Metabolic and nutritional responses of Nile tilapia juveniles to dietary methionine sources

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Abstract

Commercial diets for tilapia juveniles contain high levels of plant protein sources. Soybean meal has been utilised due to its high protein content, however, soy-based diets are limited in methionine and require its supplementation to fulfil fish requirements. DL-methionine (DL-Met) and calcium bis-methionine hydroxyl analogue (MHA-Ca) are synthetic methionine sources supplemented in aquafeeds, which may differ in biological efficiency due to structural differences. The present study evaluated the effect of both methionine sources on metabolism and growth of Nile tilapia. A growth trial was performed using three isonitrogenous and isoenergetic diets, containing plant ingredients as protein sources: DLM and MHA diets were supplemented on equimolar levels of methionine, while REF diet was not supplemented. Hepatic free methionine and one-carbon metabolites were determined in fish fed for 57 days. Metabolism of DL-Met and MHA was analysed by an in vivo time-course trial using $^{14}$C-labelled tracers. Only DL-Met supplementation significantly increased final body weight and improved feed conversion and protein efficiency ratios compared to the REF diet. Our findings indicate that methionine in DLM fed fish follows the transsulfuration pathway while in fish fed MHA and REF diets it is remethylated. The in vivo trial revealed that $^{14}$C-DL-Met is absorbed faster and more retained than $^{14}$C-MHA, resulting in a greater availability of free methionine in the tissues when fish is fed with DLM diet. Our study indicates that dietary DL-Met supplementation improves growth performance and nitrogen retention, and that methionine absorption and utilisation is influenced by the dietary source in tilapia juveniles.
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Introduction

Tilapia is the second most farmed fish worldwide, just after carp\(^1\). Nevertheless, there are still many challenges to overcome in order to farm tilapia under economic and environmentally sustainable principles. Part of the challenge is dependent on the development of sustainable feeds that will ensure an optimal nutritional status for the fish while promoting nitrogen and phosphorus retention, thus reducing nutrient excretion into the aquatic environment.

In order to increase the sustainability of aquaculture feeds, inclusion levels of plant ingredients in fish diets have increased. Soybean meal has been successfully included in aquafeeds\(^2\)\(^-\)\(^4\) due to its high protein content and relatively well-balanced amino acid profile. However, soy-based diets are limited in methionine and require its supplementation in order to fulfil fish requirements\(^5\).

Methionine (Met) is an indispensable amino acid involved in protein synthesis, transmethylation reactions and antioxidant defence. Methionine metabolism occurs mainly in the liver where L-methionine is converted into S-adenosylmethionine (SAM), a methyl donor for several reactions. Subsequently SAM donates its methyl group and is transformed into S-adenosylhomocysteine (SAH), which is rapidly converted into homocysteine. Hepatic homocysteine can be remethylated back to methionine by adding a methyl group from trimethylglycine or can irreversibly enter the transsulfuration pathway\(^6\). The transsulfuration pathway converts homocysteine into cystathionine and then cysteine. Ultimately, cysteine can be incorporated into proteins, metabolised into glutathione or oxidised to form taurine\(^7\).

DL-methionine (DL-Met) and methionine hydroxyl analogue (MHA; DL-2-hydroxy-4-methylthiobutyrate or DL-HMTBA and its calcium salt) are synthetic sources of methionine that are often supplemented to animal feeds. DL-Met is a racemic mixture of D- and L-isomers of methionine, while MHA is a racemic mixture of D- and L-isomers of methionine hydroxyl analogue\(^8\). MHA chemical structure is similar to that of methionine however, it contains a hydroxyl group instead of the amino group. Recently, studies in rainbow trout (Oncorhynchus mykiss) suggested that DL-Met and MHA uptake in the gut apical surface is facilitated by sodium-dependent transporters and mediated by proton-independent transporters across the basolateral membrane\(^9\)\(^-\)\(^10\). Data comparing the intestinal flux rates of DL-Met and MHA suggests a faster intestinal transport of the former synthetic source\(^9\)\(^,\)\(^10\)\(^-\)\(^11\).

Since animals can only metabolise L-amino acids, D-isomers of DL-Met first need to be converted to a keto-methionine intermediate (keto-methylthio-butanoic acid, KMB) and then transaminated to L-Met before becoming available\(^8\)\(^,\)\(^12\). On the other hand, both the D- and L-isomers of MHA need to be converted to KMB to become available\(^8\)\(^,\)\(^12\). These differences are likely to be reflected as differences in absorption and metabolism and may result in different biological efficiencies. Numerous studies in terrestrial animals and fish advocate that MHA
supplementation results in lower bioavailability of this compound compared to DL-Met\(^{(13-15)}\). In rainbow trout, dietary methionine hydroxy analogue calcium salt (MHA-Ca) was found to have lower bioavailability than DL-Met, resulting in 69 % lower fish weight gain, 60 % lower growth rate and 73 % lower nitrogen retention in fish\(^{(16)}\). Similarly, growth performance and feed utilisation indicators in common carp (Cyprinus carpio) fed DL-Met and MHA-Ca supplemented diets, demonstrated that MHA-Ca was 41 % to 50 % as available as DL-Met on weight-for-weight basis\(^{(17)}\). In contrast, some authors report similar efficiencies among methionine sources based on growth and feed efficiency in several aquatic animals\(^{(18-21)}\). On the other hand, a study performed in channel catfish observed that dietary MHA-Ca supplementation resulted in improved body weight, weight gain, feed conversion ratio and protein efficiency ratio\(^{(22)}\). Based on the data published, NRC\(^{(23)}\) concluded that it is reasonable to assume that the biological efficacy of MHA in fish is 75-80 % that of DL-Met on an equimolar basis (63-67 % on a weight basis). Generating data on growth, diet utilisation and methionine metabolism in a commercial relevant species such as the Nile tilapia (Oreochromis niloticus) is of paramount importance for the Aquaculture industry.

In this context, the objective of this study was to understand how the dietary source of methionine affects methionine metabolism and growth of Nile tilapia juveniles. Radiolabelled methionine sources were used in a nutrient flux assay to evaluate their influence in the amino acid metabolic pathways.

**Materials and Methods**

**Diets**

Three experimental diets were formulated to be isonitrogenous and isoenergetic (Table 1). Diet REF was a negative control, without fishmeal inclusion, formulated to be 40 % below the methionine requirement for Nile tilapia (Oreochromis niloticus)\(^{(23)}\). No methionine was supplemented to this diet. Experimental diets were the REF diet supplemented with 1.5 g/kg DL-methionine (DLM diet) and 1.8 g/kg calcium bis-methionine hydroxyl analogue (equal on molar basis to 1.5 g/kg DL-methionine; MHA diet). To minimise variability among diets, one common basal diet was formulated, and the respective supplemental methionine source was added in DLM and MHA diets. Diets were formulated to meet the minimum requirements of amino acids on digestible basis for Nile tilapia juveniles, except for methionine. Apparent digestibility coefficients (ADC) of amino acids for the ingredients used were taken from published review data\(^{(24-25)}\). Diets were supplemented with selected indispensable amino acids and di-calcium phosphate to avoid amino acid or mineral imbalances.

Upon ingredient grinding with a hammer mill (model SH1, Hosokawa-Alpine, Germany) and its mixing in a double-helixmixer, all diets (pellet sizes 1.2 and 2.0 mm) were manufactured using a
twin-screw extruder (model BC45, Clextral, France) at SPAROS Lda. (Olhão, Portugal). Upon cold extrusion, diets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, the oils were added to the pellets by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature, in a cool and aerated storage room. Proximate composition and amino acid analysis were determined in all experimental diets, as reported in Tables 1 and 2, respectively.

