongly support the role of methionine in shrimp growth performance and Met-Met ability to replace DL-methionine as feed additive. Niu, Lemme[33] and Ji, Wang[32] also verified such outcome in similar trials when whiteleg shrimps were fed with graded levels of Met-Met (0·06 to 0·3 % of the diet) for 63 and 91 d, respectively. Niu, Lemme[33] had also described a significant increase of whiteleg shrimp whole-body protein and AA content as a result of Met-Met dietary supplementation. Nevertheless, in the present trial, no differences were observed in shrimp body proximate and AA composition. Similar findings were described by Mamauag, Gao[37] for red sea bream (*Pagrus major*) fed a methionine dipeptide (0·5 % of diet). Nonetheless, Met-Met dietary spare significantly improved energy and fat whole-body

Table 11. Activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total superoxide dismutase (SOD) and catalase (CAT) in shrimp muscle, hepatopancreas and haemolymph

	Muscle								Hepatopancreas							
	ALT		AST		SOD		CAT		ALT		AST		SOD		CAT	
mU/mg protein	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
NC	114·8	10·2c	235·4	23·6b	183·7	16·8a	15·6	2·6a	24·0	2·0c	32·1	3·3b	13·1	2·6	46·7	5·9a
Met-Met 0·08 %	99·7	11·5b	236·0	13·7b	196·0	21·4a	16·8	2·1a	24·1	2·7c	32·4	6·6b	12·6	3·5	46·4	10·8a
Met-Met 0·12 %	66·9	6·9a	187·9	12·4a	262·8	23·8b	17·2	2·9a	18·9	1·9b	21·6	4·2a	15·3	4·1	44·8	3·8a
Met-Met 0·24 %	65·8	7·3a	188·0	19·9a	262·2	20·0b	30·6	3·4b	18·1	2·3ab	19·3	6·6a	14·8	2·4	47·7	5·5a
Met-Met 0·32 %	65·0	7·0a	183·5	19·6a	289·6	12·6b	37·2	4·9c	15·1	2·7a	20·7	4·8a	17·4	2·5	78·4	13·4b
P value	<0·001		<0·001		<0·001		<0·001		<0·001		<0·001		0·149		0·001	

	Haemolymph			
	ALT		AST	
mU/mg protein				
NC	6·8	1·1b	2·8	0·6b
Met-Met 0·12 %	4·5	0·7a	1·6	0·3a
Met-Met 0·24 %	4·7	0·5a	1·6	0·5a
Met-Met 0·32 %	4·0	1·2a	1·7	0·4a
P value	<0·001		0·002	

Values are presented as means ± SD (n 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

retention up to 0·12 % dietary supplementation, while protein and essential AA retention were enhanced until the 0·24 % of surplus, with the exception of methionine whose retention reached a maximum in shrimp fed Met-Met 0·12 % and then decreased at higher supplementation levels. Finally, the retention of all non-essential AA was increased in a dose–response manner. These results indicate an improved metabolic handling of the Met-Met supplemented diets (up to 0·24 % of Met-Met supplementation) for protein synthesis, with the concomitant improvement of growth (as previously discussed). Also, the present results point to the fact that methionine may be restrictive AA in the present diets that limits fat, protein and AA deposition.

AQUAVI® Met-Met presents low water solubility (data from Evonik's aqua R & D group), important for slow feeders as shrimps, but also to the ability to be slowly released in the gut[33]. The latter avoids methionine tissue imbalance and guarantees protein anabolism instead of the catabolic process[38]. In fact, the apparent digestibility of methionine was increased in Met-Met 0·32 % diet compared with NC diet. The net result of such balance is demonstrated by the higher nitrogen daily gain of shrimp fed Met-Met 0·12, 0·24 and 0·32 % and by the gradual decrease of both faecal and metabolic nitrogen daily losses.

