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TAURINE AND METHIONINE SUPPLEMENTATION IN MEAGRE (*Argyrosomus regius*) FED HIGH PLANT PROTEIN DIETS

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Estado do Paraná
fevereiro – 2018

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"Tese apresentada, como parte das exigências para obtenção do título de DOUTOR EM ZOOTECNIA, no Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá Área de concentração Produção Animal''

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‘’Seja a mudança que desejas ver no mundo’’

Mohandas Karamchand Gandhi

À minha mãe,
Solange Tarosso, meu maior exemplo de vida,
incentivadora e parceira incondicional.

A todos que, de alguma forma, se beneficiarão com o trabalho, desenvolvendo com o auxílio deste, novos resultados científicos.

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BIOGRAFIA

LORENA BATISTA DE MOURA, filha de Luiz Felipe Barreiros Batista de Moura e Solange Tarosso, nasceu em Maringá, Estado do Paraná, no dia 13 de março de 1984.

Em janeiro de 2008, se formou em ciências biológicas, pela Universidade Estadual de Maringá e em março do mesmo ano, iniciou no curso de graduação em Zootecnia, também pela Universidade Estadual de Maringá.

Em dezembro de 2010, deu início a sua jornada na pesquisa com projeto de iniciação científica, na área de aquicultura, dando continuidade a esta em 2011 como bolsista do CNPq.

Em 2012, obteve o título de Zootecnista, iniciando em seguida o curso de Pós-graduação em Zootecnia, em nível de mestrado, na área de produção animal, pela Universidade Estadual de Maringá, realizando estudos na área de nutrição de peixes.

Após obter o título de Mestre em Zootecnia, pela Universidade Estadual de Maringá, em março de 2014, ingressou no Programa de Pós-graduação em Zootecnia, em nível de doutorado, também pela Universidade Estadual de Maringá, na área de produção animal, desenvolvendo estudos na área de nutrição de peixes.

Em setembro de 2015 deu início ao doutorado sanduiche na Universidade do Porto, Portugal pelo período de um ano, quando realizou a maior parte da sua tese de doutorado coorientada pelos pesquisadores Dr. Aires Manuel Pereira de Oliva-Teles e Dr^{a} Helena Peres.

Em julho de 2017 obteve a qualificação e, em fevereiro de 2018, submeteu-se à banca examinadora para defesa da tese de doutorado e obtenção do título de doutor em produção animal pelo Programa de Pós-graduação em Zootecnia da Universidade Estadual de Maringá.

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ABSTRACT

The effect of methionine and taurine supplementation to high plant protein diet for meagre (*Argyrosomus regius*) on growth performance, whole body and liver composition, hepatic intermediary metabolism, antioxidative defence, nutrient digestibility, plasma metabolites, total bile acid content and digestive enzyme activity were assessed. For that purpose, two assays were performed in sequence. First, a 65-day feeding trial was conducted using a 180 juvenile meagres (50.0 ± 0.60g). The fish were randomly distributed in 12 (300-L) tanks. Four isoenergetic (20.0 MJ kg^{-1}) and isoproteic (420 g kg^{-1}) practical diets containing 180 g kg^{-1} of crude lipids, were formulated to contain 82% of crude protein from plant ingredients. A 2 x 2 factorial arrangement design with methionine at 0.0 and 2.6 g kg^{-1} dry diet and taurine at 0.0 and 10.0 g kg^{-1} of dry diet was used. After the feed trial sampling, the remaining fish were used for the *in vivo* digestibility trial that last 24 days. Seventy-two juvenile meagre (103 ± 21.2g), were randomly distributed into 12 (60 L) tanks designed according to Guelph system. Chromium oxide was added to the diets used in the feeding trial as external marker. Taurine supplementation improved all growth parameters evaluated ($P<0.05$) as opposed to methionine. Activity of key enzyme of amino acid catabolism and gluconeogenesis was also significantly increased by taurine supplementation ($P<0.05$). Methionine supplementation increased dry matter, protein, lipids and energy digestibility while taurine increased only lipid digestibility. Methionine supplementation did not affect plasma parameters, while taurine showed an individual effect on the increased cholesterol ($P<0.001$) and total protein content ($P<0.05$) increasing in twofold the plasma triglycerides concentration ($P<0.001$). Total bile acid content from the anterior intestine digesta was increased in twofold by taurine supplementation which also increased plasma total bile acid content. Taurine had an

individual increased lipase activity effect ($P<0.01$) while methionine when not supplemented with taurine decreased its activity ($P<0.05$). Interaction effect was also observed on the antioxidant apparatus defense. Intestinal glucose-6-phosphate dehydrogenase activity was higher when supplemented with taurine at 2.6 g kg^{-1} of methionine ($P<0.05$) and, the hepatic lipid peroxidation decreased when taurine and methionine were supplemented together ($P<0.05$). A taurine individual effect also increased hepatic glucose-6-phosphate dehydrogenase and glutathione peroxidase activity while decreased catalase activity. These results imply that the supplementation of taurine and methionine to high plant protein diets could be a good nutritional strategy to mitigate some of the limitations of using high levels of plant feed ingredients in diets for meagre (*Argyrosomus regius*), a strict carnivorous fish. By increasing nutrient digestibility and improving antioxidative defenses both amino acid showed benefitial interaction. The individual supplementation of taurine also presented to be a good nutritional strategy once significant improved growth parameters have been able to restablish bile acid production improving meagre antioxidant defenses. Thus, taurine may be classified not only as a conditionally essential amino acid for meagre fed high plant protein diets as also a functional amino acid been a good strategy to the development of sustainable aquafeeds.

Keywords: carnivorous fish; digestibility; feed efficiency, sulphur amino acids; sustainable aquafeeds

RESUMO

O presente trabalho teve como objetivo avaliar o efeito da suplementação da metionina e da taurina em dietas ricas em proteína de origem vegetal para corvinas (*Argyrosomus regius*) sobre o desempenho produtivo, composição corporal e do figado, metabolismo intermediário do fídago, defesa antioxidante, digestibilidade de nutrientes, metabolitos plasmáticos, conteúdo total de ácido biliar e atividade das enzimas digestivas. Para tanto, dois ensaios foram realizados em sequência. Um ensaio de crescimento com duração de 65 dias foi conduzido utilizando 180 juvenis de corvina (50.0 ± 0,60g). Os peixes foram distribuídos aleatoriamente em 12 tanques (300 litros cada). Quatro dietas isoenergéticas (20.0 MJ kg^{-1}) e isoproteicas (420 g kg^{-1}) foram formuladas para conter 82% de proteína de origem vegetal. A metionina foi suplementada a 0.0 e 2.6 g kg^{-1}da dieta e a taurina a 0.0 e 10.0 g kg^{-1} da dieta em um esquema fatorial 2x2. Após a amostragem do ensaio de crescimento, os peixes remanescentes foram utilizados no ensaio de digestibilidade *in vivo* com duração de 24 dias. Setenta e dois juvenis de corvina (103 ± 21,2g) foram distribuídos aleatoriamente em 12 tanques (60 litros cada) projetados de acordo com o sistema Guelph. O óxido de cromio foi adicionado às dietas utilizadas no ensaio de crescimento como marcador externo. A suplementação de taurina melhorou todos os parâmetros de crescimento avaliados (P <0,05), diferentemente da metionina. A atividade enzimática das enzimas chave do catabolismo de aminoácidos e da gliconeogênese aumentaram em resposta a suplementação de taurina (P<0.05). A suplementação com metionina aumentou a digestibilidade da matéria seca, da proteína, dos lipídios e da energia da dieta. A taurina, por outro lado, aumentou apenas a digestibilidade dos lipídios. A suplementação com metionina não afetou os parâmetros plasmáticos avaliados. No entanto, a suplementação de taurina aumentou o conteúdo de colesterol (P <0,001) e proteína total (P <0,05) dobrando a concentração de triglicerídeos (P <0,001). O teor de

ácido biliar total proveniente da digesta do intestino anterior aumentou com suplementação de taurina que, também aumentou o teor total de ácido biliar no plasma. A suplementação de taurina aumentou a atividade da lipase (P <0,01), enquanto que a metionina quando não suplementada com taurina diminuiu sua atividade (P <0,05). Também foi observada interação entre os aminoácidos suplementados sobre o status oxidativo. A atividade intestinal da glicose-6-fosfato desidrogenase foi maior quando suplementada com taurina a 2,6 g kg^{-1} de metionina (P <0,05) e, a peroxidação lipídica hepática diminuiu quando a taurina e a metionina foram suplementadas juntas (P <0,05). A suplementação apenas com taurina também aumentou a atividade hepática da glicose-6-fosfato desidrogenase e da glutationa peroxidase além de diminuir a atividade da catalase. Estes resultados implicam que a suplementação de taurina e metionina a dietas ricas em proteínas vegetais pode ser boa estratégia nutricional para mitigar algumas das limitações do uso de altos níveis de ingredientes vegetais em dietas para corvina (*Argyrosomus regius*), um peixe estritamente carnívoro. Ao aumentar a digestibilidade dos nutrientes e melhorar o aparato de defesa antioxidante, os aminoácidos apresentaram interação benéfica. A suplementação individual de taurina também se mostrou como boa estratégia nutricional, uma vez que melhorou os parâmetros de crescimento, foi capaz de restaurar a produção de ácido biliar melhorando também o status oxidativo do peixe. Assim, a taurina pode ser classificada não só um aminoácido condicionalmente essencial em dietas ricas em proteínas vegetais, como também um aminoácido funcional provando ser boa estratégia para o desenvolvimento de dietas aquícolas sustentáveis.

Palavras-chave: peixe carnívoro; digestibilidade de nutrientes; aminoácidos sulfurados, dieta sustentável.

INTRODUCTION

Substituting fishmeal (FM) in diets for carnivorous marine fish species has been considered globally to elaborate cost-effectiveness and sustainable diets. However, for carnivorous fish, only selected ingredients containing high protein content, well-balanced amino acid profile and high nutrient digestibility may be used as alternative protein sources due to its high protein requirements (Gatlin et al., 2007; Hardy, 2008). Soybean meal has been considered one of the major alternative ingredient to FM, for its high protein content and good amino acid profile (Storebakken et al., 2000). However, the presence of anti-nutritional factors and sulphur amino acid deficiency can cause reduced feed efficiency, abnormal intestinal morphology and a decrease on growth performance in some fish species fed diets based on soybean meal (van den Ingh et al., 1991; Francis et al., 2001).

The imposition of high plant protein diets to carnivorous fish can result on a shifting of their metabolic profile (Pérez-Jiménez et al., 2009), including pathways involved in protein and carbohydrate utilization (Martin et al., 2003; Vilhelmsson et al., 2004; Dias et al., 2005). However, it is highly improbable that complete replacement of FM will be possible using a single alternative protein source in carnivorous fish aquafeeds (Salze et al., 2010). The use of crystalline amino acids to restore plant protein ingredients amino acid profile has proven to be a good strategy to enhance growth performance of fish without detrimental impacts towards a sustainable aquaculture (Gaylord et al., 2007).

In this context, taurine is an abundant non-proteic amino acid in FM but limited in soybean meal. For fish, the sources of taurine are from *de novo* synthesis or dietary intake and, the major pathway for taurine synthesis is through methionine via cysteine by a series of enzymatic reactions (Griffith, 1983). During this process, L-cysteinesulphinate decarboxylase (CSD) is considered the rate-limiting enzyme for taurine biosynthesis in

the liver (de la Rosa and Stipanuk, 1985; Morris and Rogers, 1992). Despite its importance, little information has been reported about the role of taurine on metabolic pathway of carnivorous fish, specially its effects when supplemented in plant protein-based diets.

Previous studies have shown that taurine regulates several biological processes, including prevention of tissue injury by oxidative stress (Huxtable, 1992; Tas et al., 2007), blood cell volume regulation and protection of cells from osmotic stress (Han et al., 2006). Presents hypoglycemic properties (Kulakowski and Murato, 1984; Fang et al., 2002; Kaplan et al., 2004), being used as a major attractant for fish (Gaylord et al., 2006; Takagi et al., 2008) and as a promote growth supplement in aquaculture (Ferreira et al., 2015). Therefore, even though taurine is classified as a non-essential amino acid, it has been considered as a conditionally indispensable nutrient to certain carnivorous fish species fed plant protein based diets (Brotons-Martinez et al., 2004; Matsunari et al., 2005), since its deficiency can cause inferior growth performance as well as physiological abnormalities such as anemia and green discoloration of the liver, so-called ‘‘green liver’’ (Aoki et al., 2000; Goto et al., 2001; Watanabe et al., 2001).

Taurine also participates on lipid metabolism by modulating the digestion and absorption of dietary fats through its participation on bile acids synthesis, the major catabolic pathway for cholesterol (Kulakowski and Murato, 1984; Fang et al. 2002; Kaplan et al., 2004). Bile acids are constituents of bile and potent digestive surfactants that promote lipid absorption, aid the adsorption of co-lipase and lipase, and help solubilize the lipolysis products which have accumulated at the interface, into mixed micelles composing bile salts and a range of other lipids, to facilitate transport to the gut mucosal surface prior to uptake and absorption (Wilde and Chu, 2011). The taurocholic acid or taurochenodeoxycholic are the major bile acids in the liver of almost all fish species, except cyprinids (Vessey et al., 1990; Goto et al., 1996; Yeh and Hwang, 2001). Yamamoto et al. (2005) reported that plasma taurine concentration decreases a few hours after feeding taurine supplemented diets in rainbow trout, which might be due to the conjugation of bile acid to taurine for lipid digestion.

Many fish are capable of synthesizing taurine adequately to support normal physiological functions from sulfur amino acids if methionine levels meet their requirement (Yokoyama et al., 2001). However, this ability does appear to be insufficient or inexistent for other fish species (Yokoyama et al., 2001; Park et al., 2002; Matsunari et al., 2008). Therefore, the limited capacity to synthesize taurine in fish may impose a

supplementation necessity of this amino acid when its dietary intake is insufficient. Recent work has indicated that taurine can be considered as conditionally indispensable in all-plant protein-based diets to support increased growth (Gaylord et al., 2006; Takagi et al., 2008), and deficiency is associated to cause hepatomegaly and anemia (Goto et al., 2001; Takagi et al., 2008).

Meagre *Argyrosomus regius* (Asso, 1801) is a marine carnivorous migratory fish species which belongs to the Sciaenidae family with a special aquaculture potential in Spain, Egypt, France, Italy, Morocco and Turkey (Duncan et al., 2013). According to FAO (2005-2017) from 2006 to 2011, a great increase on global meagre production was observed. But, even though meagre is presently farmed in several countries in the Mediterranean basin, its production has not yet reached its full potential and rearing trials are still very limited (Velazco-Vargas et al., 2014). Meagre as a well-established culture (Roo et al., 2010; Vallés and Estévez, 2012), and do not present reproductive maturation during ongrowing period (Mananos et al., 2009). It presents many attractive attributes for the market that include large size, good processing yield, excellent taste and firm texture (Monfort, 2010), been well accepted by the consumers due to its low muscle fat content (Poli et al., 2003; Grigorakis et al., 2011).

Therefore, the current experiment was performed to evaluate the effect of the taurine and methionine supplementation on high plant protein diet for juvenile meagre on growth performance, body and liver composition, hepatic intermediary metabolism, antioxidative defense, nutrient digestibility, plasma metabolites, total bile acid content and digestive enzyme activity. As for to evaluate if taurine and methionine supplementation are good nutritional strategies to mitigate some of the limitations of using high levels of plant feed ingredients in diets for meagre, a strict carnivorous fish.

LITERATURE REVIEW

Meagre (*Argyrosomus regius*)

The family Sciaenidae includes 70 genus and 270 species (Nelson, 1994) which are distributed in the Atlantic, Indian and Pacific oceans. Meagre *Argyrosomus regius* (Asso, 1801) is a carnivorous migratory fish species of the Sciaenidae Family (Figure 1). It is distributed all through the coastal of Eastern Atlantic, from Norway to Congo, including the Mediterranean and the Black Sea (Monfort, 2010), with a special aquaculture potential in Spain, Egypt, France, Italy, Morocco and Turkey (Duncan et al., 2013).

Figure 1 - Meagre (*Argyrosomus regius*). Source: ec.europa.eu. Access in 06/2017.

Meagre aquaculture started during the 1990´s in Southern France and Italy, followed by Spain in 2004 and later by Greece, Turkey and Egypt (Monfort, 2010). According to FAO (2005-2017) from 2006 to 2011, an increase on global meagre production was observed. Currently, the main producer is Egypt, followed by Spain and Turkey, being France and Italy the most important suppliers of meagre fingerlings (Monfort, 2010).

However, even though meagre is presently farmed in several countries, its production has not reached its full potential and rearing trials are still very limited.