Growth trial

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Nile tilapia (Silver Natural Male Tilapia™) juveniles were obtained from Til-Aqua International B.V. (The Netherlands) and the experiment was conducted at Centre of Marine Sciences of Algarve (CCMAR) facilities (Faro, Portugal). CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). Upon arrival, fish were acclimatised to the new rearing facilities in a recirculating aquaculture system and were fed a commercial diet (crude protein: 340 g/kg; crude fat: 50 g/kg).

Juvenile Nile tilapia were reared in 100 litre cylindrical tanks in a recirculating aquaculture system equipped with a mechanical filter, a submerged biological filter and a UV steriliser. Photoperiod was natural (10 h light: 14 h dark), temperature averaged 25.9 ± 0.5 °C and dissolved oxygen in water was maintained above 85 % of saturation. Water quality parameters were monitored daily and adjusted when necessary; pH was maintained between 7.0 and 8.2 and the concentration of unionized ammonia and nitrites in water was 0 mg/l during the whole experimental period. Mortality was monitored daily.

Fish with an initial mean body weight of 2.3 ± 0.4 g were allocated into nine tanks at an initial density of 1.1 kg/m³ (50 fish per tank). Eight fish from the initial stock were sampled and the whole-fish were stored at -20 °C until analysis of proximate composition. Triplicate tanks were randomly assigned to one of the three dietary treatments (REF, DLM and MHA). Fish were fed to visual satiety by hand, three times a day (09.30, 12.30 and 16.30 hours) and feed intake was recorded daily for 57 days.

At the end of the trial, each tank was bulk weighed. Ten fish from each tank were euthanised with a lethal dose of anaesthetic (1.5 ml/l phenoxyethanol, Sigma-Aldrich, Spain). Whole-body, liver and viscera weight of five individual fish were recorded for calculation of biometric indexes and liver samples were snap-frozen in liquid nitrogen and kept at -20 °C until free amino acid
analysis. The other five fish were stored at -20 °C until analysis of whole-body proximate composition and amino acid content. Fish were fasted for 24 h before initial and final samplings.

**Metabolic utilisation of supplemental methionine sources**

At the end of the growth trial, to understand how different dietary methionine sources are absorbed and metabolised in tilapia juveniles, a time-course metabolic trial was performed using radiolabelled DL-Met and MHA. REF diet was not included in this trial since the aim was to assess putative differences in the metabolic flux of the methionine supplemental sources and not of the intact protein. Random fish fed the DLM or MHA diet were transferred to the nutrient flux laboratory after being fasted for 24 h.

DL-(1-14C)-methionine (14C-DL-Met; 0.00185 GBq) and (1-14C)-calcium bis-methionine hydroxyl analogue (14C-MHA; 0.00185 GBq) (Campro Scientific GmbH, The Netherlands) were used as tracers to radiolabel the experimental diets (DLM and MHA). The methodology for labelling the experimental diets was established in previous works of the authors (26-28). The tracers were diluted in freshwater Ringer solution and a known value of the tracer was dispensed using a micropipette on individual pellets of the correspondent experimental diet. The pellets were dried at 50 °C for 1 h. Eight pellets per fish, corresponding to 0.3 % body weight, were loaded into a hollow plastic tube of 1.5 mm inner diameter, and stored for subsequent tube-feeding. Prior to tube-feeding, the amount of radioactivity (disintegrations per minute; DPM) of ten individual pellets from each experimental diet labelled with the corresponding tracer was determined in a TriCarb 2910TR low activity liquid scintillation analyser (Perkin Elmer, USA) after adding the scintillation cocktail (Ultima Gold XR, Perkin Elmer).

The *in vivo* method of tube-feeding used to perform the metabolic trials was adapted from Costas (29), which was a modification of the method first described by Rust *et al.* (30) and modified by Rønnestad *et al.* (31). Fish were anaesthetised (200 mg/l MS-222 buffered with sodium bicarbonate, Sigma-Aldrich) and subsequently were taken out of the water using a fish net and placed onto a dry plastic tray. The previously loaded plastic tube containing the radiolabelled pellets was inserted into the fish mouth and the feed pellets were gently pushed directly into the oesophagus using a solid piece with a smaller diameter placed inside as a plunger. The diameter and length of the hollow plastic tube was previously tested to avoid injuring the fish oesophagus. This procedure lasted approximately ten seconds. The metabolic fate of the tracer (14C-DL-Met or 14C-MHA) was considered to represent the fate of the tracee (DL-Met or MHA-Ca) (32).

After tube-feeding, fish were placed into a tank with clean and aerated freshwater to eliminate any residual anaesthetic and were monitored for eventual pellet regurgitation. After this period, fish were transferred into individual incubation chambers containing 2 litres of freshwater at 26 °C.
Each chamber was hermetically sealed and supplied with a gentle oxygen flow. After the incubation period, oxygen flow was stopped and fish were euthanised inside the chambers by a lethal dose of anaesthetic (750 mg/l of MS-222 buffered with sodium bicarbonate). The incubation periods were 1, 2, 3, 4 or 6 h ($n = 6$-$7$ fish for each diet and incubation period). Fish was removed from the chamber and weighed.

Water samples were collected from each individual chamber to determine the amount of radioactivity (DPM) present in the incubation water. The radioactivity present in the incubation water resulted from evacuated (non-absorbed) and/or catabolised radiolabelled methionine source as CO$_2$. Viscera, liver, skin-on fillets and the rest of the fish were collected and weighed. Viscera consisted of washed digestive tract (so that no alimentary bolus was present), spleen, pancreas and perivisceral fat and will be designated from hereafter as Viscera compartment; skin-on fillets (muscle with skin), as Muscle compartment; and the rest of the fish (consisting of head, heart, kidney, bones, and fins) as Residual compartment. Viscera and Liver compartments were analysed as whole. Muscle and Residual compartments were minced using a coffee grinder until a homogeneous mixture was obtained and 0.5 g samples were taken for further analysis.

All fish tissues were incubated at 4 °C for 24 h with 6% (w/v) trichloroacetic acid (TCA), with periodical stirrings. After this period, tissue samples were taken and the TCA samples collected for radioactivity determination (from hereafter designated as Free fraction). In order to get a better insight of the metabolic flux of $^{14}$C-DL-Met and $^{14}$C-MHA, tissue samples from the 6 h incubation period were homogenised and underwent a series of extraction procedures to separate organic compounds such as protein, lipids, and other metabolites as described previously by Rocha et al. (26). Briefly, samples were transferred to a clean vial and further homogenised in distilled water using an Ultra-turrax homogeniser (IKA, Germany). Total lipids were extracted using a modified Bligh and Dyer method (33) for small volumes and total protein was extracted based on a TCA precipitation method (34). The supernatant containing non-extracted metabolites was collected for radioactivity determination. Protein pellet was resuspended in an appropriate volume of Solvable™ (Perkin Elmer, USA) and kept at 50 °C until complete solubilisation was achieved. Lipids and other metabolites are designated as Others fraction, while protein as Protein fraction. Scintillation cocktail (Ultima Gold XR, Perkin Elmer) was added to all samples and DPM were counted in a TriCarb 2910TR low activity liquid scintillation analyser (Perkin Elmer). All counts were corrected for quench and lumex. Radioactivity found in the Incubation Water and in the Free fractions of all compartments was normalised for fish or tissue weight and expressed as DPM per gram. Radioactivity present 6 h after feeding the radiolabelled nutrients in the Protein and Others fractions of all compartments was expressed as DPM.
To estimate the availability of the radiolabelled nutrient in each compartment, the area under the curve from 1 to 6 h was calculated as follows:

$$ \sum_{i=1}^{n-1} \left[ \frac{(DPM_{t+1} + DPM_t) \times (t_{i+1} - t_i)}{2} \right] $$

where $t$ is the time point, $DPM_t$ the amount of radioactivity found at $t_i$ and $n$ the total number of measures\(^{(35)}\). Area under the curve was expressed as a percentage of total cumulated (1 to 6 h) radioactivity (DPM) per dietary treatment.