Likewise, the majority of the free AA and nitrogenous metabolites analysed in the hepatopancreas were significantly increased in the highest Met-Met supplementation levels (Met-Met 0·24 and 0·32 %) compared with both NC and Met-Met 0·08 %. This is in fact true for methionine and the conditionally essential and methionine-derived AA, cysteine. Also, ornithine, thrimethylglycine and key methionine-related metabolites as homocysteine (HCys), cystathionine, S-adenosylmethionine (SAM) and S-adenosyl homocysteine (SAH) concentrations were augmented in the hepatopancreas. Accordingly, the modulation of the methionine-related pathways was also verified with the up-regulation of hepatopancreas mRNA expression of SAM-synthase (SAM-synth) and spermine synthase in the highest Met-Met dose. Also, the expression of the S-adenosyl homocystainase (SAHH) was found increased up to Met-Met 0·12 % and then significantly reduced in the following supplementation levels (discussed below). The higher free-AA hepatopancreas content was then reinforced by the decreased activity of the aminotransferases (ALT and AST) since both enzymes catalyse the transfer of the amino group of any AA to a carbonyl compound, a mechanism responsible for the formation of necessary AA in the metabolism of proteins and gluconeogenesis. Hence, the low activity of such enzymes may be indicative of an hepatopancreas reduced free-AA mobilisation and tissue damage[39]. In fact, the activity of both enzymes was also reduced in shrimp muscle and haemolymph in response to Met-Met dietary inclusion. Moreover, the higher free-AA pool observed in the hepatopancreas of shrimp fed higher levels of Met-Met supplementation could also be of advantage under osmoregulatory challenging. In most marine invertebrates, it has been described that a high intracellular FAA concentration contributes to osmoregulation in the saline environment[40]. Actually, the ability of crustaceans to maintain intracellular isosmotic homoeostasis rely on a rapid response of cells to regulate the concentration of organic osmolytes, composed essentially, by nitrogen-based compounds, as AA[41]. Through their

Table 12. Total glutathione, protein carbonyls and lipid peroxidation levels in shrimp muscle and hepatopancreas

	Muscle				Hepatopancreas					
	Protein carbonyls		Lipid peroxidation		Total glutathione		Protein carbonyls		Lipid peroxidation	
	nmol/mg		nmol MDA/mg		uM/mg protein		nmol/mg		nmol MDA/mg	
Diet	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd
NC	41·9	9·2	173·6	7·2c	6·9	3·7a	22·4	4·6	35·7	6·4
Met-Met 0·08 %	46·2	13·1	154·9	8·6b	14·5	12·8a	23·3	5·8	34·4	3·3
Met-Met 0·12 %	51·4	15·6	160·2	9·3b	26·4	4·5b	22·5	4·4	32·0	6·1
Met-Met 0·24 %	51·3	11·3	124·3	9·6a	46·1	6·2c	25·5	8·5	30·3	7·8
Met-Met 0·32 %	51·4	8·8	120·1	10·8 a	57·3	7·0d	23·3	5·0	26·7	4·1
P-value	ns		<0·001		<0·001		ns		ns	

Values are presented as means ± SD (*n* 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

accumulation or degradation[42,43], FAA function as osmoeffectors has verified for brine shrimp (*Artemia franciscana*)[44] and whiteleg shrimp[45].

Looking to the oxidative status of shrimps, the several indicators tested pointed to a higher antioxidant activity in both muscle and hepatopancreas in those fed dietary Met-Met supplementation. For instance, the activity of superoxide dismutase in muscle, responsible for the dismutation of superoxide radicals into oxygen and hydrogen, was found increased by Met-Met, concomitant with the decreased levels of lipid peroxidation. Moreover, in both muscle and hepatopancreas tissues, catalase activity, the enzyme accountable for the elimination of hydrogen peroxide to water and oxygen, was found improved in the highest supplementation levels. In agreement, the content of hepatopancreas total glutathione was significantly increased by the Met-Met. GSH is in fact a tripeptide of L-γ-glutamyl-L-cysteinyl-glycine that beyond its antioxidant role can serve as a cysteine storage form[46] in response to the increased hepatopancreas cysteine concentration in shrimp fed Met-Met 0·24 and 0·32 %. The observed results seem to point to a clear optimisation of Met dietary content for its antioxidant properties.