At present, there are a few main bottlenecks to the expansion of meagre industry. Firstly, the large variable growth rates (Duncan et al., 2013) during husbandry. Secondly, a limited genetic variation of the available broodstocks with negative implications in breeding selection programs development. Thirdly, the industry is not addressing issues in fish health, emerging diseases and parasites (Merella et al., 2009; Ternengo et al., 2010). Furthermore, no commercial diets have been developed for this fish, specially for its different life stages of grown. Currently, meagre is fed diets developed for sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) which basal formulation matches the protein and lipid requirements determined for meagre of 170^{-1} g kg crude lipids and 500 g kg^{-1} of crude protein (Chatzifotis et al., 2010, 2012). The low larval survival and the elevated fry production cost are reproductive problems still not solved. Finally, the need for a more expanded market and diversification of provided products (Monfort, 2010) beyond the whole fresh fish.

In the wild, meagre can grow up to two meters and reach more them 50 kg (FAO, 2005–2017). Meagre is characterized as a spring-summer spawning specie thus, during spring, coincident with periods of freshwater discharge, meagre enters estuarine waters in synchronous groups for spawning, until late summer (Grau et al., 2009). Juveniles leave estuaries at the end of summer and spend two to three years in coastal waters before migrating to offshore feeding areas (Duncan et al., 2013). Water temperature seems to be the most important factor determining the migrations and reproduction of meagre (FAO, 2005–2017). During winter, meagre returns to deeper waters to feed (Quéméner et al., 2002). Meagre growth is mainly achieved during summer once feeding activity is substantially reduced at 13-15 ºC. However, as a euryhaline and thermohaline species, it tolerates wide temperature changes from 2 to 38 °C and salinity variations from 5 to 42 ‰ (González- Quirós et al., 2011). Meagre is gonochoristic with fixed sex after sexual differentiation that is usually completed in 2 years of age by males (900 g) and in 3 years by female with aproximatly 1500 g (Schiavone et al., 2012) being sexually active over a potential life span of at least 20 years (Shabana et al., 2012). Broodstock should be formed from fish over 6 kg (captivity reared fish) or over 8 kg (wild fish) (Duncan et al., 2013). Meagre has induced spawning protocols for the production of viable eggs in development (Duncan et al., 2013) and do not present reproductive maturation during ongrowing period (Mananos et al., 2009). Its production cycle can be divided in three different

periods: pre-ongrowing, juvenile feeding and harvest. Pre-ongrowing consists of growing the fish up to 3–15 g mainly in land-based facilities and it lasts approximately two to four months, depending on temperature and desired juvenile size for transferring. The main problem during this phase is cannibalism which can be overcome by frequent feeding and approximately bi-monthly size grading. Flexi-bacteria and, to a lesser extent, vibrios can be also problematic during pre-ongrowing, particularly attacking fin edges (Duncan et al., 2013).

In terms of nutrient requirement for meagre, little information is known specially on amino acid requirements. Recently, several studies that were focused on the feeding of meagre under aquaculture conditions indicated that basic dietary nutrient requirements of juvenile meagres and sub-adult stages are close to those of other carnivorous marine fish species. For example, Chatzifotis et al. (2012) reported that a dietary crude protein level of 50% resulted in nearly optimal growth performance in juvenile meagre, while Martínez-Llorens et al. (2011) found that meagre fed a commercial diet with 47% crude protein and 20% crude lipid showed the best growth performance. These latter figures are similar to those being currently adopted in commercial diets for sea bream and sea bass which are in a range of 45–55% of crude proteins and 17 – 21% of crude lipids on a dry weight basis (Chatzifotis et al., 2012).

Nowadays, juvenile meagre is mainly farmed in the sea, using circular or square surface cages of 500-1000 m^3. The best temperature for its growth is between 17-26 ºC and at normal stocking density of 50 fish/m^3. The feeding periods occur in less than 24 months, when meagre reach 800 to 1200 g (FAO, 2005-2017). However, they can be frequently fed until they reach 2000 to 3000 g, a harvest size that is more suitable for fillets or slices (Jiménez et al., 2005). On the Southern Mediterranean coast of Spain, meagre can achieve 5 to 1100 g in the first 12 months and to 2500 g in 24 months, with seawater temperatures in the range of 14–26 °C (Quéméner et al., 2002; Duncan et al., 2013).

Meagre has other attractive attributes for the market that include large size, good processing yield, excellent taste and firm texture (Monfort, 2010). Meagre flesh quality is very well accepted by the consumers due to its low muscle fat content (Poli et al., 2003; Grigorakis et al., 2011), even when fed a diet with the high fat content (Poli et al., 2003; Piccolo et al., 2008). Hence, based on the flesh quality, meagre is consider as a fish species of high nutritional and dietetic value (Quéméner et al., 2002; Poli et al., 2003).

Monfort (2010) reviewed the European markets and reported that farmed meagre is

mainly sold fresh, whole or as fillets, and only small volumes are sold frozen. Over 50 % of the fish are sold at 1–2 kg, 30 % above 2 kg, and the rest below 1 kg. However, the main direction in future market development for meagre will be oriented to the production of ready-to-cook portions, as fillets, cuts or smoked fillets. Meagre is also interesting for recreational purposes, as for aquariums, considering its high adaptation to captivity and the wide range of temperatures and salinities tolerated.

In the last decade, meagre has become an increasingly important fish species to Mediterranean aquaculture. In 2011, the EU adopted an ambitious strategy to prevent the loss of biodiversity and ecosystem services in the EU by 2020. As part of the strategy, the EU aimed to identified species diversification for Aquaculture, which revealed meagre as an interesting candidate for Mediterranean aquaculture due to its easily adaptation to captivity and rapid growth rate.

Sustainable Aquafeeds

In feeds manufacture, the ingredients, usually products for using in animal feeds or by-products from human food are the main sources of energy, proteins (amino acids), fats, carbohydrates, vitamins and minerals (NRC, 2011). In this context, dietary protein is the major and most expensive component of formulated aquafeeds (Wilson, 2002).

Carnivorous fish has a high requirement for protein and generally feed for this species has high level of FM as the main protein source (Watanabe, 2002; Tacon and Metian, 2008), mainly due to its high suitable nutritive value and good palatability (Watanabe, 2002). In recent years, however, the development of feed with lesser amounts of FM for aquaculture has become an issue, once worldwide FM supply is much lower than its demand (Watanabe, 2002; Tacon and Metian, 2008). Furthermore, FM high demand usually contributes to the high final price of aquafeeds (FAO, 2016).

Aiming the reduction of FM in aquafeeds, many studies have been conducted with the objective of substituting an expensive ingredient with a less expensive alternative protein sources and to overcome the paradox of rearing fish by feeding fish to fish (Gaylord et al., 2007; Takagi et al., 2008). It is important that the new ingredient sustain comparable levels of feed intake, feed conversion efficiency, as well as growth rate and survival of fish. As results, it has been recognized that FM content in fish feed can be partially replaced by alternative protein sources (Watanabe, 2002).

However, for carnivorous fish species, only selected ingredients with high protein

content, good amino acid profile, high nutrient digestibility and general lack of antinutrients may be used as alternative protein sources, due to their high protein requirements and low plasticity in using plant protein ingredients (Gatlin et al., 2007; Hardy, 2008). Among these alternative ingredients there are by-products of animal production, single-cell organisms and vegetable feedstuffs.

A large number of plant feedstuffs are currently been incorporated into aquafeeds to support the sustainable production of various fish species, including oilseeds, legumes and cereal grains, used as protein or energy concentrates as well as novel products developed through various processing technologies (Gatlin et al., 2007; Lim and Lee, 2009; Zhanga et al., 2012; Tian et al., 2012). However, comparatively to FM, alternative plant protein sources have some nutritional disadvantages such as inadequate amino acid (AA) profile, low digestibility, low palatability, and the presence of several anti- nutritional factors, which may interfere with fish performance and health due to impaired nutrient utilization (Gatlin et al., 2007; Li et al., 2009). Particularly in mariculture of carnivorous fish species, excessive replacement of FM with alternative proteins can result in inferior growth performance as well as physiological abnormalities such as anemia and green discoloration of the liver, so-called ‘‘green liver’’ (Aoki et al., 2000; Watanabe et al., 2001; Goto et al., 2001).

Among the main plant protein ingredient currently use in aquafeeds, soybean meal stands out for presenting high protein content and a well-balenced AA profile, been considered a major alternative to FM in aquafeeds (Storebakken et al., 2000). However, it presents anti-nutritional factors and sulfur AA deficiency that can cause abnormal intestinal morphology, decrease growth performance and feed conversion impairment (Francis et al., 2001). Therefore, establishing the optimal requirement of essential amino acids in addition to the characterization of alternative protein/AA sources have been one of the main objective of fish nutrition research (Li et al., 2009; Furuya and Furuya, 2010).

It is highly improbable that complete replacement of FM will be possible with a single alternative protein source in carnivorous fish aquafeeds (Salze et al., 2010). Therefore, the supplementation of synthetic AA to restore plant protein ingredients amino acid profile has proven to be a good strategy to achieve growth performance without detrimental impacts towards a sustainable aquaculture (Furuya and Furuya, 2010). Silva et al. (2010) for example, observed that a supplementation of L- glutamine and L-glutamate on plant protein-based diets for Nile tilapia increases its weight gain and intestinal villus height.

Studies with both aquatic and terrestrial animals show that many AA regulate key metabolic pathways crucial to maintenance, growth, reproduction, and immune responses termed as ‘‘functional AA’’ by Li et al. (2009). Identification and dietary supplementation of those AA or their biologically active metabolites is expected to offset adverse effects of replacement of FM from aquafeeds, therefore restoring feed intake and growth.

Convincing evidence shows that supplementing taurine to all-plant protein diets can promote growth and feed efficiency by carnivore fish, such as rainbow trout (*Oncorhynchus mykiss*) (Gaylord et al., 2007) and Japanese flounder (*Paralichthys olivaceus*) (Kim et al., 2005a, 2005b). Furthermore, it has been shown that taurine managed to mitigate some of the anti-nutritional side effects of plant protein ingredients on carnivorous fish. Its supplementation was able to restore bile acid production disturbed by a high soybean meal-based diet (Nguyen et al., 2011a), therefore increasing lipase activity with lipid digestibility improvement (Chatzifotis et al., 2008).

Thus, the continuous growth and intensification of the currently aquaculture production depend no longer on the insertion of a FM alternative ingredient into aquafeeds but, to the total replacement of FM by an alternative sustainable protein source. If this goal is achieved, FM will be no longer the primary protein source, but rather a specialty ingredient added to enhance palatability, balance dietary AA or to supply other essential nutrients and biologically active compounds. For that purpose, the study of dietary supplementation of AA and their metabolism may provide new strategies to develop amino acid-balanced feeds that can offset environmental impacts on aquaculture animals, improve growth performance and profitability of the aquaculture industry achieving true sustainability in fish farming (Hasan, 2000).

Methionine

L-Methionine (α-amino-γ-methylthiobutyric acid) was discovered by John Howard Mueller in 1922 as a substance in an acid hydrolysate of casein. One year later, after developing an improved method to obtain a large amount of this substance from casein, Muller proposed the composition of methionine as a sulfur-containing AA (Wu, 2013) (Figure 2). In addition, since the fish do not synthesize methionine is also characterized as essential amino acids (NRC, 2011).

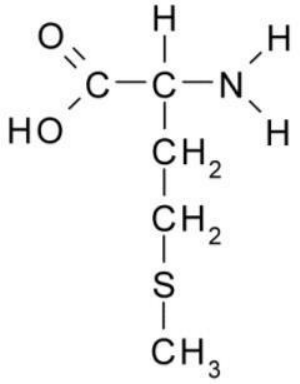

Figure 2 - Methionine structure. Source: Adapted from Wu (2013).

The major metabolic functions of methionine are its utilization for protein synthesis and its conversion to S-adenosylmethionine (SAM) via transmethylation with consequently production of homocysteine (Riedijk et al., 2007). SAM is the major donor of methyl groups in animals and a precursor in polyamine synthesis. In most cases, SAM reacts by transfer of the S-methyl group to one of several possible acceptors including glycine, forming sarcosine; guanidineacetate, forming creatine, among many others (Griffith, 1987) (Figure 3).

Homocysteine can be further catabolized to cysteine through transsulfuration or be used for methionine synthesis through remethylation (Wu, 2013). Cysteine is oxidized in animals to sulfate, thiosulfate, and sulfite and its catabolism occurs primarily in hepatocytes. Additionally, cysteine can be oxidized by formaldehyde to N-formylcysteine and, along with glutamate and glycine, is used to synthesize glutahione, a major cellular antioxidant, and coenzyme A playing a key role in cellular protein function and redox status (Riedijk et al., 2007). Finally, cysteine, through specific enzymes can be convert into taurine (Wu, 2013).

Due to cysteine anabolic pathway, the need of animals for cysteine can be met by dietary methionine and that part of the dietary requirement for methionine can be fulfilled by cysteine. In this context, cysteine remains a nonessential AA only if methionine intake is sufficient to meet the total sulfur AA requirement. These results indicate not only that cysteine is formed from methionine and, therefore, can spare methionine (Wu, 2013) but also that fish have a total sulfur AA requirement rather than a specific methionine requirement (Wilson and Halver, 1986).

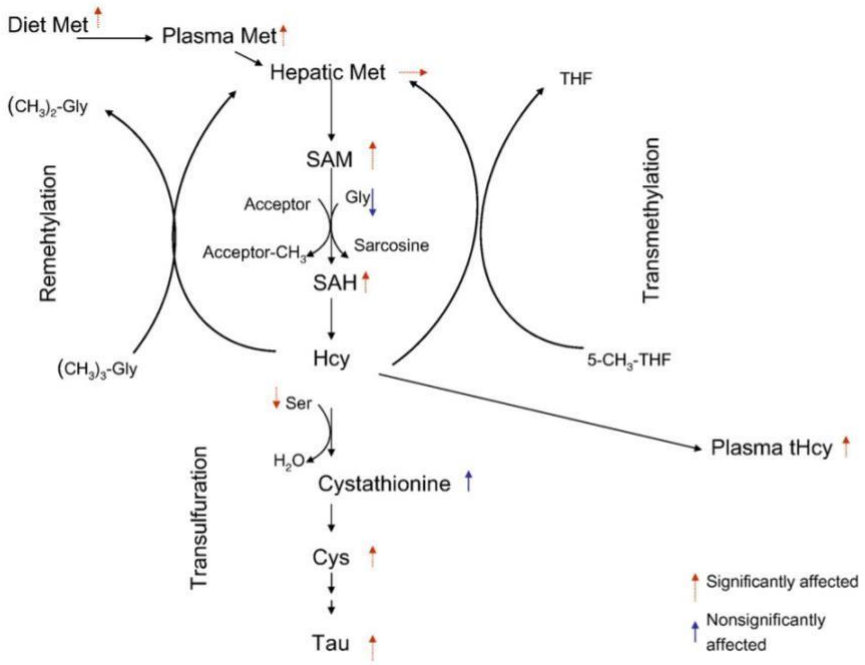

Figure 3 - Catabolism of methionine via the transulfuration pathway in animals. Source: Espe et al. (2008).

In this context, since methionine and cysteine are taurine precursors, a supplementation of this sulfur AA may spare dietary taurine requirement (Jirsa et al., 2014; Wang et al., 2014). On the other hand, the supplementation of exogenous taurine might alter the transsulphuration or the re-methylation of homocysteine to methionine (Mato et al., 2002), rendering more methyl groups available for other methyl-requiring pathways as for example polyamine synthesis (Seiler, 1990; Pegg, 2009). Polyamines are essential for cell growth and proliferation, any changes in polyamines might contribute to the growth-promoting effects reported when juvenile fish are fed diets supplemented with taurine (Gaylord et al., 2007).

Pathways of methionine transmethylation, remethylation and transsulfuration for the synthesis of cysteine and taurine are in fish despite possible quantitative differences among species (Goto et al., 2003). Interestingly, cysteine dioxygenase, one of the two rate-controlling enzymes in taurine synthesis from cysteine, is up regulated by dietary methionine at the transcriptional level, but not affected by dietary taurine (Gaylord et al., 2007). Transcription of the other enzyme, cysteine sulfinate decarboxylase, in the liver of rainbow trout is not influenced by dietary taurine, but inhibited by dietary methionine (Gaylord et al., 2007). Therefore, it is important to determine whether changes in maximum activities of enzymes measured at saturated concentrations of substrates translate into alterations in the flux of methionine to taurine (Li et al., 2009).

Methionine is usually the first limiting AA in fish diets containing high levels of plant protein sources, such as soybean meal, peanut meal, and corpa meal (Mai et al., 2006). Thus, fish growth is often reduced when fed diets containing high levels of soybean meal due mainly to this imbalance of sulfur amino acid (Elangovan and Shim 2000; Chou et al., 2004). Compared with herbivores and omnivores, carnivores require a higher percentage of dietary protein which depends largely on animal proteins ingredients. Hence, the amount of FM that can be replaced by soybean meal may be limited for carnivores fish unless other nutritional strategy is develop.