**Biochemical analysis**

Raw materials (soybean meal, soy protein concentrate, pea protein concentrate, corn meal and wheat bran) were analysed for dry matter, crude protein and amino acid content using NIR (AMINONIR\(^{®}\), Evonik Nutrition & Care, Germany) before diet formulation.

Chemical analysis followed standard procedures of the Association of Official Analytical Chemists (AOAC\(^{(36)}\)) and were run in duplicates. Before analysis, diets and pooled whole-body fish were finely ground. Moisture content was determined by drying the samples at 105 °C for 24 h and ash content by incineration in a muffle furnace at 550 °C for 6 h. Freeze-dried whole-body samples and diets were analysed for crude protein (N x 6.25) by wet chemistry (AMINOLab\(^{®}\), Evonik Nutrition & Care, Germany) using the combustion/Dumas method; crude fat by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany); gross energy by combustion in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany) calibrated with benzoic acid and phosphorus content by digestion at 230 °C in a Kjeldathem block digestion unit followed by digestion at 75 °C in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406).

Amino acid content in diets and whole-body fish samples was analysed by wet chemistry (AMINOLab\(^{®}\), Evonik Nutrition & Care, Germany) using ion exchange chromatography. Hepatic free amino acids, SAM and SAH contents were determined after homogenisation of freeze-dried samples in 0.1 M HCl on ice, centrifugation at 1500 \(x\) g at 4 °C for 15 min and deproteinization of the supernatant by centrifugal ultrafiltration (10 kDa cut-off, 2500 \(x\) g at 4 °C for 20 min). All samples were pre-column derivatised with Waters AccQ Fluor Reagent (6-aminquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA), except samples for SAM and SAH analysis, which were not derivatised. All analyses were performed by ultra-high-performance liquid chromatography (UPLC) on a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Amino acids were identified by retention times of
standard mixtures (Waters) and pure standards (Sigma-Aldrich). Instrument control, data acquisition and processing were achieved by the use of Waters Empower software.

Nutritional indicators

Growth performance parameters, somatic indexes and nutrient retention were calculated as follows:

Daily voluntary feed intake (VFI, % ABM / day) = 100 x (apparent feed intake / ABM / days), where ABM is average body mass = (final biomass + initial biomass)/2.

Feed conversion ratio (FCR) = apparent feed intake / wet weight gain.

Protein efficiency ratio (PER) = wet weight gain / crude protein intake.

Hepatosomatic index (HSI %) = 100 x (liver weight / total weight).

Viscerosomatic index (VSI %) = 100 x (viscera weight / total weight).

Protein or energy retention (% intake) = 100 x [(final body protein or energy content – initial body protein or energy content) / (protein or energy intake)].

Daily nitrogen intake (mg N / kg / day) = nitrogen intake / ABM / days.

Daily nitrogen gain (mg N / kg / day) = (final body nitrogen content – initial body nitrogen content) / ABM / days.

Daily nitrogen loss (mg N / kg / day) = daily nitrogen intake – daily nitrogen gain.

Statistical analysis

Sample size was determined based on preliminary power analysis to ensure a probability of at least 80% in the detection of treatment effects. Data are presented as means ± standard deviation. Data expressed as a percentage were arcsine square root transformed previously to the statistical analysis (37). All data were checked for normal distribution and homogeneity of variances. Differences among groups were identified by one-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test or, when the assumptions for the ANOVA failed, by Kruskal-Wallis one-way analysis of variance by ranks followed by Dunn’s multiple-comparison tests. Data from the time-course metabolic trials were subjected to linear regression analysis to understand the relationship between the tracer (14C-DL-Met or 14C-MHA) and incubation time in each compartment (Incubation Water, Viscera, Liver, Muscle and Residual compartments). Additionally, one-way ANOVA followed by planned contrasts test was performed to compare the differences between 14C-DL-Met and 14C-MHA fed fish at each time point. At 6 h time point, Mann-Whitney U test was additionally used to identify differences in the Protein and Others fractions of the several body compartments. All statistical differences were considered significant at P < 0.05. Statistical analyses were performed using the open source software R version 3.6.1.
Results

Growth performance and feed utilisation

At the end of the growth trial, survival was 100% in all treatments and fish had a 9 to 10-fold increase in body weight (Table 3). Fish fed the DLM diet were significantly heavier (28.3 ± 4.7 g) than fish fed the REF diet (24.1 ± 3.0 g) and the MHA (24.8 ± 2.7 g) diets (p < 0.05). Fish biomass gain differed marginally with the different dietary treatments (p = 0.08). Feed intake was similar among experimental groups. Fish fed the DLM diet presented a significantly lower feed conversion ratio (FCR) than fish fed the REF diet (p < 0.05). Protein efficiency ratio (PER) was significantly higher in fish fed the DLM (3.4 ± 0.1) than the REF (3.1 ± 0.1) diet (p < 0.05). Dietary treatments did not affect significantly hepatosomatic and viscerosomatic indexes (p > 0.05).

Whole-body fish composition and nutrient retention

Fish from the DLM diet presented significantly higher body protein and energy content than fish from the MHA diet (p < 0.05; Table 4). Fish whole-body moisture, ash, fat and phosphorus content were not affected by the dietary treatments (p > 0.05). Additionally, whole-body total methionine content was significantly higher in DLM fed fish (3.86 ± 0.01 g/kg) than in fish from the REF (3.54 ± 0.11 g/kg) or MHA (3.50 ± 0.14 g/kg) dietary treatments (p < 0.05). No significant differences were found for the other amino acids among experimental groups. Protein and energy retention were significantly higher for fish fed the DLM diet than for fish fed the other diets (p < 0.05; Table 4).

Dietary treatments did not influence daily nitrogen intake. DLM fish exhibited the highest daily nitrogen gain (726 ± 3 mg N/kg/day), significantly different from REF (657 ± 8 mg N/kg/day) or MHA (660 ± 22 mg N/kg/day) fed fish (p < 0.05). Moreover, fish from the DLM group presented the lowest daily nitrogen loss (667 ± 43 mg N/kg/day), which was significantly different from the REF group (p < 0.05; Fig. 1).

Methionine and one-carbon metabolites

Free methionine (Fig. 2A) was significantly higher in liver of fish fed the DLM and MHA diets than in REF fed fish (p < 0.05). S-adenosylmethionine (SAM; 1.25 to 1.35 mg SAM/g DW liver), S-adenosylhomocysteine (SAH; 0.23 to 0.27 mg SAH/g DW liver) or SAM/SAH ratio (4.79 to 5.48) were not affected by the dietary treatments (p > 0.05). Homocysteine levels (Fig. 2B) were significantly lower in DLM fish than in fish fed REF and MHA diets (p < 0.05). Cystathionine was significantly lower in fish fed the supplemented diets than in fish fed the REF diet (p < 0.05; Fig. 2C). Free cysteine content was not affected by the dietary treatments (p > 0.05; 0.17 to 0.20 mg Cys/g DW liver). Fish fed the DLM diet had more hepatic taurine content than fish fed REF and
MHA diets ($p < 0.05$). In addition, there were no significant differences in taurine content between fish fed REF and MHA diets ($p > 0.05$; Fig. 2D). Hepatic trimethylglycine was significantly lower in fish fed the DLM ($0.05 \pm 0.00 \text{ mg} \text{TMG/g DW liver}$) than the REF ($0.06 \pm 0.00 \text{ mg} \text{TMG/g DW liver}$) diet ($p < 0.05$). No significant differences in hepatic trimethylglycine content were found for fish fed the MHA diet compared to the other groups ($p > 0.05$).