Nonetheless, the expression pattern of GPX and GST was not found in agreement with the dose–response increase of GSH pool. The expression of these antioxidant enzymes increased in shrimp fed up to Met-Met 0·12 % and then dropped in the higher supplementation levels pointing to some level of response to the high methionine dietary content. The observed down-regulation of GST and GPX expression could be related to the activity of non-enzymatic and methionine-related antioxidant mechanisms. Both enzymes transcription is mostly dependent on the involvement of a pro-oxidant stimuli, as ROS and other electrophiles[47]. Since methionine and cysteine are easily oxidised AA, an increased consumption of ROS[48] is excepted with higher methionine concentration, thus rendering ROS less available as a transcriptional factor GST and GPX genes.

An integrated view of the modulated methionine-related pathways with close association with the immune and oxidative stress response

The network of mechanisms evaluated in the present work clearly displayed a modulation of the several methionine-related pathways in response to the dietary Met-Met supplementation. Hence, in this section of the discussion it is intended to correlate the observed results of hepatopancreas with the methionine pathways with close association to shrimp immune and antioxidative response (metabolic pathways and results summarised in Fig. 1).

First, it should be noted that dietary Met-Met surplus was found to be directly translated in an increase of hepatopancreas methionine content, in a dose–response manner. Afterwards, by the action of S-adenosylmethionine synthase (SAM-synth)[49], methionine can generate S-adenosylmethionine (SAM), the universal methyl donor for numerous transmethylation reactions[10]. Indeed, in response to the higher methionine availability, the amount of metabolite SAM and mRNA expression of SAM-synth was improved in shrimp fed the highest Met-Met inclusion levels. Then, SAM-derived methyl group can have different fates, pivotal in the regulation of cellular functions and proliferation, DNA methylation or even in the oxidative stress response[10,20,49–51]. One of those fates is the polyamine biosynthesis via the aminopropylation pathway, where decarboxylated SAM adds aminopropane to the forming polyamines, required for cell proliferation[17]. In the present study, the aminopropylation pathway seems to be modulated since the expression of spermine synthase, responsible for the conversion of polyamine spermidine into spermine in the polyamine biosynthesis pathway, was found up-regulated in Met-Met 0·32 %. Also, the concentration of the non-proteinogenic amino acid ornithine, regulator of polyamine biosynthesis,[52] was found augmented by the higher Met-Met dietary availability.

Moreover, SAM can be consumed through the transsulfuration route, where SAM gives place to S-adenosyl homocysteine and then by the action of S-adenosyl homocysteinase to homocysteine (HCys)[50]. In fact, HCys levels were found superior in most Met-Met dietary treatments. However, the expression levels of SAHH were only found increased up to 0·12 % of Met-Met surplus and then decreased in higher levels of supplementation. This modulation pattern may be interpreted as a negative feedback mechanism to the increasing amounts of HCys in the highest Met-Met supplementation levels. In fact, such hypothesis is backed by the higher amount of trimethylglycine in Met-Met 0·32 %, since

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Table 13. Expression (as normalised mRNA) of selected immune, antioxidant and metabolic genes in shrimp hepatopancreas