The dietary supplementation of synthetic amino acid can be a good strategy to supply further deficiency caused by a plant protein-based diet (Li et al., 2009) specially for carnivorous fish aquaculture. In this context, methionine and its derivatives are commercially produced by chemical processes and is commonly available in the DL-form, being readily absorbed and efficiently used by animals. D-Methionine must be transaminated into a-ketoacid by D-methionine oxidase, and the a-ketoacid is then converted into L- methionine by transaminases (Wu and Thompson, 1989) (Figure 4).

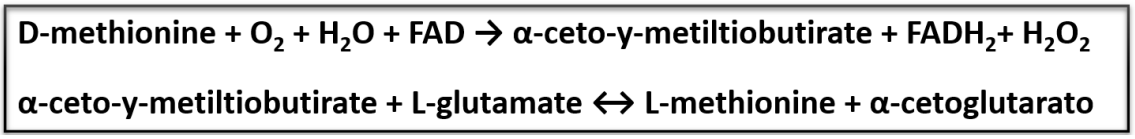

Figure 4 - Transamination of D-methionine to L-methionine. Source: adapted from Wu (2013).

The methionine dietary requirement has been estimated for several species of fish, ranging from 1.8 to 4.0% of the dietary protein (Wilson, 2002). Based on growth rate and feed conversion, methionine requirement for juvenile grouper (*Epinephelus coioides*) was determined to be 13.1 g kg^{-1} (2.73% dietary protein) based on the highest weight gain by LRP analysis and 2.6 g kg^{-1} of cystine (Luo et al., 2005). For cobia juveniles (*Rachycentron canadum*), Zhou et al. (2010) determined the requirement of methionine in 11.9 g kg^{-1} (2.64% of dietary protein), using diets with 6.7 g kg^{-1} of cystine. Thebault et al. (1985) observed optimal growth rate occurred in sea bass fed diets with 13 g kg^{-1} of methionine content, with 0.6% of its supplementation in the crystalline form.

Taurine

In 1954 taurine was identified as a product of cysteine metabolism via formation of cysteinesulfinic acid in the rat liver. Since then, although in the strict sense, taurine is not

an amino acid as it lacks a carboxyl group, it is being characterized as a non-essential β-amino acid (Wu, 2013). Taurine is also classified as a sulfur-containing AA and by so, may also be called an amino sulfonic acid. Taurine (2-aminoethanesulphonic acid), unlike most AA, is not metabolized or incorporated into protein, remaining free in the intracellular water (Han et al., 2006) (Figure 5). It is highly abundant on vertebrates organisms and, in mammals, for example, it has already been identified on the central nervous system, spinal cord, retina, liver, kidney, muscle tissue and reproductive system among others (Bolz et al., 1985; Worden and Stipanuk, 1985; Campistron et al., 1986; Chanda and Himwich, 1970; Lee et al., 1992; Huxtable, 1992; Quesada and Sturman, 1993; Bustamante et al., 2001).

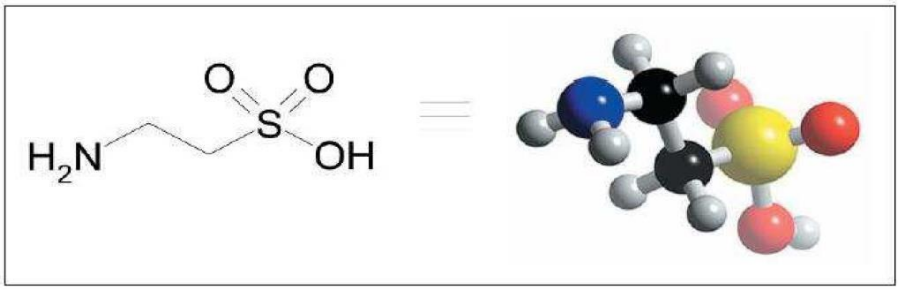

Figure 5 - Molecular structure (left) and atomic structure (right) of taurine. Source: Szymansky and Katarzyna (2008).

Previous studies have shown that taurine regulates several biological processes, including prevention of tissue injury by oxidative stress (Huxtable, 1992; Tas et al., 2007), modulation of the digestion and absorption of dietary fats besides its hypoglycemic properties (Kulakowski and Murato, 1984; Fang et al., 2002; Kaplan et al., 2004). It is also responsible for cell volume regulation by protecting cells from osmotic stress (Han et al., 2006), it has been used as a major attractant for fish in Europe (Gaylord et al., 2006; Takagi et al., 2008) and, as dietary supplement to promote growth in aquaculture (Ferreira et al., 2015).

The sources of taurine are from *de novo* synthesis or dietary intake. The major pathway for taurine synthesis is through the cysteinesulfinate pathway (Wu, 2013). Methionine turns into cystathionine by cystathionine synthetase. The transformation of cystathionine to cysteine by cystathionase is followed by cysteine oxidation to cysteinesulphinate through the activity of cysteine dioxygenase (CDO). This reaction continues by the decarboxylation of cysteinesulphinate to hypotaurine by L-Cysteinesulphinate decarboxylase (CSD) enzyme and then to taurine (Worden and Stipanuk, 1985; Griffith, 1987). During this process, CSD is considered the rate-limiting

enzyme for taurine biosynthesis in the liver (de la Rosa and Stipanuk 1985; Morris and Rogers, 1992), therefore its activity reflects the ability of the organism to synthesize taurine (Figure 6).

However, taurine cellular concentration is not only controlled by taurine biosynthetic enzymes CDO and CSD but also by taurine Na^+ and Cl^- dependent cellular transporter (TauT) (Tappaz, 2004; Voss et al., 2004). Thus, taurine intracellular concentration, important for its biological functions, is not only determined by the capacity of a given cell to synthetize taurine but to its capacity of taurine transportation from the extracellular medium (Tappaz, 2004). The biosynthetic pathway depends on the expression level and regulation of the key enzyme CSD, as well as from the availability of cysteine and methionine. Yet, the contribution of taurine transport into the intracellular medium depends not only on the regulation and expression level of the TauT, as also on the taurine level inside the cell (Warskulat et al., 1997a, 1997b; Tappaz, 2004). Most vertebrates are able to synthesize taurine from cysteine, but in different rates (Worden and Stipanuk, 1985; Yokoyama and Nakazoe, 1992; Yokoyama et al., 2001), since CSD activity is extremely variable across the species (Salze and Davis, 2015). However, this endogenous taurine synthesized by fish, cannot meet fish requirement (Gaylord et al., 2006; Kim et al., 2008a). Thus, the whole taurine body pool must result from a balance between taurine and sulfur amino acid intake from the diet, taurine synthesis by the liver and reabsorption by the kidney (Huxtable, 1992), while only a small part is reabsorbed as bile salts (Sturman et al., 1975).

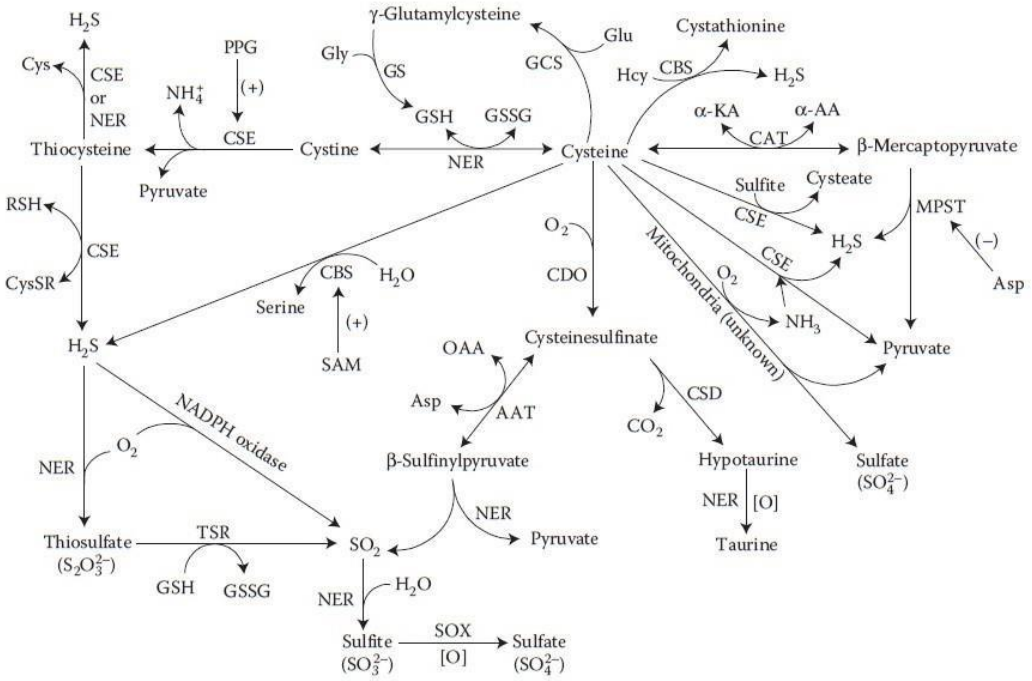

Figure 6 - Synthesis of cysteine and taurine in animal cells. Source: Wu (2013).

Dietary taurine requirements of fish have a large spread, especially affected by fish species and sizes and by dietary protein sources and levels (Salze and Davis, 2015). Rhodes and Davis (2011) reported that taurine requirement in marine finfish is in a range of 2.6 to 72.0 g kg^{-1}. Gaylord et al. (2006) aiming to evaluate taurine supplementation to plant-protein based diets observed that 0.5% of taurine on the diet increased growth rate of rainbow trout. Similar results were also found by Jirsa et al. (2014) with white sea bass (*Atractoscion nobilis*). Brotons-Martinez et al. (2004) also working with sea bass indicate that for a better growth when fishmeal and soybean meal are the primary sources of protein a 0.2% taurine in the diet is required. Qi et al. (2012) studying two different life stages suggested that 1.0% taurine in diet of juvenile turbot (*Scophthalmus maximus* L.) with 6.3 g weight and 0.5% taurine in diet of turbot with 165.9 g weight are probably optimal.

Dietary taurine deficient can cause abnormal physiological conditions and inferior feed utilization (Takagi et al., 2006, 2011). Hence, an efficacy of taurine supplementation on growth has been reported in several fish species. Chen et al. (2004) observed that growth of red sea bream (*Pagrus major*) and Japanese flounder larvae were improved by rotifer enriched with taurine. Furthermore, it was reported that growth and feed efficiency of red sea bream (Takagi et al., 2006), cobia (Lunger et al., 2007) and rainbow trout (Gaylord et al., 2006, 2007) fed low levels of FM in diets based on alternative plant

protein sources were also improved by dietary taurine supplementation. Therefore, taurine is being recently considered as a conditionally indispensable amino acids (Kim et al., 2008a, 2008b; Li et al., 2009; Wu, 2013).

Taurine is highly abundant in marine animals (Park et al., 2002) but absent in alternative protein sources such as soybean meal and cottonseed meal (Kim et al., 2008a). According to Yamamoto et al. (1998) while FM contains approximately 0.5–0.7% taurine, plant proteins contain only trace amounts of it. Thus, supplementation of taurine into plant protein-based diets can be indispensable for promoting the growth and feed utilizations of carnivore fish (Li et al., 2009) especially for fish with low CSD activity.

Sulphur amino acids on fish oxidative *status*

Free radicals are defined as molecules having an unpaired electron in the outer orbit of the electron shell. Oxygen free radicals are superoxide (O^{2-}), hydroxide (OH^{-}), peroxyl (OH^{2-}), alkoxyl and hydroperoxyl (HO_2^{-}) radicals. Nitrogen free radicals, on the other hand, are nitric oxide (NO), peroxnitrite (NO_3^{-}), nitroxyl anion (HNO), among others. Both nitrogen and oxygen free radicals can be converted to other non-radical reactive species such as H_2O_2, hypochlorous acid (HClO), hypobromous acid (HBrO) and peroxynitrite (HOONO) (Droge, 2002).

These reactive oxygen species (ROS) are generated as part of the normal aerobic cellular metabolism (Storz and Imlayt 1999). Thus, there are two facets to free radicals in biology. They can serve as signaling and regulatory molecules at physiologic levels or then can cause cell damage for their high cytotoxic oxidant capacity (Lynch et al., 2000; Saraymen et al., 2003; Gokirmak et al., 2003). When the ROS generation rate exceeds that of their removal, oxidative stress occurs which may produce deleterious effects including protein oxidation, DNA strand-break damage and peroxidation of unsaturated lipids (Martınez-Alvarez et al., 2005). Lipid peroxidation resulting from oxidation of cholesterol and fatty acids may compromise the cell membrane integrity which may induce injury of tissues so as leak of enzymes or ions. DNA damage, on the other hand, may eventually lead to cell death or abnormal cell growth. (Hoshi and Heinemann, 2001; Veena et al. 2006).

The antioxidative activity has mainly been designated as the peroxide value of oxidation products and from the oxidation rate of co-existing lipids. This process can occur through different mechanisms, such as metal chelation, activated oxygen species

scavenging, recycling of other antioxidants or repair of damaged molecules induced by oxidative stress (Halliwell and Gutteridge, 2007). In this context, effects of free radicals can be controlled enzymatically by wide range of antioxidant enzymes, such as: superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). SOD alters toxic superoxide radicals to H_2O_2. Catalase converts H_2O_2 to molecular oxygen and water as GPX which also catalyses the conversion of H_2O_2 to water (Peng et al., 2014). Thus, all these ways of action are a part of an adequate protection systems made by enzymatic and non-enzymatic antioxidant compounds that organisms possess in order to avoid or repair the damage that these compounds may cause in tissues (Halliwell and Gutteridge, 2007). This is particularly relevant in aquaculture, since the oxidative damage of fish tissues is directly associated to animal welfare (Sargent et al., 2002; Senso et al., 2007) (Figure 7).

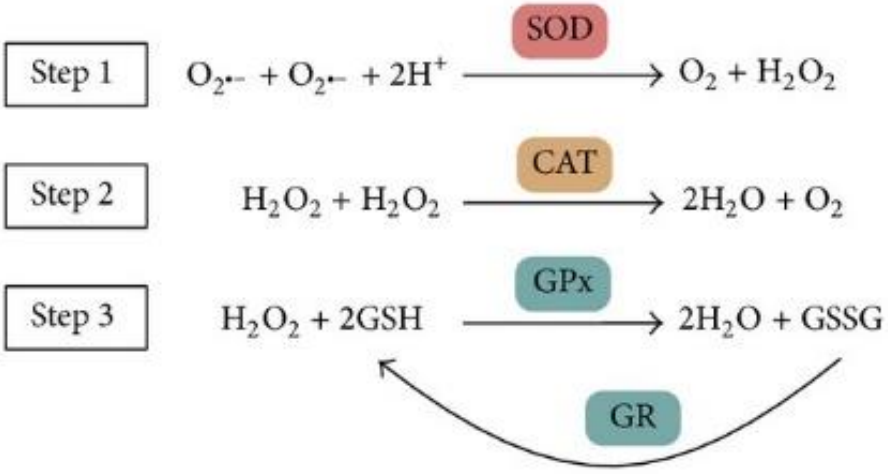

Figure 7 - Main enzymatic antioxidant defense system *in-vivo* and their reactions on scavenging free radicals. Source: Peng et al. (2014).

As the antioxidant capacity of a compound occurs through different mechanisms the total antioxidant activities of chemical compounds can not be evaluated by any single method. Thus, several methods can be used to test the antioxidant activities of various chemical compounds as the activity of the key antioxidant defense system mentioned above. *In vivo*, there is a high degree of interaction among these endogenous antioxidants. Depending on the order of their corresponding redox potentials, it is common for one antioxidant to regenerate another one from its oxidized species (Coutinho et al., 2016).

Sulphur-containing amino acid as cysteine, methionine and taurine presents among other functions high antioxidant action. The net positive charge present on sulphur amino

acids or on their residues may protect cells by scavenging free radical. Compounds containing sulphur can reduce Cu^{2+} or Fe^{2+} to Cu^{+} and Fe^{2+}, respectively, while being oxidized to disulfides which can be easily reduced back via glutathione (Hoshi and Heinemann, 2001). The reduced metal ions can be then reoxidize by reaction with a superoxide (Lynch et al., 2000; Pfanzagl et al., 2003).

Methionine is an efficient scavenger of almost all oxidizing molecules. It is particularly susceptible to oxidation by ROS and then converted to methionine sulphoxide (MeSOX). However, this compound is usually reduced back to methionine via MeSOX reductase if the MeSOX was not further oxidated thus, this reaction can be physiologically reversible (Hoshi and Heinemann, 2001). It appears that the cycle of methionine oxidation and reduction represents a natural scavenging system for ROS (Figure 8).