Moreover, total free amino acids were significantly higher in DLM fish liver ($108.42 \pm 2.90 \text{ mg/g DW liver}$) than in liver of fish fed the REF ($86.48 \pm 3.29 \text{ mg/g DW liver}$) and the MHA ($86.08 \pm 1.81 \text{ mg/g DW liver}$) diets ($p < 0.05$). Similarly, the sum of free indispensable (IAA) and dispensable amino acids (DAA) were significantly higher in liver of fish fed the DLM diet than in liver of fish fed the other diets ($p < 0.05$; Fig. 3).

**Metabolic utilisation of supplemental methionine sources**

The amount of radiolabelled tracer found in *Incubation Water* had a linear increase during the time-course for both DLM and MHA treatments (Table 5; $p < 0.05$). Also, at the end of the time-course (6 h) a higher amount of $^{14}$C-MHA was present in the *Incubation Water* compartment than of $^{14}$C-DL-Met ($p < 0.05$; Fig. 4). Furthermore, the area under the curve for the MHA diet was 2.4-fold higher than for the DLM diet, indicating a higher evacuation and/or catabolism of $^{14}$C-MHA compared to $^{14}$C-DL-Met by juvenile tilapia (Table 5).

After 1 h of diet ingestion, the amount of tracer found in *Viscera* (Fig. 5A) and *Liver* (Fig. 5B) *Free* fractions was higher in the DLM than in the MHA fish, although only significantly different for the *Liver Free* fraction ($p < 0.05$; Fig. 5B). The $^{14}$C-DL-Met presented a linear decrease during the incubation period in both compartments ($p < 0.05$; Table 5). On the other hand, this linear pattern was not found in fish fed MHA diet. The amount of tracer in *Viscera Free* fraction increased up to 4 h and then decreased at 6 h to values slightly below the values determined for the DLM fed fish (Fig. 5A). After 4 h of ingestion, the amount of tracer found in *Viscera Free* fraction of MHA fed fish was significantly higher than in fish fed the DLM diet ($p < 0.05$; Fig. 5A). Regarding the $^{14}$C-MHA in the *Liver Free* fraction, a plateau was observed from 2 h until the end of the incubation period (Fig. 5B). The area under the curve in the *Viscera* and *Liver Free* fractions were similar for both dietary treatments, indicating a similar bioavailability of both methionine sources along the experimental period (Table 5).

The presence of $^{14}$C-DL-Met in the *Residual Free* fraction presented a peak at 2 h after diet ingestion (Fig. 5C), while for $^{14}$C-MHA a linear increase was observed during the whole incubation period ($p < 0.05$; Table 5). The amount of tracer found in the *Residual Free* fraction of DLM fed fish was significantly higher than in fish fed the MHA diet ($p < 0.05$; Fig. 5C) at 3 h after diet ingestion. Similar to the *Residual Free* fraction, the amount of tracer found in the *Muscle Free*
fraction (Fig. 5D) of MHA fed fish exhibited a linear increase with time (p < 0.05; Table 5), while for the $^{14}$C-DL-Met a peak was found 2 h after diet ingestion. The amount of $^{14}$C-DL-Met found in Muscle Free fraction was significantly higher than that of $^{14}$C-MHA (p < 0.05) at 2 and 3 h after diet ingestion. The area under the curve for the Residual and Muscle Free fractions were 1.3 and 1.9-fold higher in DLM fed fish than in fish fed MHA diet, respectively, indicating a higher bioavailability of the methionine source in the fish fed the DLM diet (Table 5).

At the end of the incubation period (6 h) a significantly higher amount of tracer was determined in the Viscera and Muscle Protein fractions from DLM fed fish (Fig. 6; p < 0.05) No significant differences between treatments were detected in Liver and Residual Protein fractions (p > 0.05). Regarding the Others fraction (lipids and other metabolites), there were no significant differences between treatments in all compartments (p > 0.05). The amount of tracer present in the Liver Others fraction was the lowest (333 and 222 DPM for fish fed the DLM and MHA diets, respectively), and the highest in the Residual Others fraction (2858 and 3037 for DLM and MHA fed fish, respectively).

Discussion

The in vivo method using radiolabelled tracers ($^{14}$C-DL-Met and $^{14}$C-MHA) was utilised to gain a deeper understanding on how different methionine sources may affect the dietary methionine utilisation by Nile tilapia juveniles. The amount of tracer found in the Incubation Water increased with time in both treatments, moreover the analysis of the area under the curve from 1 to 6 h after ingestion, revealed a higher amount of $^{14}$C-MHA than of $^{14}$C-DL-Met indicating a greater evacuation of unabsorbed MHA and/or higher catabolism of this methionine source compared to DL-Met. The solubility of MHA-Ca in water is higher than that of DL-Met$^{(38)}$, however, since the labelled pellets were placed directly into the fish oesophagus and were not in contact with water, the radioactivity present in the Incubation Water was the result of the evacuation of unabsorbed tracer. Similarly, in broiler chicks fed $^{14}$C-DL-Met or $^{14}$C-MHA a higher amount of tracer was present in the excrements of $^{14}$C-MHA fed birds than in excrements of birds fed the $^{14}$C-DL-Met$^{(39-40)}$. DL-Met and MHA uptake is mediated by sodium-dependent transporters across the brush border membrane$^{(9-10)}$, however, MHA is also partly transported by diffusion$^{(41)}$. In rainbow trout, it has been demonstrated that MHA presents a slower intestinal absorption rate than DL-Met$^{(10)}$. This indicates that MHA stays longer in the intestine being more prone to bacterial degradation$^{(42)}$ that would lead to a further decrease in the intestinal absorption of MHA and its evacuation. Combined, these two factors may contribute to the increased amount of unabsorbed $^{14}$C-MHA relative to $^{14}$C-DL-Met in the Incubation Water.
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In our study, *Viscera* and *Liver Free* fractions of DLM and MHA fed fish presented a similar cumulative bioavailability of $^{14}\text{C}}$-DL-Met and $^{14}\text{C}}$-MHA for the 6 h-period. However, a comparison between the two dietary treatments revealed different patterns of tracer flux in these compartments throughout the *in vivo* trial. Fish fed the DLM diet presented a peak in *Liver Free* fraction 1 h after feeding while no peak was observed during the 6 h time-course for MHA fed fish. The D-isomer of DL-Met and L- and D-isomers of MHA need to be converted to L-methionine to become available to the animal via a two-step process$^{(8,43)}$; however, the L-isomer present in DL-Met is readily available. Therefore, the results from the *in vivo* metabolic trial suggest a faster availability of DL-Met to metabolic processes than of MHA.

In fish fed the DLM diet, the tracer was available to the *Residual Free* fraction (*Free* fractions in head, heart, kidney, bones and fins) faster than in MHA fed fish. Moreover, for the 6 h trial, the cumulative methionine source bioavailability in *Residual* compartment was higher in DLM than in MHA fed fish. Although the liver is the major site of conversion of D-Met and of D- and L-MHA to L-methionine, this process also occurs in the kidney$^{(8,39,44)}$. This may explain the high amount of tracer found in the *Residual Free* fraction, as this compartment includes the kidney among other tissues. Other studies have reported higher amounts of $^{14}\text{C}}$-DL-Met than of $^{14}\text{C}}$-MHA recovered in the kidney of broilers$^{(39-40)}$. In a study where chicken kidney homogenates were incubated with $^{14}\text{C}}$-labelled L-Met, DL-Met or MHA, DL-Met was found to be the substrate most readily converted into the intermediate KMB, revealing that the majority of the KMB produced in the kidney would result from the oxidation of the D- and not the L-isomer of DL-Met$^{(44)}$. Further studies are necessary to confirm if in fish the kidney plays an important role in the conversion of D- to L-Met.