Gene	NC		Met-Met 0·08 %		Met-Met 0·12 %		Met-Met 0·24 %		Met-Met 0·32 %		P-Value
	Mean	sd	Mean	sd	Mean	sd	Mean	sd	Mean	sd	
crustin	3·5E-05	8·3E-05	2·6E-03	5·8E-03	2·3E-03	4·5E-03	3·6E-04	9·3E-04	2·6E-05	6·5E-05	ns
pen3	1·3E-02	1·9E-02	5·18E-02	9·5E-02	8·2E-02	8·4E-02	9·2E-03	2·2E-02	3·5E-04	8·2E-04	ns
hemocy	2·5E + 00	2·5E + 00	3·2E + 002·3E + 00		2·0E + 00	2·8E + 00	1·7E + 00	2·6E + 00	4·9E-01	6·8E-01	ns
lys-like	3·0E + 01	3·4E + 01ab	7·6E + 01	9·1E + 01b	3·6E + 01	6·2E + 01ab	1·3E + 01	2·0E + 01ab	2·3E + 00	2·7E + 00 a	0·047
lectin	2·4E + 02	2·2E + 02	3·2E + 02	3·0E + 02	2·3E + 02	2·7E + 02	1·8E + 02	3·6E + 02	3·4E + 01	3·9E + 01	ns
casp3	4·7E-04	9·8E-04	2·1E-03	2·6E-03	7·5E-04	1·7E-03	4·2E-04	4·0E-04	2·6E-05	1·8E-05	ns
rab	9·0E-04	1·0E-03	1·1E-02	3·0E-02	2·6E-03	5·2E-03	2·7E-04	4·5E-04	9·4E-06	1·1E-05	ns
thdox	3·6E-02	4·2E-02	1·6E-01	1·8E-01	1·1E-01	1·5E-01	7·4E-02	8·1E-02	6·2E-02	1·5E-01	ns
gpx	1·7E-03	1·4E-03a	5·3E-03	6·3E-03ab	8·1E-03	7·7E-03b	3·0E-03	4·9E-03ab	5·7E-04	8·2E-04a	0·021
gst	4·2E-03	5·6E-03a	1·5E-02	1·3E-02ab	2·3E-02	2·5E-02b	1·1E-02	1·2E-02ab	1·3E-03	2·4E-03a	0·019
sam-synth	1·2E-03	2·9E-03a	4·0E-04	9·4E-04a	2·5E-03	5·5E-03a	8·8E-03	1·2E-02a	2·5E-02	2·6E-02b	0·005
sms	1·9E-05	2·5E-05a	2·7E-04	6·0E-04a	2·4E-04	6·7E-04a	2·6E-04	7·6E-04a	1·7E-03	1·6E-03b	0·004
odc-antizyme	5·7E-07	8·4E-07	3·0E-07	4·1E-07	1·4E-08	2·3E-08	5·4E-08	1·3E-07	8·9E-09	2·0E-08	ns
sahh	6·9E-02	7·2E-02a	2·6E-01	3·0E-01b	6·5E-01	3·0E-01c	2·5E-02	4·8E-02b	3·6E-03	5·3E-03a	<0·001

Values are presented as means ± SD (n 5). Data were analysed by a one-way ANOVA, with methionine supplementation level as independent variable. When appropriate, means were compared by the Student–Newman–Keuls test. Statistical significance was tested at 0·05 probability level. Different lower-case letters stand for differences among dietary treatments.

it works as a methyl donor for the remethylation of HCys back to methionine in the methionine cycle[53,54]. Nevertheless, HCys can follow the transsulfuration course to form cystathionine and the conditionally essential AA cysteine[54]. Actually, the amount of both molecules was found increased by Met-Met spare. Cysteine can then take part in the formation of the antioxidant molecule glutathione[55], whose activity was found increased in a dose–response manner to the Met-Met availability. Nevertheless, and as previously discussed, the expression of the free radical scavengers GST and GPX were found increased only up to Met-Met 0·12 % and then found down-regulated to the basal levels presented by the NC dietary treatment. Is here hypothesised that such response could be the result of a negative feedback mechanism to the high amount of glutathione and its precursor, cysteine. Similar response was previously observed for SAHH mRNA expression. Methionine contribution to oxidative stress homeostasis is further demonstrated by the higher catalase and low aminotransferases activity in the hepatopancreas that point to an improved protection to oxidative damage and minor tissue damage.