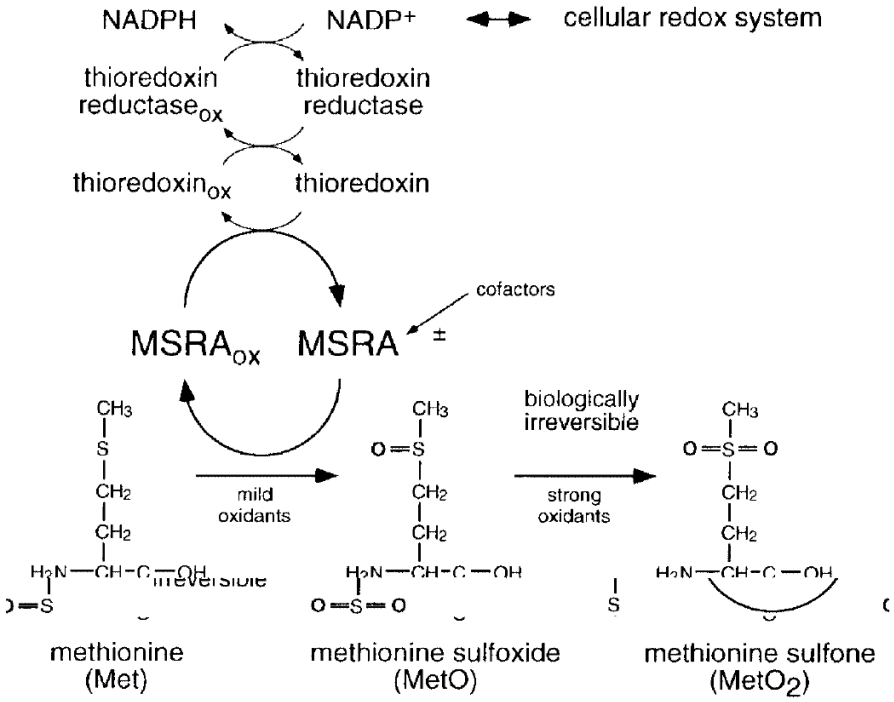

Figure 8 - Oxidation and reduction of methionine residues. Souce: Hoshi and Heinemann, 2001

Studies with rats indicated that methionine improved SOD, GPX and CAT activities in the heart of male Sprague–Dawley rat (Seneviratne et al., 1999), as well as GR activities in tertbutylhydroperoxide induced brain synaptosomes of albino rat (Slyshenkov et al., 2002). Methionine can also promote antioxidant action indirectly as a cysteine precursor which in turn is the main limiting amino acid for glutathione (GSH) synthesis, an important antioxidant molecule that acts as cofactor of GPX on ROS reduction (Lu, 2000; Wu et al., 2004). Thus, methionine can indirectly affect GSH

generation with repercussions on cells oxidative status (Wu et al., 2004; Li et al., 2007; Métayer et al., 2008). GSH is a substrate for GSH-transferases and peroxidases, enzymes that catalyses the reactions for detoxification of ROS. Depletion of GSH, the major cellular antioxidant, results in increased vulnerability of the cell to oxidative stress. GSH levels of tissue are not regulated by synthesizing enzymes alone, but rather by the combination of a sulphur containing amino acid supply and metabolism. Sulphur-containing amino acid plays a role in determining the flux of cysteine catabolism and GSH synthesis. Cysteinr or sulphur amino acids supplementation is an effective method of restoring GSH status (Kim et al., 2003).

Taurine as methionine is often associated with antioxidative cytoprotective actions. Taurine can partially scavenge reactive oxygen species (ROS) and prevent changes in membrane permeability following oxidant injury, but it does not act as a chelator of lead (Neal et al., 1999). Hypochlorous acid (HOCl), for example, is produced during the leukocyte's (usually neutrophil granulocyte) respiratory burst via the myeloperoxidase pathway. It is a cytotoxic oxidant used to kill pathogens, but it is equally toxic to the host's cells. Taurine reacts with HOCl to produce the stable oxidant taurine chloramine (Tau-Cl), thereby reducing the oxidative stress (Salze and Davis, 2015). In addition, taurine has also been shown to reduce lipid peroxidation levels (Bosgelmez and Guvendik, 2004; Zhang et al., 2004; Nandhini et al., 2005; Aydogdu et al., 2007) and elevate GSH hepatic levels in rats.

Taurine and vitamin E treatment maintained GSH levels and increased the activity of GPX, the levels and increased the activity of GPX, the levels of SOD and CAT, and directly scavenges superoxide radicals. GSH in turn is a major component of the cellular antioxidant system playing an important role in the antioxidation of ROS and free radicals (Ebrahim and Sakthisekaran, 1997; Atmaca, 2004). In fish, the relationship between taurine and oxidative stress was hypothesized in jaundiced *Seriola quinqueradiata* (Sakai et al., 1998). Taurine supplementation also led to a restored catalase activity and reduced lipid peroxidation levels in *Totoba macdonaldi* (Bañuelos-Vargas et al., 2014). Finally, it has also been shown in *Trachinotus carolinus* that was maintained on a taurine-deficient diet for 16 days: a significant decrease in hepatic mitochondrial protein content and mitochondrial activity was reported to be strongly correlated with a decrease in taurine content (Salze et al., 2014). As antioxidants *in vivo* can destroy free radicals the diet supplementation with antioxidants may offer increased protection against damage (Lu and Liu, 2002; Fang et al., 2002; Gokirmak et al., 2003; Saraymen et al., 2003).

Taurine on lipid metabolism

The aim of the lipid digestion is to hydrolyze lipid soluble triglyceride into amphiphilic products such as fatty acids and monoglycerides which can then be transported away from the oil phase into aqueous phase micelles ready to be uptaken by the epithelial cells. Most dietary fats are consumed in the form of triglycerides and are emulsified into droplets. In order for the lipases to access triglycerides, the enzyme has to adsorb onto the surface of the fat droplets assistant by its cofactor co-lipase, to hydrolyze triglycerides producing fatty acids and monoglycerides through the process of lipolysis (Lowe, 1997; Wilde and Chu, 2011). In this context, because of the buildup of lipolysis, product at the interface during lipolysis (Patton and Carey, 1979) can cause lipase inhibition thus; biosurfactants are required to remove these products (Crandall and Lowe 2001; Reis et al., 2009). Bile plays a critical role in this process by aiding fat digestion.

Bile is an alkaline secretion produced by the liver cells working not only as digestive but also as an excretory fluid. As an excretory fluid, bile contains substances that can not be eliminated efficiently in urine because they are insoluble or protein bound like bile acids, bilirubin, cholesterol, heavy metals such as iron and cooper, lipophilic steroids and drug metabolites. As a digestive fluid, bile contains bile acids, potent digestive surfactants that promote lipid absorption (Hofmann, 1999). It can be secreted directly into the proximal intestine or be stored in the gallbladder when it is not immediately needed. The main bile acids are cholic acid, deoxycholic acid and chenodeoxycholic acid (Wilde and Chu, 2011). During its production, bile acid is conjugated with taurine or glycine (e.g. taurocholic acid, glycodeoxycholate) making them more water-soluble at acidic pH and, with increased resistance to precipitation by Ca^{2+} (Hofmann, 1999). The amphiphilic character of bile acid will allow its capacity to form micelles (la Mesa et al., 1985).

Bile acids are produced in the liver and derived from cholesterol. They have an essentially steroid structure composing by four rings with a side chain terminating in carboxylic acid, to which the taurine or glycine becomes conjugated (Hofmann, 1999). The 7 alpha- hydroxylation of cholesterol is the rate-limiting step in the conversion of cholesterol into bile acids (Hofmann, 1999). They are stored in the gallbladder, as part of the bile, and released into the duodenum in response to the presence of food. Their primary aim is to form micelles which help solubilize lipolysis products, displacing them from the interface allowing lipolysis to continue (Maldonado-Valderrama et al., 2008; Reis et al.,

2009).

In this context, taurine plays a well-recognized role in fat digestion as a conjugator with bile acids in the liver. Its conjugated bile acid taurocholic acid or taurochenodeoxycholic are the major bile acids in the liver of almost all fish species, except cyprinids (Vessey et al., 1990; Goto et al., 1996; Yeh and Hwang, 2001) (Figure 9). Salze et al. (2012) reported that taurine supplementation enhanced the digestive enzyme activities of cobia larvae, while other studies observed an effect of taurine on the conjugated bile acid composition in Japanese flounder (Kim et al., 2005a). An increased on the 7 alpha-hydroxylase activity, with a consequently enhancement on bile acid concentration, was also observed (Fujihara et al., 1978; Kibe et al., 1980). In addition, taurine supplementation to the soy protein concentrate diet enhanced the utilization of dietary lipid via improving the metabolism of bile salts (Goto et al., 1996).

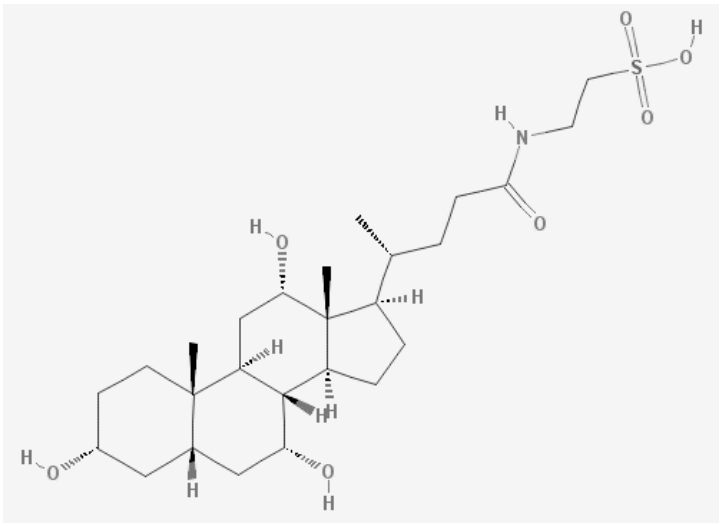

Figure 9 – Molecular estructure of Taurocholic acid. Source: PubChem Compound Database (accessed June 2, 2017).

Previous studies have found that fish fed an soybean meal based diet developed digestive physiological disorders, such as insufficient bile acid levels in the gallbladder and intestine, low digestive enzyme activity in the intestine and inferior lipid digestion with consequently low growth (Nguyen et al., 2011a, 2011b). The authors reported that this result could be caused by the dietary taurine deficiency. In this case, the supplementation of taurine becomes indispensable to maintain normal physiological condition and growth performance of fish (Gaylord et al., 2007; Takagi et al., 2011).

Taurine also seems to have a lipid-lowering effect (Murakami et al., 1999, 2000, 2002). An increased conjugation of bile acids with taurine results in an elevated excretion

of cholesterol, suggesting a potential hypocholesterolemic effect of taurine (Cherif et al., 1996, 1998; Nandhini et al., 2002).

Digestive Enzymes

Digestive enzymes are crucial for digestive processes, allowing protein, carbohydrate and fat degradation into smaller and simple molecules. These molecules can then be absorbed and transported into tissues, by the circulatory system, and used for growth, tissues repair and reproduction (Furné et al., 2005). There are several factors that affect the activity of digestive enzymes. These include diet composition (Santigosa et al., 2008; Chatzifotis et al., 2008; Cedric, 2009), age (Kuz´mina, 1996) and environment conditions (Zhi et al., 2009). Thus, quantifying the activity of digestive enzymes is a useful way to provide information on the nutritional value of diets and possible interaction between anti-nutritional factors and digestive enzymes of fish when fed formulated diets (Refstie et al., 2006; Corrêa et al., 2007).

All fish species seem to possess the enzymatic apparatus necessary to hydrolyze and absorb simple and complex carbohydrates. Digestion and absorption take place by the same routes in herbivores, omnivores and carnivores species. The α-amylase (EC 3.2.1.1) is a key enzyme for carbohydrate digestion. It acts on complex polysaccharides, like starch and glycogen, hydrolyzing them up into maltotriose and maltose, a combination of branched oligosaccharides and some glucose (Papoutsoglou and Lyndon, 2003). In fish, amylase has been identified in pancreatic juice, in the stomach and in the intestines but the main producers seem to be the pancreas and the liver (Klahan et al., 2009). Herbivorous and omnivorous species appear to digest starchy components of vegetable feedstuffs more efficiently than carnivorous species (De Almeida et al., 2006; Al-Tameemi et al., 2010). Thus, feeding habits have great impact on amylase activity (Horn et al., 2006; Corrêa et al., 2007; Caruso et al., 2009).

The main digestive process of lipids involves their extracellular hydrolysis in the intestine and cecal lumen by a variety of lipases and colipases (Higgs and Dong, 2000). Lipase (E.C.3.1.1.3) catalysis the breakdown of triacylglycerol into diacylglycerol and monoacylglycerol (Savona et al., 2011). Its activity in fish has been found in pancreas extracts, pyloric ceca and upper intestine but can extend to the distal part of the intestine, decreasing progressively its activity (Klahan et al., 2009). However, the pyloric ceca and anterior intestines seem to be the primordial sites of lipid hydrolysis (Halver and Hardy,

2002). Despite of all fat-digestive enzymes are known to act in alkaline media (Tramati et al., 2005; Klahan et al., 2009), in some species like the Siberian sturgeon (*Acipenser baerii*) there is hydrolysis by lipase in the stomach (Halver and Hardy, 2002). In *Diplodus puntazzo*, lipase activity was detected in all regions of the gut, indicating a uniform distribution in the entire gut system (Tramati et al., 2005).

Several authors suggested that lipase presence is greater in carnivorous than in omnivores or herbivores fish because carnivorous species consume fat-rich food (Tengjaroenkul et al., 2000; Furné et al., 2005). This fact might suggest that the type of diet could influence the production of lipases in adult fish (Kuz'mina, 2008). The largest class of lipids present in fish diets is triacylglycerol class. The lipase activity is directly related to triglycerides (TG) and phospholipids (PL) levels present in the diet as shown in sea bass (*Dicentrarchus labrax*) larvae fed diets which contain different levels of these lipid fractions (Cahu et al., 2003; Zambonino Infante and Cahu, 2007; Savona et al., 2011). The digestive proteases catalyze the hydrolytic degradation of proteins (Garcia- Carreno and Hernandez-Cortes, 2000). Proteases also activate the released zymogens of a large number of digestive enzymes into their active form (Bureau et al., 2002). In the EC system for the enzymatic nomenclature, all proteases (peptide hydrolases) belong to the subclass 3.4, which is divided into 3.4.11-19 (exoproteases) and 3.4.21-24 (endoproteases) (Nissen, 1993). Endoproteases disrupt in the middle of polypeptide chain, while exoproteases hydrolyze the free ends of peptide chains (Bureau et al., 2002). The exopeptidases, in special aminopeptidases, are mostly intracellular or membrane bound and cuts amino acids from the amino end of a peptide chain one at a time (Bureau et al., 2002). The high catalytic efficiency at low temperatures and low thermal stability are some of the differences in properties of proteases from marine and terrestrial animals (Klomklao et al., 2005).

In fish digestive organs proteases such as pepsin, gastricsin, trypsin, chymotrypsin, collagenase, elastase, carboxypeptidase and carboxyl esterase have been found (Dimes, 1994; Simpson, 2000) although the two major groups of proteases are pepsin and trypsin. Pepsin is chacarterized as the major acidic protease in fish stomach and it is the first proteolytic enzyme to break large peptide chains (Tengjaroenkul et al., 2000). Trypsin and chymotrypsin, on the other hand, are the major alkaline proteases in the intestine (Natalia et al., 2004; Caruso et al., 2009). The expression of proteases in higher trophic level species increases with age (Kuz'mina, 2008) and, in lower trophic level species these enzymatic activities are brought down (Ugolev and Kuz'mina, 1994)

with distribution that varies between species and organs. Usually, carnivorous fish species have a short intestine, with higher protease activity than herbivores (Lazzari et al., 2010) and the level of proteolytic activity seems to be related to the fish species growth rate (Hidalgo et al., 1999). Some authors suggested that vegetable protein sources included in the diet can decrease proteolytic activity (Venou et al., 2003; Santigosa et al., 2008).

The pancreas secretes enzymes with alkaline protease activity as inactive proenzymes mixed with digestive juice with a basic pH. A peptide located in the active site needs to be released for the activation of this enzyme. In fish, this family of alkaline enzymes includes the serine proteases (trypsin, chymotrypsin, elastase and collagenase). In pancreatic tissue most of the trypsin and chymotrypsin are present as trypsinogen and chymotrypsinogen. Trypsinogen is activated by enterokinase with a release of a peptide located at the active site. The active trypsin then activates other digestive enzymes like chymotrypsin (Bureau et al., 2002). These enzymes become active in existing pyloric caeca and proximal intestine and hydrolyze the protein (Santigosa et al., 2008).

Thus, evaluate if taurine and methionine supplementation are good nutritional strategies to mitigate some of the limitations of using high levels of plant feed ingredients in diets for meagre could contribute to the development of an innovative low-FM environmental friendly diet, through correct use of sustainable plant resources. Consequently, contributing to establish a real sustainable aquaculture, both from an economic and ecological perspectives.