After being converted, the methionine originating from the different dietary methionine sources is transported to the rest of the body to be utilised by the fish. As a consequence of the faster absorption and hepatic metabolism, 2 h after feeding a higher amount of tracer was observed in the *Muscle Free* fraction of DLM fish, implying that dietary methionine is available earlier for utilisation when fish are fed this source. Also, cumulative methionine bioavailability in *Muscle Free* fraction was higher in DLM than in MHA fed fish. Therefore, one can consider that methionine is readily available for protein synthesis faster and for a longer period in DLM fed fish than in fish fed the MHA diet. In fact, 6 h after feeding, the amount of tracer found in the *Protein* fractions of all compartments of DLM fed fish was higher than in MHA fed fish, with significant differences detected in *Viscera* and *Muscle* compartments. This indicates that DLM fish exhibited a faster and more effective incorporation of methionine into muscle protein. Although the amount of tracer present in the *Liver Protein* fraction of DLM fish was higher than in fish fed the MHA diet, the difference was not significant due to a high variability within treatments. The vital physiological
functions of the liver in detoxification, protein synthesis and digestion, constantly producing metabolites, may account for this variability.

In both dietary treatments the amount of tracer retained in the lipid fraction, the major component of the Others fraction, was very low. Metabolic studies in gilthead seabream\(^{26,45}\) and Senegalese sole\(^{46}\) using a \(^{14}\)C-amino acid mixture have previously demonstrated that absorbed amino acids were preferentially used for protein synthesis and only a small proportion were converted into lipids.

At the end of the growth trial, the content of hepatic free amino acids and one-carbon metabolites was determined in fish fed control and supplemented diets. The content of total hepatic free amino acids in fish fed the DLM diet was significantly higher than in fish fed the REF or the MHA diets, due to an increase in both indispensable and dispensable amino acids. Since these are not postprandial results, it indicates a higher bioavailability of amino acids for metabolic purposes in Nile tilapia fed diets supplemented with DL-Met. Methionine supplementation caused an increment of free methionine in liver, independently of the source. Methionine dietary supplementation studies in Nile tilapia\(^{47}\) and Atlantic salmon (Salmo salar)\(^{48-49}\) were unable to observe a similar effect of dietary supplementation in hepatic methionine levels, possibly due to the fact that these studies report postprandial results unlike the current study where fish were sampled 24 h after feeding, hence the result of basal metabolism. The increase in free methionine levels in liver indicates a higher availability for protein synthesis, transmethylation reactions and antioxidant defence in fish fed supplemented diets (DLM and MHA diets) compared with fish fed the non-supplemented diet (REF diet). In the current study, DLM and MHA fed fish presented similar levels of hepatic methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), while homocysteine was significantly lower in fish fed the DLM diet in comparison to the other dietary treatments. This suggests that the different methionine sources follow different metabolic pathways.

In neonatal pigs, to meet methionine requirements, protein synthesis is favoured over methionine transmethylation and the methionine pool is conserved by increasing homocysteine production and suppressing transsulfuration\(^{50}\). In the present study, the hepatic free amino acid analysis indicate that fish fed MHA and REF diets probably remethylate homocysteine back to methionine, with the addition of a methyl group from trimethylglycine. On the contrary, DLM fed fish seem to divert methionine to the transsulfuration pathway, resulting in a significantly higher hepatic taurine content in DLM compared to MHA fed fish. Similarly, feeding Atlantic salmon with soy-based diets supplemented with DL-Met also resulted in an increase in the transsulfuration pathway and consequently higher hepatic taurine content\(^{48}\). These results indicate a stimulation of the transsulfuration pathway in fish fed the DLM diet.
DLM and MHA fed fish had a similar intake of dietary methionine, cysteine and taurine. The experimental diets were soy-based, hence low in taurine. In fact, taurine content in all diets was below 0.1 g/kg. Taurine is an end-product of methionine metabolism and of the transsulfuration pathway. Therefore, although methionine and cysteine hepatic levels were similar in fish fed DLM and MHA diets, a higher hepatic taurine content was found in the former, probably due to a higher availability of methionine, as indicated by the metabolic trials. Higher hepatic taurine content is beneficial for the fish as taurine is involved in numerous physiological functions. In fish, taurine plays important roles in bile salt formation\(^\text{(51-52)}\), lipid digestion\(^\text{(27)}\), osmoregulation\(^\text{(53)}\) and antioxidant defence\(^\text{(54-56)}\) and it also increases amino acid retention\(^\text{(27,57)}\). In Nile tilapia, it has been demonstrated that dietary taurine supplementation improves growth performance\(^\text{(58)}\). In the current work, taurine status in DLM fed fish might have partially contributed to the improvement in growth performance observed in the growth trial. Diet supplementation with DL-Met increased fish body weight when compared to REF and MHA fed fish. Relative to the REF diet, DLM diet produced more 214 g in biomass gain, whereas MHA diet produced only more 124 g (on tank basis). This indicates that on equimolar basis MHA is only 58 % as efficient as DL-Met in terms of biomass gain. DL-Met supplementation improved feed conversion and protein efficiency ratios when compared to the basal diet (REF), while MHA fed fish presented intermediate results. These results are in agreement with previous studies in fish\(^\text{(13,16-17,59)}\), demonstrating that DL-Met supplementation improves growth and promotes protein accretion more efficiently in Nile tilapia.

In the metabolic trial, it was established that the distinct methionine sources are utilised differently by Nile tilapia juveniles in the short-term. Residual and Muscle Free fractions shown higher availability of methionine in DLM than in MHA fed fish and 6 h after feeding a greater amount of tracer in the Muscle Protein fraction was found in the former. These differences were also reflected in the long-term. At the end of the experimental period, protein retention in DLM fed fish was higher than in fish fed the MHA diet. Protein and total methionine whole-body content were also higher in fish fed the DLM diet than in fish from the MHA group. The differences found between DLM and MHA fed fish in total methionine content and availability explain the differences in protein content, reinforcing that Nile tilapia ultimately utilise DL-Met more efficiently for protein deposition than MHA.

The augmented protein retention was ultimately reflected in the nitrogen balance. All diets were isonitrogenous and feed intake was similar in all treatments, resulting in similar nitrogen intake amongst treatments. However, DLM fed fish presented the highest nitrogen gain and the lowest nitrogen losses, indicating that these fish were more efficient in retaining nitrogen than MHA fed fish. Similar results have been reported in studies performed with rainbow trout juveniles\(^\text{(16)}\), where the relative bioavailability of MHA was compared to DL-Met by dose-response trials regarding...
growth performance and nutrient retention. Lower nitrogen losses are related to higher protein digestibility and/or lower catabolism, resulting in lower nitrogen release to the environment. The in vivo metabolic trial revealed that the amount of tracer present in the Incubation Water increased with time and was lower in fish fed the DLM diet than in MHA fed fish. Consequently, in the long-term trial this is reflected in the nitrogen balance, indicating that dietary DL-Met supplementation contributes to a reduction in the environmental impact of Nile tilapia farming.

In conclusion, dietary methionine sources influence methionine absorption and utilisation in Nile tilapia juveniles. The in vivo study indicated that DL-Met is more retained than MHA probably due to a faster absorption rate as well as a greater availability of free methionine in the tissues to be utilised by Nile tilapia. Additionally, methionine from the different sources appear to follow distinct metabolic pathways; while methionine from the DL-Met seems to be transsulfurated, methionine from MHA and REF diets is probably remethylated to methionine to maintain the free methionine pool. In the long-term, dietary DL-Met supplementation of soy-based diets improved growth performance and nitrogen retention in Nile tilapia, reducing the environmental impact and contributing towards a more sustainable industry.

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Conflict of Interest
KM is a full-time employee of Evonik Operations GmbH.

Authorship
C.A., S.E. and K.M. conceived the experiment. K.M. formulated the experimental diets. M.C. and R.C. conducted the experimental trials and laboratory analysis. R.T. conducted laboratory and data analysis, and wrote the manuscript under the supervision of S.E. and C.A. All authors contributed to and approved the manuscript.
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References

1. FAO (2020) The State of World Fisheries and Aquaculture 2020. Sustainability in Action. Rome.

2. Carter CG & Hauler RC (2000) Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, Salmo salar L. Aquaculture 185, 299-311.