In summary, AQUAVI® Met-Met efficiency in promoting zootechnical performance in a practical formulation scenario for the whiteleg shrimp (*Penaeus vannamei*) is once again confirmed. It can be concluded that graded dietary increase of methionine dipeptide up to 0·24 % for 74 d may result in significant gains on the growth performance, feed efficiency, nutrient and nitrogen gain and shrimp survival. Moreover, for the first time, it was showed that Met-Met dietary spare leads to an improvement of free-AA pool and nitrogen metabolites concentration in the hepatopancreas and reduces the signs of oxidative stress in both hepatopancreas, muscle and haemolymph. Finally, in a closer look to the methionine-related pathways passive to be altered by Met-Met spare, a clear modulation of the described antioxidant and cell proliferation routes was detected. Actually, despite the activation of control feedback mechanisms in response to higher surplus levels, homeostasis seems to be maintained that is clearly showed by the growth performance demonstrated by those diets.

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K. M. and J. D. conceived the experiments and formulated the experimental diets; J. D. produced the experimental diets; S. F. B., C. T., M. V. and R. S. conducted the experimental trial; M. M. and J. D. performed most laboratory techniques and M. M. wrote the manuscript under the supervision of J. D., B. C. and K. M. All authors contributed to and approved the manuscript.

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Table 14. Exponential (Ex), liner broken-line (LBL) and quadratic broken-line (QBL) models estimation of methionine (met) and met + cysteine (Cys) requirements for maximum final body weight (FBW), biomass gain and feed conversion ratio (FCR) after 74 d of feeding

			Met			Met + Cys		
Model	Response variable	Equation	Estimated requirement (% DM)	P value	R2	Estimated requirement (% DM)	P value	R2
Exponential (Ex)	FBW	$Y = 9{\cdot}49 + 6{\cdot}0\ (1-e^{(-9{\cdot}57 \times (X-\beta 3))})$	0·87	<0·01	0·88	1·34	<0·01	0·88
	Biomass gain	$Y = 1014{\cdot}8 + 904{\cdot}3\ (1-e^{(-9{\cdot}545 \times (X-\beta 3))})$	0·87	<0·01	0·90	1·34	<0·01	0·90
	FCR	$Y = 2{\cdot}51 - 1{\cdot}26\ (1-e^{(-6{\cdot}49 \times (X-\beta 3))})$	1·02	<0·01	0·90	1·49	<0·01	0·90
Linear broken-line (LBL)	FBW	$Y = 15{\cdot}01-35{\cdot}74\ (0{\cdot}72-Met)$ for Met < 0·72; $Y = 15{\cdot}01$ for Met ≥ 0·72	0·72	<0·01	0·91	1·19	<0·01	0·91
	Biomass gain	$Y = 1845-5415\ (0{\cdot}72-Met)$ for Met < 0·72; $Y = 1845$ for Met ≥ 0·72	0·72	<0·01	0·93	1·19	<0·01	0·93
	FCR	$Y = 1{\cdot}43-5{\cdot}71\ (0{\cdot}75-Met)2$ for Met < 0·75; $Y = 1{\cdot}43$ for Met ≥ 0·75	0·75	<0·01	0·92	1·22	<0·01	0·92
Quadratic broken-line (QBL)	FBW	$Y = 15{\cdot}07-98{\cdot}84\ (0{\cdot}80-Met)^2$ for Met < 0·80; $Y = 15{\cdot}07$ for Met ≥ 0·80	0·80	<0·01	0·89	1·27	<0·01	0·89
	Biomass gain	$Y = 1854-14\ 940{\cdot}6\ (0{\cdot}80-Met)^2$ for Met < 0·80; $Y = 1854$ for Met ≥ 0·80	0·80	<0·01	0·90	1·27	<0·01	0·90
	FCR	$Y = 1{\cdot}42-11{\cdot}39\ (0{\cdot}87-Met)^2$ for Met < 0·87; $Y = 1{\cdot}42$ for Met ≥ 0·87	0·87	<0·01	0·91	1·34	<0·01	0·91
Average model requirement	FBW		0·80			1·27		
	Biomass gain		0·80			1·27		
	FCR		0·88			1·35		

$\beta 3$: Met (0·56) or Met + Cys (1·03).