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OBJECTIVES

To evaluate the potential of taurine and methionine supplementation as a nutritional strategy to mitigate some of the limitations of using high levels of plant feed ingredients in diets for meagre (*Argyrosomus regius*).

Especific Objectives

To evaluate the effect of the taurine and methionine supplementation on plant protein-based diet for juvenile meagre on:

- Growth performance, feed utilization and whole-body composition;
- Metabolic enzymes activities;
- Oxidative status and serum biochemical parameters;
- Nutrient digestibility and digestible enzymes;
- Total bile acid content of plasma and intestinal digesta.

CHAPTER I

Taurine and methionine supplementation as a nutritional strategy for growth promotion and antioxidant defense improvement of meagre (*Argyrosomus regius*) fed high plant protein diet

(According to Aquaculture Standards)

Abstract

Methionine and taurine supplementation effect to a plant protein-based diet for meagre (*Argyrosomus regius*) was assessed on growth performance, metabolic hepatic enzymes activity and on antioxidative status. Four isoenergetic and isoproteic practical diets were formulated to contain 82% of protein from plant ingredients. A 2 x 2 factorial design was used with methionine at 7.5 and 10.0 g kg^{-1} dry diet, supplemented or not with taurine at 10.0 g kg^{-1} dry diet. A 65-day feeding trial was conducted using a 180 juvenile meagres (50.0 ± 0.60g). Dietary taurine supplementation improved growth and feed utilization. ALT and AST activities increased only with taurine supplementation, while GDH activity was unaffected by dietary treatment. Fructose-1,6-bisphosphatase activity was affected by both amino acids supplementation. Interaction effect was observed on intestinal G6PDH activity, been higher when supplemented with taurine at 10.0 g kg^{-1} of methionine and, on hepatic lipid peroxidation, which decreased when taurine and methionine were supplemented together. Taurine supplementation increased hepatic G6PDH while decreased CAT activity. Dietary methionine supplementation was inefficient to overcome the dietary taurine limitation and thus, taurine should be incorporated in plant protein-based diets, being an efficient nutritional strategy for the mitigation of growth limitations of meagre fed high plant ingredients. The supplementation of both amino acids improved oxidative status being able to mitigate diet-induced stress response caused by high levels of plant ingredients. Moreover, growth performance was not improved by increasing dietary methionine which suggests that meagre requirements are met with 7.5 g kg^{-1} dietary methionine.

Keywords: carnivorous fish; intermediary metabolism; sustainable aquafeed, sulfur amino acid

1. Introduction

Fish are reported to be able to adapt to different nutritional conditions. Thus, many studies have focused on the comprehension of fish plasticity toward the improvement of nutrient utilization and the fish performance in aquaculture systems (Espe et al., 2012; Banuelos-Varga et al., 2014).

Substituting fishmeal in diets for carnivorous fish species has been considered globally to elaborate cost-effectiveness and sustainable diets. However, for carnivorous fish, only selected ingredients containing high protein content, well-balanced amino acid profile and high nutrient digestibility may be used as alternative protein sources due to its high protein requirements (Gatlin et al., 2007). The imposition of high plant protein diets to carnivorous fish can result on a shifting of their metabolic profile, including pathways involved in protein and carbohydrate utilization (Vilhelmsson et al., 2004; Dias et al., 2005; Messina et al., 2007).

The use of limiting crystalline amino acids to restore plant protein ingredients amino acid profile has proven to be a good strategy to enhance growth performance of fish (Kaushik et al., 2004; Gaylord et al., 2007; Li et al., 2009). Furthermore, studies with aquatic animals have also showed that many amino acids which regulate key metabolic pathways are crucial not only to maintenance and growth, but also to reproduction, immune and oxidative status responses, can be able to mitigate nutritional stress termed as ‘‘functional amino acid’’ (Li et al., 2009).

In this context, besides being required for protein synthesis, methionine is an essential amino acid which serves as a methyl donor for several methylation reactions, including DNA, presenting an antioxidant and an improve immune response effect (Feng et al., 2011; Kuang et al., 2012; Wu, 2013). Furthermore, methionine is usually the first limiting amino acid in fish diets containing high levels of plant protein sources (Kaushik and Seiliez, 2010). On the other hand, taurine, a non-proteic amino acids, is also abundant in fishmeal but limiting in plant feedstuffs. It is involved in numerous physiological processes, such as bile acid conjugation, glucose uptake by the cells, osmoregulation, cell membrane stabilization, and anti-inflammatory events (Dokshina et al., 1976; Foos and Wu, 2002). Also acting as a non-enzymatic antioxidant, a cell membrane stabilizer, and an anti-inflammatory promoter (Zhang et al. 2004; Salze and Davis, 2015).

Many fish are capable of synthesizing taurine adequately to support normal physiological functions from sulfur amino acids if methionine levels meet their

requirement (Goto et al., 2001; Salze and Davies, 2015). However, this ability does appear to be inexistent or insufficient to meet requirements for other fish species (Park et al., 2002; Matsunari et al., 2008). Therefore, the limited capacity to synthesize taurine in fish may impose a supplementation necessity of this amino acid when its dietary intake is insufficient. Recent work has indicated that taurine can be considered as conditionally indispensable in plant protein based diets to support increased growth (Gaylord et al., 2006; Takagi et al., 2008; Salze and Davies, 2015).

Meagre (*Argyrosomus regius*) is a marine carnivorous fish species with a special aquaculture potential in the Mediterranean (Duncan et al., 2013). It grows fast and it is well accepted by the consumers (Mananos et al., 2009; Monfort, 2010). According to FAO (2005-2017) from 2006 to 2011, a great increase on global meagre production was observed. However, even though meagre is presently farmed in the Mediterranean basin, its production has not reached its full potential yet and rearing trials are still very limited (Velazco-Vargas et al., 2014).

Therefore, the present study aimed to evaluate the effect of taurine and methionine supplementation to a practical plant protein-based diet on growth performance, feed efficiency, whole body composition, metabolic enzymes activities and antioxidative status. As for to evaluate the potential of both amino acid as a nutritional strategy to mitigate developmental restriction and diet-induced stress response caused by high levels of plant ingredients on diets for juvenile meagre.

2. Materials and methods

2.1. Experimental design and diets

Four isoproteic (420.0 g kg^{-1} crude protein) and isoenergetic (180.0 g kg^{-1} crude lipids) practical plant protein-based diets were formulated to meet nutritional requirements of juvenile meagre (Martínez-Llorens, et al. 2011, Velazco-Vargas, et al. 2014) and, to contain 82% of protein from plant origin and 18% from fishmeal. A 2 x 2 factorial arrangement of treatments was used with methionine at 7.5 and 10.0 g kg^{-1} dry diet supplemented or not with 10.0 g kg^{-1} dry diet of taurine. Fish were randomly distributed to 12 circulars 300 L-tanks and diets were randomly assigned to each treatment, which consisted on three replicates of 15 fish per replicate.

All dietary ingredients were finely ground and well mixed. Mixtures were then dry pelleted without steam using a laboratory pellet mill (California Pellet Mill,

Crawfordsville, IN, USA) through 3.0 mm die. Crystalline amino acids were coated with agar before mixing with the other ingredients. After dried in an oven for 24h at 35ºC, pellets were sieved and stored in a freezer until use, at -10 ºC. The ingredients and proximate composition of the experimental diets are presented in Table 1, and the AA composition of the experimental diets is presented in Table 2.

Table 1
Ingredients (% dry weight) and proximate composition of the experimental diets

Ingredients	Diet			
	CT	TAU	MET	MET+TAU
Fishmeal[1]	8.0	8.0	8.0	8.0
CPSP[2]	2.0	2.0	2.0	2.0
Soybean meal[3]	22.6	23.2	22.4	22.4
Wheat gluten meal[4]	5.0	5.0	5.0	5.0
Corn gluten meal[5]	15.0	15.0	15.0	15.0
Pea protein[6]	4.0	2.9	4.0	3.2
Wheat meal[7]	20.3	19.8	20.3	20.0
Fish oil	14.8	14.8	14.8	14.8
Vitamin premix[8]	1.5	1.5	1.5	1.5
Choline chloride	0.5	0.5	0.5	0.5
Mineral premix[9]	1.0	1.0	1.0	1.0
Dibasic calcium phosphate	3.8	3.8	3.8	3.8
Agar	1.0	1.0	1.0	1.0
Binder[10]	1.0	1.0	1.0	1.0
DL-Metionine[11]	0.0	0.0	0.26	0.26
Taurine[11]	0.0	1.0	0.00	1.0
Proximate analysis (% dry matter)				
Dry matter	86.7	91.7	88.6	93.2
Crude Protein	43.0	41.6	43.0	41.8
Crude Lipid	17.7	17.7	17.6	18.0
Ash	7.7	8.1	7.7	7.9
Gross Energy (kJ kg^{-1})	22.5	22.7	22.4	22.7

[1]Crude protein: 71.0%; Crude Lipid: 11.3%; Sorgal, S.A. Ovar, Portugal;
[2]Soluble fish protein concentrate, Crude protein: 80.4%; Crude Lipid: 15.7%;
[3]Crude protein: 56.4%; Crude lipid: 2.5%; Sorgal, S.A. Ovar, Portugal;
[4]Crude protein: 84.8%; Crude Lipid: 1.7%; Sorgal, S.A. Ovar, Portugal;
[5]Crude protein: 80.1%; Crude Lipid: 4.1%; Sorgal, S.A. Ovar, Portugal;
[6]Crude protein: 84.0%; Crude Lipid: 2.7%; Cosucra, Belgium;
[7]Crude protein: 11.5%; Crude Lipid: 3.2%; Sorgal, S.A. Ovar, Portugal;
[8]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400;
[9]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet);
[10]Aquacube. Agil, UK;
[11]Feed-grade amino acids, Sorgal, S.A. Ovar, Portugal.

Table 2
Determined amino acid composition (dry matter %) of the experimental diets

	CT	TAU	MET	MET+TAU
Methionine	0.75	0.75	0.97	0.98
Arginine	2.87	2.75	2.83	2.79
Histidine	1.03	1.04	1.00	1.00
Isoleucine	1.84	1.72	1.76	1.76
Leucine	4.10	4.10	4.01	4.03
Lysine	2.35	2.30	2.34	2.29
Threonine	1.55	1.57	1.58	1.48
Valine	1.99	1.89	1.97	1.92
Phenylalanine	2.65	2.61	2.64	2.54
Taurine	0.13	1.24	0.13	1.24
Tyrosine	1.78	1.70	1.77	1.70
Alanine	2.79	2.84	2.82	2.81
Aspartic acid	3.91	3.77	3.74	3.73
Glutamic acid	6.35	6.00	6.26	6.11
Glycine	2.88	2.79	2.84	2.81
Serine	2.41	2.29	2.34	2.27
Proline	2.39	2.31	2.15	2.27
Cysteine	0.60	0.65	0.60	0.65
Tryptophan	0.44	0.46	0.44	0.45

2.2. Fish and experimental conditions

The experiment was conducted by certified scientists (category C FELASA) according to the recommendations of the European Union Directive 2010/63/EU on the protection of animals for scientific purpose.

The growth trial was performed at the Marine Zoology Station, Porto University, Portugal, in a thermoregulated recirculating water system equipped with a battery of fiberglass tanks supplied with continuous flow of filtered seawater (6.0 L min^{-1}). Temperature was regulated to 22.0 ± 0.9^{o}C, salinity of 35.5 ± 0.8 g L^{-1} and dissolved oxygen kept near saturation (7.0 mg L^{-1}) by blowers and diffusers that supplied air to each tank and biological filter. The photoperiod was maintained to a 12 h light/12 h dark regime, provided by artificial illumination.

Juvenile Meagres (*Argyrosomus regius*) were obtained from the Portuguese Institute of the Sea and the Atmosphere-IPMA, Algarve, Portugal, and after transportation to the experimental facilities were submitted to a quarantine period. During this period, fish were fed a commercial diet two times a day. The feeding trial was conducted with triplicate groups of 15 fish of 50.0 ± 0.6g mean initial body weight and lasted for 65-day trial. During the trial fish were fed to apparent satiation, twice a day (8.00 a.m and 16.00 p.m). All fish in each tank were bulk-weighed at the beginning of the trial, after one day of

starvation, and at the end of the trial four hours after the morning meal. For that purpose, fish were slightly anesthetized with 0.3 ml L^{-1} ethylene glycol monophenyl ether.

2.3. Sampling

Three fish from the initial stock population were pooled for initial whole body composition analysis.

At the end of the experiment two samples were taken. The first sampling was made 4 h after the morning meal. Three fish from each tank were anesthetized with 0.3 ml^{-1} ethylene glycol monophenyl ether and then euthanized by cord section and dissected on chilled trays. Livers were collected and divided into two parts to measure key metabolic enzymes activities of amino acid catabolism and glucose metabolism, antioxidant enzymes activities and to determine liver composition.

The activity of key antioxidant enzymes was also determined in anterior intestine. For that purpose, the digestive tract, from the three fish, was excised and free from surrounding adipose and connective tissues. The intestine was divided in three different portions: anterior, middle and distal. The distal part was distinguished from de mid intestine by the increase in intestinal diameter, darker mucosa and annular rings. The anterior and medium portions were obtained by division of the remaining intestine into two parts. The anterior intestine represents the portion, with the pyloric caeca, directly after the stomach.

All tissues collected during sampling procedures were snap frozen in liquid nitrogen and stored at -80ºC until analysis. A second sampling procedure was performed 24 h after the first sampling in order to assure the fish had no digesta in the gut. Three fish from each tank were pooled for whole-body composition, viscera and liver weight for determination of hepatosomatic (HSI) and visceral indices (VI).

2.4. Analytical methods and calculations

Chemical analysis of the experimental diets and whole fish body was done following Association of Official Analytical Chemists methods (AOAC, 2000). Dry matter was determined by drying in an oven at 105°C until constant weight; ash by incineration in a muffle furnace at 450°C for 16 h; protein content (N×6.25) according to the Kjeldahl method, using a Kjeltec digester and distillation units (Tecator Systems, Höganäs, Sweden; model 1015 and 1026, respectively); lipids by petroleum ether extraction in a SoxTec extraction system (Tecator Systems, Höganäs, Sweden; extraction unit model

1043 and servisse unit model 1046); and dietary energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR model 6200, PARR Instruments, Moline, IL, USA).

Experimental diets were analyzed for amino acids composition according to Peres and Oliva-Teles (2009). Samples were hydrolyzed for 23 h with 6 N hydrochloric acid at 110°C, under nitrogen atmosphere and derivatized with phenylisothiocyanate (PITC; Pierce) reagent before separation by high-pressure liquid chromatography (HPLC) in a PicoTag Amino Acid Analysis System (Waters, Bedford, MA, USA), (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487). External amino acids standards (Pierce NC10180), plus taurine standard (5 mM), were prepared along with the samples and norleucine was used as internal standard. Chromatographic peaks were analyzed with the Breeze software (Waters). Trp was measured by a spectrophotometric method as described by De Vries et al., 1980.

The main growth performance variables were calculated as follows: daily weight gain (WG) = {[(final weight – initial weight) x 1000/(final weight + initial weight)/2]}/days; feed conversion rate (FCR) = dry feed intake/ wet weight gain; daily growth index (DGI) = [(final weight$^{1/3}$ - initial weight$^{1/3}$)/days] x 100; protein efficiency ratio (PER) = wet weight gain/crude protein intake. Visceral index (VI) = (viscera weight/body weight) × 100; hepatosomatic index (HIS) = (liver weight/body weight) × 100. Nitrogen (N), Lipid (L) and Energy (E) retention = (((final body weight x final body N, L or E content) - (initial body weight x initial body N, L or E content))/(N, L or E intake)) x 100.

2.5. Liver composition

Hepatic crude lipid was determined gravimetrically by chloroform: methanol (2:1) extraction according to Folch et al. (1957). Hepatic glycogen was converted first to glucose as described by Roehring and Allred (1974), and glucose released from glycogen was measured using a commercial kit (Pointe Scientific, Inc., USA).

2.6. Enzymes activities

A frozen sample of liver was homogenized (dilution 1:7) in ice-cold buffer (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.25 mM saccharose, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 5 mM K_2HPO_4, 1mM dithiothreitol

(DTT), pH 7.4). After being centrifuged at 1,000 g at 4 °C for 10 min, supernatants were centrifuged again at 15,000 g at 4 °C for 20 min. Resultant supernatant was collected for enzyme activity measurements.

All enzyme assays were carried out at 37 °C and the changes in absorbance were monitored to determine the enzyme activity using a microplate reader (ELx808; Bio-Tek Instruments). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The enzymatic reactions were initiated by the addition of the tissue extract.

The specific assay conditions for each enzyme were as follows: glutamate dehydrogenase (GDH; EC1.4.1.2) activity was measured using 10 mM of L-glutamic acid, as described previously by Bergmeyer (1974). Alanine aminotransferase (ALT; EC 2.6.1.2) and aspartate aminotransferase (AST; EC 2.6.1.1) activities were assayed with kits from Spinreact (ALT/GPT, ref. 41,282; AST/GOT, ref. 41,272). Enzyme activities were measured at 340 nm.

Hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activities were determined according to Vijayan et al. (1990). Reaction mixture contained 71.4 mM imidazole–HCl buffer (pH 7.4), 50 mM ATP, 100 mM $MgCl_2$, 8 mM NADP, 2 units mL-1 G6PD and 10 mM (HK) or 1 M (HK-IV) glucose. Pyruvate kinase (PK; EC 2.7.1.40) activity was determined by reaction mixture contained 71.4 mM imidazole–HCl buffer (pH 7.4), $MgCl_2$ 100 mM, ClK 2 M, NADH 3mM, ADP 20mM, LDH and PEP 40mM as substrate. Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) activity was determined according to Morales et al. (1990). Reaction mixture contained 71.4 mM imidazole-HCl buffer (pH 7.4), 100 mM $MgCl_2$, 240 mM 2-mercaptoethanol, 10 mM NADP, 2 units mL-1 G6PD, 2 units mL-1 PGI and 0.5 mM fructose-1,6-bisphosphate.

Superoxide dismutase (SOD; EC 1.15.1.1) activity 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 50mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.012 mM cytochrome C, and 0.025 IU mL^{-1} xanthine oxidase. Activity is reported in units of SOD 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 (CAT; EC 1.11.1.6) activity 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.

Glutathione peroxidase (GPX; EC 1.11.1.9) activity was measured following the method of Flohé and Günzler (1984). The GSSG generated by GPX was reduced by GR, and NADPH oxidation was monitored at 340 nm. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.1), 1 mM EDTA, 3.9 mM GSH, 3.9 mM sodium azide, 1 IU mL^{-1} glutathione reductase, 0.2 mM NADPH and 0.05 mM H_2O_2.

Glutathione reductase (GR; EC 1.6.4.2) activity was assayed as described by Morales et al. (2004), measuring the oxidation of NADPH at 340 nm. Reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63 mM NADPH, and 0.16 mM GSSG.

Glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was measured as previously described by Morales et al. (2004), using a reaction mixture containing 50 mM imidazole-HCl buffer (pH 7.4), 5 mM MgCl2, 2 mM NADP and 1 mM glucose-6-phosphate.

Except for SOD, whose units of expression have been already described, enzyme activity is expressed as nmol per mg of soluble protein (specific activity). Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard. All enzymes activity measurements were performed with a microplate spectrophotometer reader (Multiskan™ GO; Thermo Scientific, Lisbon, Portugal). All the reagents used to perform enzymatic analysis were purchased from Sigma-Aldrich (Química, S.L., Sintra, Portugal).

Lipid peroxidation levels were determined based on the concentration of malondialdehyde 250 (MDA) following the methodology described by Buege and Aust (1978). An aliquot of supernatant 251 from the homogenate (100 µL) was mixed with 500 µL of a previously prepared solution containing 252 15% (w/v) TCA (Sigma), 0.375% (w/v) thiobarbituric acid (Sigma), 80% (v/v) HCl 0.25 N and 0·01% 253 (w/v) butylated hydroxytoluene (Sigma). The mixture was heated to 100 °C for 15 min. After being 254 cooled to room temperature and centrifuged at 1500g for 10 min, the absorbance was measured at 255 and 535 nm in the supernatant. MDA concentration was expressed as nmol MDA per g of wet tissue, 256 calculated from a calibration curve.

2.7. Statistical analysis

Growth performance, feed utilization, liver and whole body composition and hepatic and intestinal enzymatic activities data were analyzed in two-way analysis of variance

(ANOVA), with dietary taurine and methionine as fixed factors using the probability level of $P<0.05$ for rejection of the null hypothesis. All statistical analysis was performed using the SPSS 21.0 software package for Windows.

3. Results

No interaction effects were observed between dietary methionine and taurine supplementation on growth performance and feed utilization (Table 3). Methionine had no effect on meagre growth performance; however, taurine supplementation improved all growth parameters evaluated. Fish accepted all diets promptly, but dietary taurine supplementation promoted a decrease on daily feed intake while improving feed conversion and protein efficiency ratios. Nitrogen, lipid and energy retention were also higher in fish fed taurine supplemented diets.

Table 3
Growth performance and feed utilization of meagre fed the experimental diets

Treatment	FBW[1]	WG[2]	FI[3]	FCR[4]	DGI[5]	PER[6]	NR[7]	ER[7]
MET level (%)								
0.75	116.30	12.04	13.38	1.19	1.82	2.10	35.80	33.38
1.0	124.20	12.94	13.08	1.09	1.99	2.36	41.33	37.72
TAU level (%)								
0.1	105.85	10.97	14.81	1.37	1.60	1.70	30.05	29.54
1.2	134.65	14.01	11.65	0.91	2.20	2.76	47.08	41.56
SEM	5.478	0.573	0.664	0.085	0.113	0.217	3.580	2.474
Two-Way ANOVA[8]				*P – values*				
MET effect	0.287	0.307	0.730	0.385	0.248	0.450	0.119	0.267
TAU effect	0.003	0.012	0.006	0.003	0.002	0.012	0.015	0.011
MET x TAU	0.360	0.385	0.601	0.501	0.274	0.918	0.849	0.935

Values presented as means (n=3) and pooled standard error of the mean (SEM).
Met (Methionine); Tau (Taurine).
[1]Final body weight (FBW, g); [2]Weight gain (WG; g/kg/day) = {[(final weight – initial weight) x 1000/(final weight + initial weight)/2]}/days; [3]Feed intake (FI, (g/kg/day); [4]Feed conversion ratio (FCR) = dry feed intake/ wet weight gain; [5]Daily growth index (DGI)) = [(final weight$^{1/3}$ – initial weight$^{1/3}$)/days] x 100; [6]Protein efficiency ratio (PER) = wet weight gain/crude protein intake; [7]Nitrogen (NR) and Energy (ER) retention (% N or E intake) = (((final body weight x final body N or E content) - (initial body weight x initial body N or E content))/(N or E intake)) x 100.
[8]Two-way ANOVA non-significant for $P>0.05$.

Dietary supplementation of taurine and methionine did not affect hepatosomatic indices, liver and whole body composition of meagre (Table 4).

Table 4
Whole body and liver composition, hepatosomatic and visceral indexes of Meagre fed the experimental diets

Treatment	Whole Body					Index		Liver	
	DM[2]	CP[3]	CL[4]	EN[5]	Ash	HSI[6]	VI[7]	Lipid	GLY[8]
Met level (%)									
0.75	27.97	16.30	7.42	6.89	4.12	1.66	7.57	14.96	8.19
1.0	27.61	16.58	7.10	6.86	4.08	1.74	7.83	18.93	8.78
Tau level (%)									
0.1	28.00	16.53	7.34	6.92	4.18	1.68	7.31	16.08	8.34
1.2	27.58	16.35	7.18	6.83	4.06	1.72	8.09	17.81	8.63
SEM	0.162	0.134	0.232	0.066	0.056	0.517	0.091	1.727	0.633
Two-way ANOVA[9]	*P – values*								
Met effect	0.269	0.354	0.563	0.802	0.661	0.831	0.686	0.802	0.235
Tau effect	0.197	0.551	0.773	0.575	0.063	0.519	0.856	0.777	0.558
Met x Tau	0.271	0.907	0.646	0.282	0.130	0.737	0.536	0.533	0.218

Values presented as means (n=3 for whole body analysis; n=9 for indexes and liver composition) and pooled standard error of the mean (SEM);
Met (Methionine); Tau (Taurine);
[1]Dry Matter (DM: %); [2]Crude Protein (CP: %); [3]Crude Lipid (CL: %); [4]Energy (EN: kJ g^{-1}); [6]Hepatosomatic indexes (HIS: %); [7]Visceral indexes (VI: %); [8]Glycogen (GLY);
[9]Two-way ANOVA non-significant for P>0.05.

Activities of key hepatic enzymes of intermediary metabolism in fish fed the experimental diets are presented in Table 5. ALT and AST activities increased with dietary taurine supplementation, while GDH activity was not significantly affected by dietary treatment. Fructose-1,6-bisphosphatase activity was increased with dietary taurine supplementation but decreased with dietary methionine supplementation. Glycolytic enzymes (HK, GK, PK) activities were unaffected by dietary treatments.

Table 5
Liver glycolytic, gluconeogenic and amino acid catabolic enzyme activities (nmol mg protein^{-1}) of Meagre fed the experimental diets

Treatment	Enzymes						
	HK[1]	GK[2]	PK[3]	FBPase[4]	AST[5]	ALT[6]	GDH[7]
Met level (%)							
0.75	2.89	6.44	18.05	27.29	1093.64	308.15	25.81
1.0	2.84	6.40	23.59	22.08	967.29	323.59	24.76
Tau level (%)							
0.1	2.97	6.58	21.81	21.12	915.07	279.47	24.06
1.2	2.76	6.26	19.82	28.26	1145.86	352.27	26.52
SEM	0.127	0.259	2.074	1.266	51.188	5.876	0.700
Two-way ANOVA[8]	*P - values*						
Met effect	0.833	0.945	0.197	0.018	0.176	0.616	0.440
Tau effect	0.440	0.548	0.639	0.002	0.017	0.023	0.077
Met x Tau	0.408	0.185	0.724	0.245	0.062	0.830	0.167

Values presented as means (n=9) and pooled standard error of the mean (SEM).
Met (Methionine); Tau (Taurine);
[1]Hexokinase (HK); [2]Glucokinase (GK); [3]Pyruvate kinase (PK); [4]Fructose-1,6-bisphosphatase (FBPase); [5]Aspartate aminotransferase (AST); [5]Alanine aminotransferase (ALT); [7]Glutamate dehydrogenase (GDH); [8]Two-way ANOVA non-significant if P>0.05.

Antioxidant enzyme activities of intestine and liver the peroxidation content of lipid are presented in Table 6 and 7, respectively. No individual effect was observed by the supplemented amino acids on intestinal antioxidant enzyme activity (Table 6). However, interaction effect was observed on intestinal G6PDH activity being higher when supplemented with taurine at 10.0 g kg^{-1} of methionine.

Table 6
Intestine antioxidant enzymes activity levels (nmol mg protein^{-1}) and lipid peroxidation content of Meagre fed the experimental diets

Treatment	G6PDH[3]	GR[3]	GPX[3]	SOD[3,4]	CAT[3]	LPO[3]
MET level (%)[1]						
0.0	70.21	21.92	22.74	229.07	81.54	57.13
0.26	71.37	23.16	25.94	244.87	69.85	55.66
TAU level (%)[2]						
0.0	65.82	22.07	25.33	231.53	77.66	66.30
1.0	76.08	23.07	23.39	243.20	73.48	47.92
SEM[5]	4.708	0.939	1.290	14.415	4.343	3.736
Two-way ANOVA[6]			*P – values*			
MET effect	0.298	0.258	0.118	0.313	0.411	0.321
TAU effect	0.033	0.768	0.172	0.991	0.334	0.203
MET x TAU	0.023	0.222	0.506	0.227	0.964	0.492

Splitting MET x TAU interaction for G6PDH activity

	TAU[1] level (%)	MET[2] level (%)		SEM[5]
		0.0	0.26	
Intestine G6PDH[4]	0.0	76.69[a]	63.03[a]	4.708
	1.0	75.56[a]	94.03[b]	4.708

[1]Methionine (MET); [2]Taurine (TAU);
[3]Glucose-6-phosphate dehydrogenase (G6PDH); Glutathione peroxidase (GPX); Glutathione reductase (GR); Superoxide dismutase (SOD); Catalase (CAT); Lipid Peroxidation (LPO);
[4]SOD: U SOD mg protein^{-1};
[5]Values presented as means (n=9) and pooled standard error of the mean (SEM);
[6]Two-way ANOVA. If interaction was significant (P<0.05), one-way ANOVA was performed for each dietary methionine level considering significant differences if P<0.05.

On the other hand, as opposed to methionine, taurine supplementation significantly increased hepatic G6PDH and GPx activity (P<0.01), decreasing CAT enzyme activity (P<0.001). Interaction effect was observed on hepatic lipid peroxidation which decreased when taurine and methionine were supplemented together (Table 7).

Table 7
Liver antioxidant enzymes activity levels (nmol mg protein^{-1}), lipid peroxidation content and green liver occurrence of Meagre fed the experimental diets

Treatment	G6PDH[3]	GR[3]	GPX[3]	SOD[3,4]	CAT[3]	LPO[3]
MET level (%)[1]						
0.0	37.80	4.84	85.58	368.66	67.48	22.39
0.26	34.91	4.70	97.71	449.46	64.22	18.47
TAU level (%)[2]						
0.0	31.26	4.85	78.68	400.49	77.03	23.03
1.0	41.45	4.68	104.61	435.64	54.68	18.12
SEM[4]	1.707	0.143	4.536	24.031	3.041	1.906
Two-way ANOVA[4]			*P – values*			
MET effect	0.310	0.626	0.132	0.205	0.511	0.220
TAU effect	0.001	0.541	0.002	0.474	0.000	0.368
MET x TAU	0.123	0.081	0.233	0.678	0.425	0.035

Splitting MET x TAU interaction for peroxidation content

	TAU[1] level (%)	MET[2] level (%)		SEM[5]
		0.0	0.26	
Liver	0.0	28.61[a]	16.67[b]	1.906
LPO[3]	1.0	16.19[b]	20.06[a]	1.906

[1]Methionine (MET); [2]Taurine (TAU);
[3]Glucose-6-phosphate dehydrogenase (G6PDH); Glutathione peroxidase (GPX); Glutathione reductase (GR); Superoxide dismutase (SOD); Catalase (CAT); Lipid Peroxidation (LPO);
[4]SOD: U SOD mg protein^{-1};
[5]Values presented as means (n=9) and pooled standard error of the mean (SEM);
[6]Two-way ANOVA. If interaction was significant (P<0.05), one-way ANOVA was performed for each dietary methionine level considering significant differences if P<0.05.

4. Discussion

Fishmeal contains approximately 0.3–0.7% of taurine while plant proteins ingredients contain only trace amounts of it (Yamamoto et al., 1998). In addition, methionine is usually one of the main limiting amino acid in fish diets containing high levels of plant protein sources (Kaushik and Seiliez, 2010). Thus, in the development of plant protein-based diets for carnivorous fish, decreased growth performance is most likely due to sulfur amino acid deficiency (Tulli et al., 2010; Coutinho et al., 2017; Salze et al., 2017a). Both taurine and methionine supplementation to low fishmeal or plant-based diets were shown to enhance fish performance in some species (Salze and Davis, 2015; Koven et al., 2016; Li et al., 2016; Salze et al., 2017b; Martins et al., 2018), while not in others (Kim et al., 2008; Coutinho et al., 2017). Takagi et al. (2006) reported that yellowtail (*Seriola quinqueradiata*) fed non-fishmeal diets supplemented with methionine to levels equivalent to fishmeal were incapable of supporting optimal fish growth, and dietary taurine supplementation was required for health achievement. Gaylord et al. (2007) evaluating methionine and taurine supplementation on rainbow trout (*Oncorhynchus*

mykiss) fed all-plant protein diets observed that the weight gain was higher for fish fed diets supplemented with taurine without methionine. The authors concluded that the supplementation of an all-plant protein diets for trout with taurine is beneficial and that methionine supplementation does not spare this need.

Guroy et al. (2017) evaluated the effects of dietary protein levels for meagre fed plant protein based diets supplemented with different concentrations of methionine and lysine, raised at experimental conditions similar to the present study. The authors concluded that the optimum dietary protein for fish growth was of 44% crude protein in diets containing 37.1 g kg^{-1} and 12.2 g kg^{-1} of lysine and methionine, respectively. Although in the present study diets were formulated to contain only 42% of crude protein presenting lower concentrations of methionine of 7.5 g kg^{-1} and 9.8 g kg^{-1} and lysine at 23 g kg^{-1} all growth parameters were higher than those observed by the former authors. These results imply on the absence of published information on the nutritional requirements of meagre at present.

In the present study, the lowest values for growth performance were observed in fish fed the diet non-supplemented with taurine. Dietary methionine supplementation to that diet did not improve growth, which was only improved by taurine supplementation. This indicates that the basal diet contained already an adequate content of dietary methionine, which suggests that this amino acid was not a limiting factor in the present diets as opposed to taurine. Contrary to present results regarding dietary methionine supplementation, recent research revealed that the supplementation of lysine and methionine to high plant protein diets could enhance growth performance and digestive capacity of fish (Gan et al., 2012; Rolland et al., 2015). Takagi et al. (2001) also claimed that growth performance of juvenile red sea bream (*Pagrus major*) was significantly enhanced by soy protein concentrate-based diet supplemented with methionine or a mix of methionine and lysine.