3. Glencross BD, Carter CG, Duijster N et al. (2004) A comparison of the digestibility of a range of lupin and soybean protein products when fed to either Atlantic salmon (Salmo salar) or rainbow trout (Oncorhynchus mykiss). Aquaculture 237, 333-346.

4. Nguyen TN, Davis DA & Saoud IP (2009) Evaluation of alternative protein sources to replace fish meal in practical diets for juvenile tilapia, Oreochromis spp. J World Aquac Soc 40, 113-121.

5. Gatlin DM, Barrows FT, Brown P et al. (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac Res 38, 551-579.

6. Selhub J (1999) Homocysteine metabolism. Annu Rev Nutr 19, 217-246.

7. Stipanuk MH (2004) Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. Annu Rev Nutr 24, 539-577.

8. Dibner J (2003) Review of the metabolism of 2-hydroxy-4-(methylthio) butanoic acid. World Poult Sci J 59, 99-110.

9. To VPTH, Masagounder K & Loewen ME (2019) SLC transporters ASCT2, B^0AT1-like, y^+LAT1, and LAT4-like associate with methionine electrogenic and radio-isotope flux kinetics in rainbow trout intestine. Physiol Rep 7, e14274.

10. To VPTH, Subramaniam M, Masagounder K et al. (2020) Characterization of the segmental transport mechanisms of DL-methionine hydroxy analogue along the intestinal tract of rainbow trout with an additional comparison to DL-methionine. Comp Biochem Physiol A Mol Integr Physiol 249, 110776.

11. Maenz DD & Engele-Schaan CM (1996) Methionine and 2-hydroxy-4-methylthiobutanoic acid are transported by distinct Na^+-dependent and H^+-dependent systems in the brush border membrane of the chick intestinal epithelium. J Nutr 126, 529-536.

12. Zhang S, Wong EA & Gilbert ER (2015) Bioavailability of different dietary supplemental methionine sources in animals. Front Biosci 7, 478-490.

13. Keembiyehetty CN & Gatlin DM (1997) Performance of sunshine bass fed soybean-meal-based diets supplemented with different methionine compounds. Progr Fish-Cult 59, 25-30.

14. Kelly M, Grisdale-Helland B, Helland SJ et al. (2006) Refined understanding of sulphur amino acid nutrition in hybrid striped bass, Morone chrysops♀×M. saxatilis♂. Aquac Res 37, 1546-1555.
15. Sauer N, Emrich K, Piepho HP et al. (2008) Meta-analysis of the relative efficiency of methionine-hydroxy-analogue-free-acid compared with DL-methionine in broilers using nonlinear mixed models. Poul Sci 87, 2023-2031.

16. Powell CD, Chowdhury MAK & Bureau DP (2017) Assessing the bioavailability of L-methionine and a methionine hydroxy analogue (MHA-Ca) compared to DL-methionine in rainbow trout (Oncorhynchus mykiss). Aquac Res 48, 332-346.

17. Zhou Y, He J, Su N et al. (2021) Effects of DL-methionine and a methionine hydroxy analogue (MHA-Ca) on growth, amino acid profiles and the expression of genes related to taurine and protein synthesis in common carp (Cyprinus carpio). Aquaculture 532, 735962.

18. Goff JB & Gatlin DM (2004) Evaluation of different sulfur amino acid compounds in the diet of red drum, Sciaenops ocellatus, and sparing value of cystine for methionine. Aquaculture 241, 465-477.

19. Forster IP & Dominy WG (2006) Efficacy of three methionine sources in diets for Pacific white shrimp, Litopenaeus vannamei. J World Aquac Soc 37, 474-480.

20. Pan FY, Feng L, Jiang WD et al. (2016) Methionine hydroxy analogue enhanced fish immunity via modulation of NF-kappaB, TOR, MLCK, MAPKs and Nrf2 signaling in young grass carp (Ctenopharyngodon idella). Fish Shellfish Immunol 56, 208-228.

21. Zhou F, Wang YQ, Bei YJ et al. (2018) Assessing the efficacy of three methionine sources in low protein and low fish meal diet for Chinese soft-shelled turtle, Pelodiscus sinensis. Aquacult Int 26, 15-26.

22. Zhao JX, Li XQ, Leng XJ et al. (2017) Comparative study on the utilization of different methionine sources by channel catfish, Ictalurus punctatus (Rafinesque, 1818). Aquac Res 48, 3618-3630.

23. NRC (2011) National Research Council, Nutrient Requirements of Fish and Shrimp, Committee on the Nutrient Requirements of Fish and Shrimp. Washington, DC: National Academies Press.

24. Konnert GDP & Masagounder K (2017) Apparent digestibility of protein, amino acids and energy in tilapia. In Digestibility Report Aqua, 1-12: Evonik Nutrition and Care GmnH.

25. Konnert GDP, Schrama JW & Masagounder K (2017) Formulating tilapia diets on digestible basis: A review of published apparent digestibility coefficients. In World Aquaculture 2017, Cape Town, South Africa.

26. Rocha F, Dias J, Geurden I et al. (2016) Dietary glucose stimulus at larval stage modifies the carbohydrate metabolic pathway in gilthead seabream (Sparus aurata) juveniles: An in vivo approach using 14C-starch. Comp Biochem Physiol A Mol Integr Physiol 201, 189–99.
27. Richard N, Colen R & Aragão C (2017) Supplementing taurine to plant-based diets improves lipid digestive capacity and amino acid retention of Senegalese sole (Solea senegalensis) juveniles. *Aquaculture* **468**, 94–101.

28. Teodósio R, Engrola S, Colen R *et al.* (2020) Optimizing diets to decrease environmental impact of Nile tilapia (*Oreochromis niloticus*) production. *Aquacult Nutr* **26**, 422–431.

29. Costas B (2011) Stress mitigation in sole (Solea senegalensis) through improved nitrogen nutrition: amino acid utilization, disease resistance and immune status. PhD Thesis, Universidade do Porto.

30. Rust MB, Hardy RW & Stickney RR (1993) A new method for force-feeding larval fish. *Aquaculture* **116**, 341–352.

31. Rønnestad I, Rojas-García CR, Tonheim SK *et al.* (2001) *In vivo* studies of digestion and nutrient assimilation in marine fish larvae. *Aquaculture* **201**, 161–175.

32. Conceição LEC, Morais S & Rønnestad I (2007) Tracers in fish larvae nutrition: A review of methods and applications. *Aquaculture* **267**, 62–75.

33. Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.

34. Panchout F, Letendre J, Bultelle F *et al.* (2013) Comparison of protein-extraction methods for gills of the shore crab, *Carcinus maenas* (L.), and application to 2DE. *J Biomol Tech* **24**, 218–223.

35. Pruessner JC, Kirschbaum C, Meinschmid G *et al.* (2003) Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology* **28**, 916–931.

36. AOAC (2006) *Official methods of analysis*. Gaithersburg, MD: Association of Official Analytical Chemists.

37. Ennos R (2012) *Statistical and data handling skills in biology*. Harlow, UK: Pearson Education.

38. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (2012) Scientific opinion on DL-methionine, DL-methionine sodium salt, the hydroxy analogue of methionine and the calcium salt of methionine hydroxy analogue in all animal species; on the isopropyl ester of methionine hydroxy analogue and DL-methionine technically pure protected with copolymer vinylpyridine/styrene in dairy cows; and on DL-methionine technically pure protected with ethylcellulose in ruminants. *EFSA Journal* **10**, 2623.

39. Saunderson L (1985) Comparative metabolism of L-methionine, DL-methionine and DL-2-hydroxy 4-methylthiobutanoic acid by broiler chicks. *Br J Nutr* **54**, 621–633.
40. Lingens G & Molnar S (1996) Studies on metabolism of broilers by using $^{14}$C-labelled DL-methionine and DL-methionine hydroxy analogue Ca-salt. *Arch Anim Nutr* **49**, 113–124.