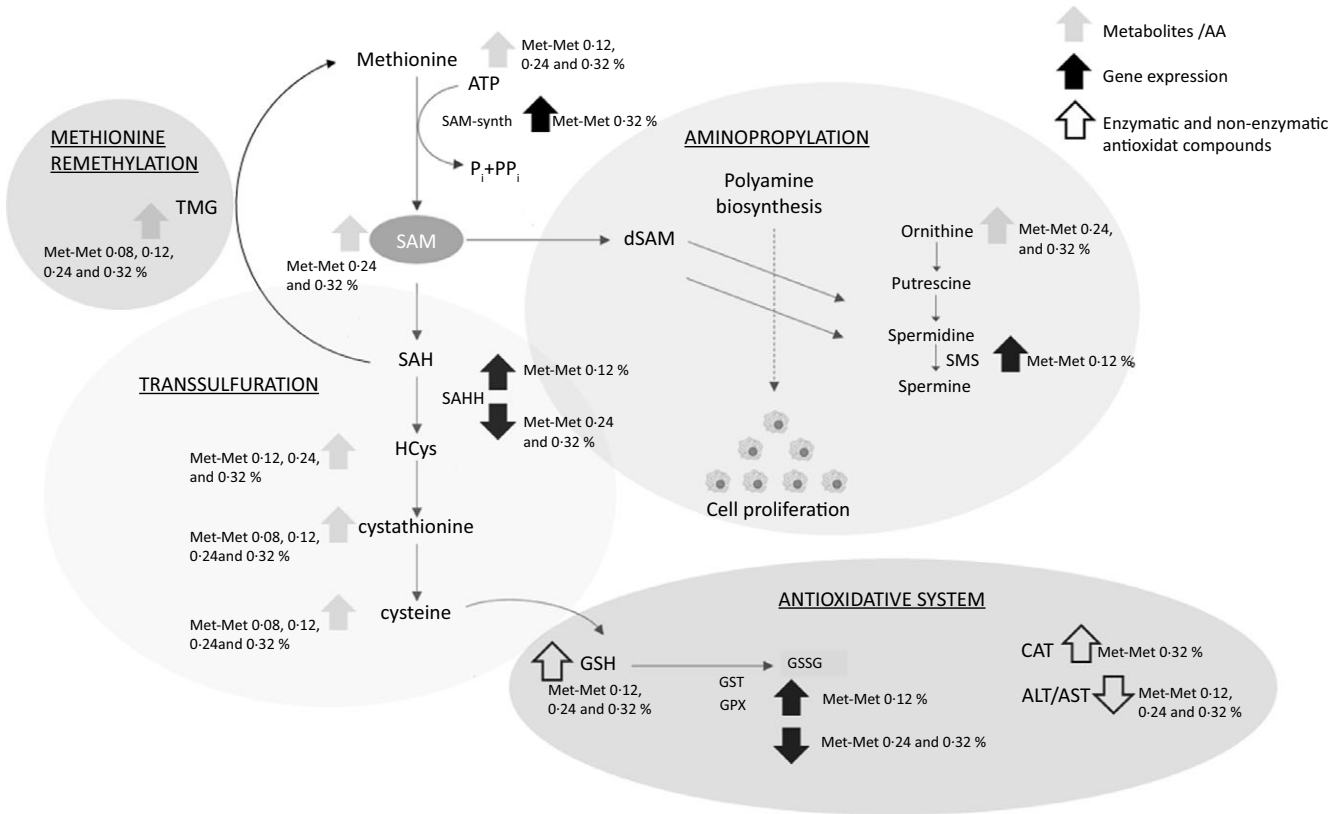

Fig. 1. Methionine-associated routes modulated in the hepatopancreas in response to Met-Met dietary supplementation. ATP: adenosine triphosphate; SAM: S-adenosylmethionine; TMG: trymethylglycine; SAH: S-adenosylhomocysteine; HCys: homocysteine; SAHH: S-adenosyl homocysteine hydrolase; dSAM: decarboxylated S-adenosylmethionine; SMS: spermine synthase; GSH: glutathione; GSSG: glutathione oxidase; GST: glutathione transferase; GPX: glutathione peroxidase; CAT: catalase; ALT/AST: alanine and aspartate aminotransferases.

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