By contrast, a great effect was observed by taurine supplementation indicating an increased growth performance effect of taurine on meagre fed plant protein-based diets. An increased weight gain in addition to a decreased feed conversion by meagre fed taurine supplemented diet indicates that the growth benefit could be due to an improvement in feed utilization. In a study similar to the present one but with another carnivorous marine species, the European sea bass (*Dicentrarchus labrax*), showed that fish coped well with a plant-based diet, not benefiting from a dietary methionine or taurine supplementation (Coutinho et al., 2017). On the contrary, Martins et al. (2018) also in European sea bass fed a plant-based diet estimated a

dietary taurine requirement of 5 g kg^{-1} of dry diet. These two last studies are conflicting and clearly suggest that deeper studies on taurine requirement in fish are required.

Other results on growth performance have been recorded by carnivorous fish fed plant protein-based diet when supplemented with taurine (Takagi et al., 2006; Gaylord et al., 2006, 2007; Jirsa et al., 2014; Salze et al., 2017b), suggesting that taurine may be conditionally indispensable for this group of fish fed plant protein based diets. Takagi et al. (2008) evaluating growth performance of yellowtail (*Seriola quinqueradiata*) fed all-plant protein diet, confirmed taurine as an essential nutrient for maintaining physiological conditions and normal growth. The authors also suggested that the hepatomegaly of fish observed was due to dietary taurine deficiency; however, no increased hepatosomatic index was observed in the present study or in that of Coutinho et al. (2017) and Martins et al. (2018).

Taurine may also interfere on lipid metabolism of fish (El-Sayed, 2014; Salze and Davis, 2015). However, in the present study no effect of taurine supplementation was observed on lipid body content and on hepatosomatic index. Similar results were observed by Coutinho et al. (2017) in European sea bass fed diets identical to those used in the present study, and in turbot (*Scophthalmus maximus* L.) fed high plant protein diet supplemented with 10.0 g kg^{-1} of taurine (Yun et al., 2012). Gaylord et al. (2006) reported that no effect was observed for whole body lipid in rainbow trout fed 5.0 to 15.0 g kg^{-1} of taurine supplemented diets. Different results were however observed by Li et al. (2016), which described a whole-body lipid and a hepatosomatic index decrease in yellow catfish (*Pelteobagrus fulvidraco*) fed with increasing dietary taurine levels. Thus, the effect of taurine on the mechanism involved in lipid storage of fish needs further elucidation.

In the present study, dietary methionine supplementation had no effect on the activity of key enzymes from amino acid catabolism (GDH, AST, and ALT) which could also suggest that the supplementation level of methionine was close to meagre requirement thereby not enough to activate these enzymes. As opposed to methionine, taurine dietary supplementation increased significantly AST and ALT activity while only slightly increased GDH activity. This result may imply that the nitrogen compounds produced by the available amino acid carbon skeletons transaminated are not excreted through ammonia production. In this case, the absent of GDH significantly increased activity, not following AST and ALT activity, may imply that meagre metabolism could be using this nitrogen compound at an alternative pathway like, for example, to the endogenous synthesis of other amino acids (Wu, 2013).

Therefore, the taurine growth promote effect observed may be also a result of its capacity to modulate the activity of GDH.

While the supplementation of methionine decreased FBPase activity, taurine supply significantly increased gluconeogenesis metabolism by increasing the FBPase activity. Taurine supplementation possibly increased FBPase activity for providing compounds from amino acid catabolism for gluconeogenesis through the increased AST and ALT activity. On the other hand, the decreased FBPase effect of methionine supplementation may suggest that the unsupplemented diet could be limiting in methionine and that the supplementation of 2.6 g kg^{-1} could be getting close to meagre requirement although this metabolic effect was not reflected on growth parameters.

Despite of the known antioxidant effect of methionine, in the present study no individual effect of this amino acid on any of the antioxidant enzymes activity evaluated was observed. These results suggest that the value of supplemented methionine, 2.6 g kg^{-1}, may not be sufficient to promote a significant difference once it did not reflect in any antioxidative status improvement on juvenile meagre.

In the present study, similar to the results observed by Bañuelos-Vargas et al. (2014) evaluating the effect of fishmeal replacement by soy protein concentrate with taurine supplementation on totoaba juveniles (*Totoaba macdonaldi*), an individual effect of taurine supplementation increased hepatic G6PDH. An increased on intestinal G6PDH enzyme activity by supplementation of taurine at 10.0 g kg^{-1} of methionine was also observed. Since G6PDH is the main supplier of the reducing power molecule NADPH, for both lipogenesis and antioxidant response (Scott et al., 1991; Morales et al., 2004; Perez-Jimenez et al., 2012), the G6PDH increased activity by taurine supplementation alone or by the interaction of both supplemented amino acid suggest a NADPH enhanced level that could improve the entire antioxidant system of both, liver and intestine.

It has been proved that high dietary plant protein diet increased superoxide anion production (Guerreiro et al., 2015), inducing oxidative stress in fish. In the present study, we have observed in the liver a downregulation of CAT by dietary taurine. This result associated with the increased of G6PDH activity agrees with the taurine role in the reduction of oxidants production in the first place (Jong et al., 2012; Salze and Davis, 2015), increasing NADPH production, thereby reducing the need for high antioxidant enzymes activities. It also reveals that the low dietary taurine levels increased the liver's susceptibility to oxidative damage, attesting for these amino acid involvements in

antioxidative processes (Métayer et al., 2008; Salze and Davis, 2015).

The hepatic GPX increased activity by fish fed taurine supplemented diets indicates that GPX was the major route for reducing H_2O_2 to molecular oxygen and water. This result may be related to the overall concentration of H_2O_2 generated, as it is known that CAT is more active when H_2O_2 production is high, whereas GPX is induced by low H_2O_2 levels (Halliwell and Gutteridge, 2007). Such low H_2O_2 levels are probably present in fish fed taurine supplemented diets, as in this group no significantly SOD activities were noticed. Taurine also affected hepatic LPO levels when supplemented with methionine, thus contributing to the improvement of the antioxidant defense strategy of meagre fed high plant protein diets.

It is worth pointing out that both methionine and taurine present an individual antioxidative effect. However, when supplemented together, an interaction between them is revealed affecting the antioxidative status of fish. Thus, a specific proportion of both amino acids should be determined to accomplish the best antioxidative status improvement effect. Hernández et al. (2017) evaluating the effects of taurine and methionine supplementation ratio to plant proteins based diets on antioxidant capacity of rainbow trout (*Oncorhynchus mykiss*) fingerlings, observed interaction between both amino acids concluding that to improved liver antioxidant activity a diet should be supplemented with a ratio of 3:1, taurine:methionine. Thus, studies to determine the best taurine:methionine ratio for meagre are required since both amino acids present proved interaction.

In conclusion, these results reveal that dietary supplementation of 10 g kg^{-1} of taurine, is an efficient nutritional strategy to mitigate growth limitations of meagre fed high levels of plant feed ingredients. They also reveal that the supplementation of both amino acid is an efficient nutritional strategy for the improvement of the antioxidative defense of juvenile meagre once it could mitigate the diet-induced stress response suggesting that they could also work as functional amino acids for meagre fed high plant protein diet. Increase of dietary methionine from 7.5 to 10 g kg^{-1} did not affect growth performance suggesting that dietary methionine needs of meagre are met with the lower dietary methionine tested.

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CHAPTER II

Effect of taurine and methionine supplementation on nutrient utilization of juvenile meagre (*Argyrosomus regius*) fed high plant protein diets

(According to Aquaculture Research Standards)

Abstract:
Methionine and taurine supplementation effect on nutrient digestibility, plasma metabolites, total bile acid content, and digestive enzyme activity were assessed in meagre (103 ± 21.2 g) juveniles fed high plant protein diets. Four isoenergetic and isoprotein diets were formulated to contain 82% of plant protein and 18% from fishmeal. A 2 x 2 factorial design was used with methionine at 7.5 and 10 g kg^{-1} supplemented or not with 10.0 g kg^{-1} of taurine. Dietary methionine supplementation increased dry matter, protein, lipids and energy digestibility while dietary taurine supplementation just increased lipids digestibility. Lipase activity was improved by dietary taurine supplementation, no other effects related to diet composition have been noticed in the other digestive enzymes activity. Dietary taurine supplementation also increased total bile acid content in the anterior intestine and plasma, and plasmatic levels of cholesterol, total proteins and triglycerides.

Keywords: carnivorous fish; diet digestibility; total bile acid, sustainable aquafeeds

1 INTRODUCTION

Fishmeal replacement by alternative feedstuffs is of upmost importance for a sustainable development of aquaculture (Gatlin et al., 2007; Hardy, 2010; Oliva-Teles et al., 2015). Plant feedstuffs are at present the most economic and environmentally sound alternatives. However, plant feedstuffs present a number of challenges associated with limited amino acids content, indigestible oligosaccharides, low phosphorus availability, antinutritional factors, and poor palatability (Francis et al., 2001; Gatlin et al., 2007).

Dietary supplementation with synthetic sulphur amino acids has proven to be a good strategy to restore plant protein amino acid profile and promote growth performance without environmental impacts (Gaylord et al., 2007; Li et al., 2009). Methionine is a sulphur amino acid that is one of the first limiting essential amino acids in plant feedstuffs, and taurine is a non-protein sulfur amino acid that lacks in most plant feedstuffs. Methionine is required for protein synthesis and serves as methyl donor for several methylation reactions, including DNA methylation, and as precursor of polyamines, L-carnitine, and cysteine (Espe et al., 2011; Wu, 2013). Taurine, a 2-aminoethane sulfonic acid, is an abundant free amino acid in fish and marine organism, including seaweed, shellfish, shrimp, crabs and squid (Yamamoto et al., 1998; Wu, 2013; Salze & Davis, 2015). Even though taurine is classified as non-essential amino acid, it has been considered as conditionally essential nutrient for several carnivorous fish species fed plant protein based diets (Takagi et al., 2008; Jirsa et al., 2014). Taurine body pool results from a balance between taurine and sulfur amino acid intake, taurine synthesis by the liver from methionine via cysteine, by a series of enzymatic reactions, and its reabsorption by the kidney (Han et al., 2006; Wu, 2013). The ability of fish to synthesize taurine from sulphur amino acids depends however of species and fish life stage (Yokoyama et al., 2001; Haga et al., 2015).

Previous studies have shown that taurine regulates several biological processes, has been used as a major attractant (Gaylord et al., 2006; Takagi et al., 2008) and as a growth promoter supplement in aquaculture (Ferreira et al., 2015). Taurine participates on lipid metabolism by modulating the digestion and absorption of dietary fats through its participation on bile acids synthesis, which are the major catabolic pathway for cholesterol (Wilde & Chu, 2011). Bile acids are constituents of bile and are potent digestive surfactants that promote lipid absorption and aid the adsorption of co-lipase and

lipase. They also help to solubilize lipolysis products into mixed micelles composed of bile salts and a range of other lipids, to facilitate transport to the gut mucosal surface prior to uptake and absorption (Wilde & Chu, 2011). The taurocholic acid and taurochenodeoxycholic are the major bile acids produced in the liver of almost all fish species, except cyprinids (Vessey et al., 1990; Goto et al., 1996; Yeh & Hwang, 2001).

Meagre *Argyrosomus regius* (Asso, 1801) is a carnivorous migratory fish species, with a special aquaculture potential in Spain, Egypt, France, Italy, Morocco and Turkey (Duncan et al., 2013). It presents relatively easy larval rearing (Vallés & Estévez, 2012) and does not present reproductive maturation during on-growing (Mananos et al., 2009). Favorable attributes for the market include large size, high processing yield, good taste and firm texture (Monfort, 2010). However, even though meagre is presently farmed in several countries in the Mediterranean basin, its production has not yet reached its full potential and nutritional are still very limited (Velazco-Vargas et al., 2013).

A few studies have been focused on the benefit of taurine supplementation on lipid metabolism, which could be one of the growth promoter effect of taurine on carnivorous fish fed plant protein-based diets. Therefore, the aim of the present study was to determine the effects of dietary methionine and taurine supplementation on meagre fed a practical plant protein-based diet on nutrient digestibility, total bile acid content, digestible enzymes, and serum metabolites. As for to evaluate the potential of dietary supplementation with both sulphur amino acid as a nutritional strategy to mitigate the negative effects of plant-based diets for juvenile meagre.

2 MATERIAL AND METHODS

2.1 Diets

Four isoprotein (420 g kg^{-1} crude protein) and isoenergetic (180 g kg^{-1} crude lipids) practical plant protein-based diets were formulated to contain 82% of protein from plant origin and 18% from fishmeal and with fish oil as only lipid source. A 2 x 2 factorial arrangement of treatments was use with methionine at 7.5 and 10 g kg^{-1} dry diet supplemented or not with taurine at 10 g kg^{-1} dry diet. All dietary ingredients were finely ground and well mixed. Mixtures were then dry pelleted without steam using a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through 3 mm die. After dried in an oven for 24h at 35°C, pellets were sieved and stored in a freezer until use.

The ingredients and proximate composition of the experimental diets are presented in Table 1, and the AA composition of the experimental diets is presented in Table 2.

TABLE 1 Ingredients and proximate composition (% dry matter) of the experimental diets

Ingredients	Diet			
	CT	TAU	MET	MET+TAU
Fishmeal[1]	8.0	8.0	8.0	8.0
CPSP[2]	2.0	2.0	2.0	2.0
Soybean meal[3]	22.6	23.2	22.4	22.4
Wheat gluten meal[4]	5.0	5.0	5.0	5.0
Corn gluten meal[5]	15.0	15.0	15.0	15.0
Pea protein[6]	4.0	2.9	4.0	3.2
Wheat meal[7]	20.3	19.8	20.3	20.0
Fish oil	14.8	14.8	14.8	14.8
Vitamin premix[8]	1.5	1.5	1.5	1.5
Choline chloride	0.5	0.5	0.5	0.5
Mineral premix[9]	1.0	1.0	1.0	1.0
Dibasic calcium phosphate	3.8	3.8	3.8	3.8
Agar	1.0	1.0	1.0	1.0
Binder[10]	1.0	1.0	1.0	1.0
DL-Metionine[11]	0.0	0.0	0.26	0.26
Taurine[11]	0.0	1.0	0.00	1.0
Proximate analysis (% dry matter)				
Dry matter	86.7	91.7	88.6	93.2
Crude Protein	43.0	41.6	43.0	41.8
Crude Lipid	17.7	17.7	17.6	18.0
Ash	7.7	8.1	7.7	7.9
Gross Energy (kJ kg^{-1})	22.5	22.7	22.4	22.7

[1]Crude protein: 71.0%; Crude Lipid: 11.3%; Sorgal, S.A. Ovar, Portugal;
[2]Soluble fish protein concentrate, Crude protein: 80.4%; Crude Lipid: 15.7%;
[3]Crude protein: 56.4%; Crude lipid: 2.5%; Sorgal, S.A. Ovar, Portugal;
[4]Crude protein: 84.8%; Crude Lipid: 1.7%; Sorgal, S.A. Ovar, Portugal;
[5]Crude protein: 80.1%; Crude Lipid: 4.1%; Sorgal, S.A. Ovar, Portugal;
[6]Crude protein: 84.0%; Crude Lipid: 2.7%; Cosucra, Belgium;
[7]Crude protein: 11.5%; Crude Lipid: 3.2%; Sorgal, S.A. Ovar, Portugal;
[8]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400;
[9]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet);
[10]Aquacube. Agil, UK;
[11]Feed-grade amino acids, Sorgal, S.A. Ovar, Portugal.

TABLE 2 Determined amino acid composition (dry matter %) of the experimental diets

	CT	TAU	MET	MET+TAU
Methionine	0.75	0.75	0.97	0.98
Arginine	2.87	2.75	2.83	2.79
Histidine	1.03	1.04	1.00	1.00
Isoleucine	1.84	1.72	1.76	1.76
Leucine	4.10	4.10	4.01	4.03
Lysine	2.35	2.30	2.34	2.29
Threonine	1.55	1.57	1.58	1.48
Valine	1.99	1.89	1.97	1.92
Phenylalanine	2.65	2.61	2.64	2.54
Taurine	0.13	1.24	0.13	1.24
Tyrosine	1.78	1.70	1.77	1.70
Alanine	2.79	2.84	2.82	2.81
Aspartic acid	3.91	3.77	3.74	3.73
Glutamic acid	6.35	6.00	6.26	6.11
Glycine	2.88	2.79	2.84	2.81
Serine	2.41	2.29	2.34	2.27
Proline	2.39	2.31	2.15	2.27
Cysteine	0.60	0.65	0.60	0.65
Tryptophan	0.44	0.46	0.44	0.45

2.2 Fish and experimental conditions

The experiment was conducted by certified scientists (category C FELASA) according to the recommendations of the European Union Directive 2010/63/EU on the protection of animals for scientific purpose.