41. Maenz DD & Engele-Schaan CM (1996) Methionine and 2-hydroxy-4-methylthiobutanoic acid are partially converted to nonabsorbed compounds during passage through the small intestine and heat exposure does not affect small intestinal absorption of methionine sources in broiler chicks. *J Nutr* **126**, 1438–1444.

42. Drew MD, Van Kessel AG & Maenz DD (2003) Absorption of methionine and 2-hydroxy-4-methylthiobutanoic acid in conventional and germ-free chickens. *Poult Sci* **82**, 1149–1153.

43. Dibner JJ & Knight CD (1984) Conversion of 2-Hydroxy-4-(Methylthio) butanoic to L-Methionine in the Chick : A Stereospecific Pathway. *J Nutr* **114**, 1716–1723.

44. Dupuis L, Saunderson CL, Puigserver A *et al.* (1989) Oxidation of methionine and 2-hydroxy 4-methylthiobutanoic acid stereoisomers in chicken tissues. *Br J Nutr* **62**, 63–75.

45. Rocha F, Dias J, Geurden I *et al.* (2016) High-glucose feeding of gilthead seabream (*Sparus aurata*) larvae: Effects on molecular and metabolic pathways. *Aquaculture* **451**, 241–253.

46. Navarro-Guillén C, Yúfera M & Engrola S (2017) Ghrelin in Senegalese sole (*Solea senegalensis*) post-larvae: Paracrine effects on food intake. *Comp Biochem Physiol A Mol Integr Physiol* **204**, 85–92.

47. Michelato M, Furuya WM & Gatlin DM (2018) Metabolic responses of Nile tilapia (*Oreochromis niloticus*) to methionine and taurine supplementation. *Aquaculture* **485**, 66–72.

48. Espe M, Hevrøy EM, Liaset B *et al.* (2008) Methionine intake affect hepatic sulphur metabolism in Atlantic salmon, *Salmo salar*. *Aquaculture* **274**, 132–141.

49. Espe M, Andersen SM, Holen E *et al.* (2014) Methionine deficiency does not increase polyamine turnover through depletion of hepatic S-adenosylmethionine in juvenile Atlantic salmon. *Br J Nutr* **112**, 1274–1285.

50. Bauchart-Thevret C, Stoll B, Chacko S *et al.* Sulfur amino acid deficiency upregulates intestinal methionine cycle activity and suppresses epithelial growth in neonatal pigs. *Am J Physiol Endocrinol Metab* **296**, 1239–1250.

51. Kim SK, Matsunari H, Takeuchi T *et al.* (2007) Effect of different dietary taurine levels on the conjugated bile acid composition and growth performance of juvenile and fingerling Japanese flounder *Paralichthys olivaceus*. *Aquaculture* **273**, 595–601.

52. Kim SK, Matsunari H, Nomura K *et al.* (2008) Effect of dietary taurine and lipid contents on conjugated bile acid composition and growth performance of juvenile Japanese flounder *Paralichthys olivaceus*. *Fisheries Sci* **74**, 875–881.
53. Takagi S, Murata H, Goto T et al. (2006) Hemolytic suppression roles of taurine in yellowtail *Seriola quinqueradiata* fed non-fishmeal diet based on soybean protein. *Fisheries Sci* **72**, 546–555.

54. Li M, Lai H, Li Q et al. (2016) Effects of dietary taurine on growth, immunity and hyperammonemia in juvenile yellow catfish *Peleobagrus fulvidraco* fed all-plant protein diets. *Aquaculture* **450**, 349–355.

55. Coutinho F, Simões R, Oliva-Teles A et al. (2017) Effects of dietary methionine and taurine supplementation to low-fish meal diets on growth performance and oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* **479**, 447–454.

56. Martins N, Magalhães R, Castro C et al. (2019) Taurine modulates hepatic oxidative status and gut inflammatory markers of European seabass (*Dicentrarchus labrax*) fed plant feedstuffs - based diets. *Amino Acids* **51**, 1307–1321.

57. Pinto W, Figueira L, Ribeiro L et al. (2010) Dietary taurine supplementation enhances metamorphosis and growth potential of *Solea senegalensis* larvae. *Aquaculture* **309**, 159–164.

58. Al-Feky SSA, El-Sayed AFM & Ezzat AA (2016) Dietary taurine enhances growth and feed utilization in larval Nile tilapia (*Oreochromis niloticus*) fed soybean meal-based diets. *Aquacult Nutr* **22**, 457–464.

59. Keembiyehetty CN & Gatlin DM (1995) Evaluation of different sulfur compounds in the diet of juvenile sunshine bass (*Morone chrysops ♀ x M. saxatilis ♂*). *Comp Biochem Physiol A Mol Integr Physiol* **112**, 155–159.
Figure 1 Daily nitrogen (N) balance in Nile tilapia juveniles fed the experimental diets for 57 days. Values are presented as means ± standard deviation (n = 3). Different letters within the same compartment indicate significant differences (p < 0.05) among diets.
Figure 2 Free methionine (A), homocysteine (B), cystathionine (C) and taurine (D) content in liver of Nile tilapia juveniles fed the experimental diets for 57 days. Values are presented as means ± standard deviation (n = 3). Different letters indicate significant differences (p < 0.05) among diets.
Figure 3 Sum of free indispensable (Sum IAA) and dispensable (Sum DAA) amino acids in liver of Nile tilapia juveniles fed the experimental diets for 57 days. Values are presented as means ± standard deviation (n = 3). Different letters within the same compartment indicate significant differences (p < 0.05) among diets.
Figure 4 Radioactivity (DPM/g of fish) in the Incubation Water compartment at 1, 2, 3, 4 and 6 hours after tube-feeding experimental diets labelled with $^{14}$C-DL-Met or $^{14}$C-MHA. Values are presented as means ± standard deviation ($n = 6$-$7$ fish for each diet and incubation period). Different letters at the same time-point indicate significant differences ($p < 0.05$) between diets.
Figure 5  Radioactivity (DPM/g of tissue) in the Viscera (A), Liver (B), Residual (C) and Muscle (D) Free fractions at 1, 2, 3, 4 and 6 hours after tube-feeding experimental diets labelled with $^{14}$C-DL-Met or $^{14}$C-MHA. Values are presented as means ± standard deviation ($n = 6$-$7$ fish for each diet and incubation period). Different letters at the same time-point indicate significant differences ($p < 0.05$) between diets.
Figure 6 Radioactivity (DPM) in the Viscera, Liver, Residual and Muscle Protein fractions at 6 hours after tube-feeding experimental diets labelled with $^{14}$C-DL-Met or $^{14}$C-MHA. Values are presented as means ($n = 6$–7 fish for each diet). Asterisks denote significant differences ($p < 0.05$) between diets within the same compartment.
TABLE 1 Formulation and proximate composition of the experimental diets (g/kg diet).

| Ingredients                                | REF | DLM | MHA |
|--------------------------------------------|-----|-----|-----|
| Soybean meal                               | 350.0 | 349.5 | 349.4 |
| Soy protein concentrate                     | 92.5 | 92.4 | 92.3 |
| Corn meal                                  | 299.2 | 298.8 | 298.7 |
| Pea protein concentrate                     | 77.8 | 77.7 | 77.7 |
| Wheat bran                                 | 66.3 | 66.2 | 66.2 |
| Soybean oil                                | 48.0 | 47.9 | 47.9 |
| Fish oil                                   | 20.0 | 20.0 | 20.0 |
| Di-calcium phosphate                        | 30.0 | 30.0 | 29.9 |
| Vit-Min premix                              | 10.0 | 10.0 | 10.0 |
| L-Lysine sulfate                            | 3.1  | 3.1  | 3.1  |
| L-Threonine                                 | 1.9  | 1.9  | 1.9  |
| L-Tryptophan                                | 0.6  | 0.6  | 0.6  |
| L-Histidine                                 | 0.6  | 0.6  | 0.6  |
| DL-Methionine                               | 0.0  | 1.5  | 0.0  |
| Calcium bis-methionine hydroxyl analogue    | 0.0  | 0.0  | 1.8  |

Analysed proximate composition (as fed basis)

|                      | REF | DLM | MHA |
|----------------------|-----|-----|-----|
| Dry matter           | 941.6 | 919.7 | 950.5 |
| Ash                  | 58.3 | 61.2 | 62.8 |
| Crude protein        | 326.4 | 323.1 | 323.9 |
| Crude fat            | 88.2 | 89.3 | 95.3 |
| Total phosphorus     | 7.3  | 7.9  | 8.1  |
| Gross energy (MJ/kg) | 19.0 | 18.6 | 19.2 |

\[a\] Solvent extracted dehulled soybean meal: 457 g/kg crude protein (CP), 31 g/kg crude fat (CF), CARGILL, Spain.

\[b\] Soycomil P: 620 g/kg CP, 7 g/kg CF, ADM, The Netherlands.

\[c\] Corn meal: 81 g/kg CP, 37 g/kg CF, Casa Lanchinha, Portugal.

\[d\] Lysamine GPS: 840 g/kg CP, 10 g/kg CF, ROQUETTE Frères, France.

\[e\] Wheat bran: 149 g/kg CP, 40 g/kg CF, Cerealis Moagens S.A., Portugal.

\[f\] Henry Lamotte Oils GmbH, Germany.
Sopropèche, France.

DCP: 168 g/kg phosphorus, 209 g/kg calcium, Premix Lda, Italy.

PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg kg/diet): cobalt carbonate, 0.65 mg; 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; calcium carbonate, 1.86 g; excipient wheat middlings.
TABLE 2 Amino acid and calcium bis-methionine hydroxyl analogue (MHA-Ca) content of experimental diets (g/kg diet).

| Analysed values | Dietary treatments |
|-----------------|--------------------|
|                 | REF    | DLM    | MHA    |
| Lysine          | 17.8   | 19.7   | 19.1   |
| Methionine      | 4.4    | 5.6    | 4.4    |
| Cysteine        | 4.4    | 4.3    | 4.4    |
| Threonine       | 13.5   | 13.6   | 13.8   |
| Arginine        | 23.0   | 23.3   | 23.9   |
| Isoleucine      | 13.9   | 14.0   | 14.3   |
| Leucine         | 25.2   | 25.2   | 25.7   |
| Valine          | 15.2   | 15.2   | 15.7   |
| Histidine       | 8.3    | 8.5    | 8.7    |
| Phenylalanine   | 15.9   | 16.0   | 16.3   |
| Glycine         | 13.3   | 13.3   | 13.7   |
| Serine          | 16.0   | 15.7   | 16.2   |
| Proline         | 17.1   | 16.7   | 17.1   |
| Alanine         | 14.4   | 14.3   | 14.7   |
| Aspartate       | 34.2   | 34.4   | 35.4   |
| Glutamate       | 56.7   | 55.9   | 57.3   |
| MHA-Ca*         | 0.0    | 0.0    | 1.3    |
| Taurine         | < 0.1  | < 0.1  | < 0.1  |

* Active content of MHA in the MHA diet is 1.1 g/kg (equal on molar basis to DL-Met) since MHA-Ca active form is 84%.
TABLE 3 Growth performance, somatic indexes and feed utilisation of Nile tilapia juveniles fed the experimental diets for 57 days.

|                          | REF     | SD   | DLM    | SD   | MHA    | SD   |
|--------------------------|---------|------|--------|------|--------|------|
| Final body weight (g)    | 24.1    | 3.0  | 28.3   | 4.7  | 24.8   | 2.7  |
| Biomass gain (g/tank)    | 928     | 85   | 1142   | 55   | 1052   | 118  |
| Daily voluntary feed intake (% average biomass/day) | 2.8     | 0.1  | 2.7    | 0.1  | 2.7    | 0.1  |
| Feed conversion ratio (FCR) | 1.0    | 0.0  | 0.9    | 0.0  | 0.9    | 0.0  |
| Protein efficiency ratio (PER) | 3.1    | 0.1  | 3.4    | 0.1  | 3.3    | 0.1  |
| Hepatosomatic index (HSI, %) | 1.53   | 0.47 | 1.34   | 0.33 | 1.30   | 0.20 |
| Viscerosomatic index (VSI, %) | 9.47   | 0.64 | 9.07   | 1.02 | 8.74   | 1.37 |

Initial body weight = 2.3 ± 0.4 g for all dietary treatments (n = 150).

Values are presented as means ± standard deviation (n = 15 for final body weight, HSI and VSI; n = 3 for the remaining parameters). Different superscripts within the same row indicate significant differences (p < 0.05) among diets. Absence of superscripts indicates no significant differences.
### TABLE 4 Whole-body composition and protein and energy retention of Nile tilapia juveniles fed the experimental diets for 57 days.

| Body composition (g/kg wet weight) | Dietary treatments |          |          |          |
|-----------------------------------|--------------------|----------|----------|----------|
|                                   | REF                | DLM      | MHA      |          |
|                                   | Mean   | SD     | Mean   | SD     | Mean   | SD     |
| Moisture                          | 717.2  | 2.8    | 709.8  | 3.1    | 725.8  | 12.6   |
| Ash                               | 36.3   | 2.3    | 38.3   | 0.5    | 35.8   | 0.5    |
| Protein                           | 147.8$a^{ab}$ | 3.3    | 155.9$a$ | 0.7    | 145.1$b$ | 5.4    |
| Fat                               | 82.2   | 3.3    | 79.4   | 1.9    | 74.2   | 4.1    |
| Phosphorus                        | 4.7    | 0.2    | 5.1    | 0.1    | 4.9    | 0.2    |
| Energy (MJ/kg)                    | 6.6$a^{ab}$ | 0.1    | 6.8$a$ | 0.1    | 6.3$b$ | 0.3    |

**Retention (% intake)**

|                                   |          |          |          |
|                                   | REF    | DLM    | MHA    |
| Protein                           | 44.7$b$ | 2.2     | 52.2$a$ | 1.7    | 47.8$b$ | 0.8    |
| Energy                            | 35.7$b$ | 1.2     | 40.6$a$ | 1.4    | 36.1$b$ | 0.6    |

Initial body composition: moisture = 742.8 g/kg WW; ash = 40.0 g/kg WW; protein = 164.6 g/kg WW; fat = 47.7 g/kg WW; phosphorus = 5.9 g/kg WW; energy = 5.5 MJ/kg WW.

Values are presented as means ± standard deviation (n = 3). Different superscripts within the same row indicate significant differences (p < 0.05) among diets. Absence of superscripts indicates no significant differences.
TABLE 5 Linear regression analysis and area under the curve (1-6 h) of the free fractions analysed in the time-course metabolic trial.

| Compartments     | DLM  | MHA  |
|------------------|------|------|
| Incubation Water | <0.001 | <0.001 |
| Viscera          | 0.015 | 0.489 |
| Liver            | 0.010 | 0.222 |
| Residual         | 0.219 | 0.002 |
| Muscle           | 0.821 | 0.005 |

Area under the curve* (% total cumulated radioactivity DPM)

| Compartments     | DLM  | MHA  |
|------------------|------|------|
| Incubation Water | 2.0  | 4.7  |
| Viscera          | 54.5 | 60.0 |
| Liver            | 29.7 | 26.1 |
| Residual         | 8.3  | 6.3  |
| Muscle           | 5.5  | 2.9  |

* Please refer to Metabolic utilisation of supplemental methionine sources section for further details.