The digestible trial was performed at the Marine Zoology Station, Porto University, Porto, Portugal, for 24 days, in a thermoregulated recirculating water system equipped with 12 circular 60 L fiberglass tanks, designed according to Cho et al. (1982), and with a feces settling column connected to the outlet of each tank.

Meagre (*Argyrosomus regius*) juveniles were obtained from the Portuguese Institute of the Sea and the Atmosphere-IPMA, Algarve, Portugal, and after transportation to the experimental facilities were submitted to a quarantine period. During this period, fish were fed a commercial diet two times a day. Then, 72 fish with mean initial weight of 103 ± 21.2 g were randomly distributed into the 12 rectangular 60 l-tanks. Diets were randomly assigned to triplicate tanks with 6 fish per replicate, and fish were fed to apparent satiation, three times a day (10.00 a.m.; 12.30 and 15.00 p.m.). The first 7 days of the experimental period were used for fish adaptation to the diets, and then feces were collected for 24 days. Before the morning meal, feces accumulated in each settling column were collected, centrifuged at 3,000 *g*, pooled for each tank, and stored at -20 °C

until analysis. Thirty minutes after the last daily meal, tanks, water pipes, and settling columns were thoroughly cleaned to remove excess feed and feces.

During the trial, water-flow was established at a rate of about 4.5 L min^{-1}, temperature was regulated to 22 ± 0.9 ºC, salinity was 37.5 ± 0.8 g L^{-1} and dissolved oxygen was kept near saturation (7.0 mg L^{-1}) by air diffusers in each tank. Fish were subject to a 12 h light/12 h dark photoperiod regime provided by artificial illumination.

2.3 Sampling

At the end of the digestible trial, to ensure that intestine was full at sampling time, fish were fed in a continuous manner. Before euthanized, all six fish from each tank had their blood collected from the caudal vein with a heparinized syringe. Blood was immediately centrifuged at 10,000 g for 10 min and plasma aliquots of 1 mL were frozen at −80 °C until analysis.

Then, all fish were immediately euthanized by cord section and dissected on chilled trays. The digestive tract of three fish from each tank was excised and freed from surrounding adipose and connective tissues. The intestine was divided in three different portions: anterior, middle and distal. The distal part was distinguished from de mid intestine by the increase in intestinal diameter, darker mucosa, and annular rings. The anterior and medium portions were obtained by division of the remaining intestine into two parts. The anterior intestine represents the portion, with the pyloric caeca, directly after the stomach. Clamps at the end of each intestine region were placed to prevent mixing of intestine contents. A pool of intestine contents was then collected from anterior and mid intestine, and immediately stored at -80ºC until bile acid content analysis.

Three other fish were used for the enzymatic activity measurement. The digestive tract was handled in the same way as described above. Then, the anterior intestine was stored at 80°C, until analyses.

2.4 Proximate analysis

Chemical analysis of the experimental diets and feces was done following Association of Official Analytical Chemists methods (AOAC, 2000). Briefly, dry matter was determined by drying in an oven at 105°C until constant weight; ash by incineration in a muffle furnace at 450°C for 16 h; protein content (N×6.25) according to the Kjeldahl method, using a Kjeltec digester and distillation units (Tecator Systems, Höganäs,

Sweden; model 1015 and 1026, respectively); lipids by petroleum ether extraction in a SoxTec extraction system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046). Dietary energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR model 6200, PARR Instruments, Moline, IL, USA). Chromium oxide content in diets and feces was quantified by acid digestion, according to Furukawa & Tsukahara (1966).

Experimental diets were analyzed for amino acids composition according to Peres & Oliva-Teles (2009). Briefly, samples were hydrolyzed for 23 h with 6 N hydrochloric acid at 110 °C, under nitrogen atmosphere and derivatized with phenylisothiocyanate (PITC; Pierce) reagent before separation by high-pressure liquid chromatography (HPLC) in a PicoTag Amino Acid Analysis System (Waters, Bedford, MA, USA), (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487). External standards were prepared along with the samples and norleucine was used as internal standard. Chromatographic peaks were analyzed with the Breeze software (Waters).

2.5 Apparent digestibility coefficients

The apparent digestibility coefficient (ADC) of lipids, dry matter, protein, and energy of the test diets were calculated according to the following equation:

$$ADC = 100 - \left[100.\left(\frac{\%I_r}{\%I_f}\right).\left(\frac{\%N_f}{\%N_r}\right)\right]$$

Where I_r = percentage of chromic oxide in the diet; I_f = percentage of chromic oxide in the feces; N_r = percentage of nutrients or energy in the diet; N_f = percentage of nutrients or energy in the feces (Cho et al., 1982).

2.6 Digestible enzymes activities

Anterior intestine samples were homogenized (dilution 1:5) in ice-cold buffer (100mMTris– HCl, 0.1 mM EDTA and 0.1% (v/v) TritonX-100, pH7.8). Homogenates were centrifuged at 30,000 g for 30 min at 4°C and the resultant supernatants were kept in aliquots and stored at −80 °C until use.

The specific assay conditions for each enzyme were as follows. Total protease activity was measured by the casein-hydrolysis method described by Hidalgo et al. (1999). The reaction mixture containing casein (1% w/v; 0.125 mL), buffer (0.1 M Tris–

HCl, pH 9.0; 0.125 mL) and homogenate supernatant (0.05mL) was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 0.3 mL trichloroacetic acid (TCA) (8% w/v) solution. After being kept for 1 h at 4 °C, samples were centrifuged at 1,800 g for 10 min and the supernatant absorbance measured at 280 nm. A control blank for each sample was prepared adding the supernatant from the homogenates after the incubation period. Tyrosine solution was used as standard.

α-Amylase (EC 3.2.1.1) activity was measured at 405 nm by the rate of 2-chloro-4-nitrophenol formation (molar extinction coefficient, 12.9 mM−1cm−1) at 37 °C using a commercial kit from Spinreact, Girona, Spain (ref. 41,201).

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) activity was determined using a Spinreact kit (ref. 1001274). The rate of methylresorufin (molar extinction coefficient, 60.65 $mM^{-1}cm^{-1}$) formation was quantified photometrically (580 nm; 37 °C) and it is proportional to the concentration of catalytic lipase present in the sample homogenate.

All enzymes activity measurements were performed with a microplate spectrophotometer reader (Multiskan™ GO; Thermo Scientific, Lisboa, Portugal). All reagents used to perform enzymatic analysis were purchased from Sigma-Aldrich (Química, S.L., Sintra, Portugal). Enzyme activities were expressed as specific activity. One unit (U) of activity was defined as μmol of product generated per minute of hepatic soluble protein (specific activity). Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

2.7 Total bile acids

Total bile acids content from anterior and middle intestine digesta, plasma, and gallbladder was determined using a Spinreact kit (ref. 1001030). In the presence of Thio-NAD, the enzyme 3-α hydroxysteroid dehydrogenase (3-α HSD) converts bile acids to 3-keto steroids and Thio- NADH. The reaction is reversible and 3-α HSD can convert 3-keto steroids and Thio- NADH to bile acids and Thio-NAD. In the presence of excess NADH, the enzyme cycling occurs efficiently and the rate of formation of Thio-NADH is determined by measuring specific change of absorbance at 405 nm.

2.8 Plasma metabolites

Plasma glucose, cholesterol, triacylglycerides, and total proteins were analyzed using enzymatic colorimetric kits from Spinreact, Girona, Spain (glucose kit, ref.

1001191; cholesterol kit, ref. 1001091; triacylglycerides kit, ref. 1001312, total lipids kit, ref. 1001270 and total proteins kit, ref. 1001291).

2.9 Statistical analysis

Differences among variables were evaluated by two-way analysis of variance (ANOVA) with dietary taurine and methionine as fixed factors and deemed significant at $P<0.05$. Fish were used as statistical units where significant interactions occurred between the main effects comparisons were performed within methionine level for taurine effects using Tukey's multiple range tests. All statistical analyses were performed using the SPSS 21.0 software package for Windows.

3 RESULTS

Dietary methionine supplementation increased all ADC parameters analyzed, while dietary taurine supplementation only improved the ADC of lipids (Table 3).

TABLE 3 Apparent digestibility coefficients (ADC; %) of the experimental diets

Treatment	Dry Matter	Protein	Lipids	Energy
MET[1] level (%)				
0.0	54.66	88.32	79.61	71.61
0.26	61.94	91.19	84.66	75.42
TAU[2] level (%)				
0.0	56.71	89.75	80.13	72.56
1.0	59.89	89.76	84.13	74.47
SEM[3]	1.245	0.469	1.020	0.772
Two-way ANOVA[4]		*P – values*		
MET effect	0.006	0.003	0.002	0.011
TAU effect	0.090	0.979	0.004	0.080
MET x TAU	0.594	0.735	0.171	0.269

[1]Methionine (MET); [2]Taurine (TAU).
[3]Values presented as means (n=3) and pooled standard error of the mean (SEM).
[4]Two-way ANOVA non-significant if $P>0.05$.

Total bile acid content in the plasma and intestine was unaffected by dietary methionine supplementation but increased in fish fed the dietary taurine supplemented diets, and this increase was statistically significant in the anterior intestine and plasma (Table 4).

TABLE 4 Total bile acid content (mg g^{-1}) of Meagre intestinal digesta and plasma (µmol L^{-1}) fed the experimental diets

Treatment	Anterior Intestine	Middle Intestine	Plasma
MET[1] level (%)			
0.0	19.97	44.35	67.23
0.26	20.77	43.66	69.11
TAU[2] level (%)			
0.0	13.51	29.79	60.51
1.0	27.75	55.58	74.96
SEM[3]	3.30	6.44	5.53
Two-way ANOVA[4]		*P - values*	
MET effect	0.837	0.842	0.733
TAU effect	0.038	0.324	0.025
MET x TAU	0.729	0.789	0.766

[1]Methionine (MET); [2]Taurine (TAU).
[3]Values presented as means (n=3) and pooled standard error of the mean (SEM).
[4]Two-way ANOVA non-significant if P>0.05.

Total protease and amylase activities were not affected by diet composition (Table 5). Lipase activity was affected by dietary taurine supplementation, but there was an interaction with dietary methionine level. Lipase activity was higher in fish fed taurine supplemented diet only in the high methionine diet.

TABLE 5 Digestive enzyme activity (U mg protein^{-1}) of Meagre fed the experimental diets

Treatment	Protease	Lipase	Amylase
MET[1] level (%)			
0.0	106.97	1.63	9.14
0.26	113.52	1.66	9.89
TAU[2] level (%)			
0.0	105.32	1.47	9.52
1.0	115.17	1.83	9.51
SEM[3]	3.000	0.130	0.420
Two-way ANOVA[4]		*P - values*	
MET effect	0.145	0.989	0.377
TAU effect	0.201	0.008	0.982
MET x TAU	0.804	0.040	0.057
Splitting MET x TAU interaction for lipase activity			
	TAU[2] level (%)	MET[1] level (%)	
		0.0	0.26
Lipase	0.0	1.59±0.31[a]	1.32±0.35[b]
	1.0	1.67±0.36[a]	1.94±0.33[a]

[1]Methionine (MET); [2]Taurine (TAU).
[3]Values presented as means (n=3) and pooled standard error of the mean (SEM).
[4]Two-way ANOVA non-significant if P>0.05.

Dietary methionine supplementation did not affect the plasma parameters measured (Table 6) while dietary taurine supplementation increased plasma cholesterol, total proteins, and triglycerides concentration.

TABLE 6 Plasma biochemistry values (mg dl^{-1}) of Meagre fed the experimental diets

Treatment	Cholesterol	Total proteins	Triglyceride
MET[1] level (%)			
0.0	0.18	0.19	0.36
0.26	0.18	0.19	0.34
TAU[2] level (%)			
0.0	0.16	0.18	0.26
1.0	0.20	0.19	0.44
SEM[3]	0.004	0.008	0.025
Two-way ANOVA[4]			
MET effect	0.589	0.577	0.641
TAU effect	0.000	0.022	0.000
MET x TAU	0.836	0.237	0.925

[1]Methionine (MET); [2]Taurine (TAU);
[3]Values presented as means (n=3) and pooled standard error of the mean (SEM);
[4]Two-way ANOVA non-significant for P>0.05;

4. DISCUSSION

In the development of a practical plant protein-based diet for carnivorous fish special attention should be given to sulfur amino acids deficiency, as replacing plant protein for fishmeal usually results in reduction of methionine and taurine intake (Chatzifotis et al., 2008; Jirsa et al., 2010; Oliva-Teles et al., 2015). Taurine is a non-protein bound amino acid and therefore, taurine requirement for growth is not associated with its incorporation in tissue proteins, as it is the case with other amino acids such as methionine, but rather to its metabolic/regulatory or other secondary functions (Chatzifotis et al., 2008; Salze & Davis, 2015).

Lipids usually constitute a considerable fraction of energy intake of marine carnivorous fish, since they use carbohydrates as energy source inefficiently compared to non-carnivorous fish (Stone, 2003; Enes et. al., 2009). The general digestive process of lipids involves their extracellular hydrolysis in the stomach, intestine, and ceca lumen by a variety of lipases and colipases (Wilde & Chu, 2011). In this context, bile acids are known to play a very important role in lipid digestion and absorption, besides its critical role on fish triglyceride lipase activity (Tocher, 2003). Bile acids are produced in the liver and are derived from cholesterol conjugated with taurine or glycine (e.g. taurocholic acid, glycodeoxycholate) to produce amphiphilic molecules, such that they will form micelles (Wilde & Chu, 2011). Taurocholic acid or taurochenodeoxycholic are the major bile acids of almost all teleost fish species, except cyprinids (Vessey et al., 1990; Goto et al., 1996; Yeh & Hwang, 2001).

In fish, dietary taurine supplementation has been reported to increase conjugated bile acid (Goto et al., 1996; Kim et al., 2008; Iwashita et al., 2009), and so the activity of bile salt activated lipase improving lipid digestibility and absorption (Chatzifotis et al., 2008; Kim et al., 2008). Lipase has been described as the main lipase on fish lipid digestion. It is secreted by pancreas and it is involved in the digestion and absorption of dietary cholesterol esters and neutral lipid esters (Wilde & Chu, 2011). It catalysis the breakdown of triacylglycerol into diacylglycerol and monoacylglycerol (Savona et al., 2011). In the present study, besides its effect on bile acid synthesis taurine was also shown to increase lipase activity. Such effect of taurine was also observed in other studies (Gjellesvik et al., 1989, 1992; Chatzifotis et al., 2008; Rueda-López et al., 2017). Interestingly, the increase of lipase activity was only observed in fish fed the high methionine diets, indicating a potential synergistic effect of methionine surplus. Overall,

this resulted in an improvement of the ADC of lipids due to both dietary methionine and taurine supplementation.

Due to the buildup of lipolysis, products at the interface during lipolysis can cause lipase inhibition; thus, as the main intestinal biosurfactants, bile acids are required to remove these products (Crandall & Lowe, 2001; Reis et al., 2009). This process could explain the increased effect on lipase activity by taurine supplementation observed on the present study. In addition, the possibility that other enzymes, as bile acid activated lipase, contributed to the observed activities cannot be excluded as bile acids are known to protect nonspecific or bile salt dependent lipase from proteolytic degradation (Vahouny et al., 1965; Albro & Latimer, 1974).

In the present study, plasma and intestine total bile acids were higher in fish fed diets supplemented with taurine. This suggests an overcoming effect of taurine on the biosynthesis and secretion of hepatic bile acids or an entero-hepatic circulation of bile acids, as described by Yamamoto et al. (2007) and Nguyen et al. (2015) in fish fed soybean meal rich diets supplemented with taurine. Digestive physiological disorders, such as insufficient bile acids levels in the gallbladder and intestine, low digestive enzyme activity in the intestine, and lower lipid digestion in fish fed soybean meal based diet was also reported by Nguyen et al. (2011a; 2011b). As in this study, dietary taurine supplementation has been reported to increase conjugated bile acid by other authors in different fish species (Matsunari et al., 2005; Gaylord et al., 2006; Takagi et al., 2006; Kim et al., 2008; Yun et al., 2012). Fish fed taurine supplemented diets also showed the highest triglycerides, cholesterol, and total protein content. On the contrary, Li et al. (2016) reported that such metabolites were highest in fish fed taurine deficient diets. Our results are however in line with the improvement of lipid digestion and absorption observed in fish fed the taurine supplemented diets.

In conclusion, our results indicate that the supplementation of methionine to a plant protein-based diet improved nutrient digestibility in juvenile meagres while dietary taurine supplementation further improved lipid digestibility. The effects of dietary taurine supplementation were associated to increase of total bile acid content. Present results suggest that taurine is an essential amino acid for juvenile meagres fed plant protein-based diets.

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CLOSING REMARKS

Once the benefits of the taurine and methionine supplementation to high plant protein diets have been stablish it is important to identify the requirement of both amino acid in the present experiment conditions in the first place.

As interections between both amino acid were observed, studies testing diferent levels of methionine and taurine in a factorial arrengment design are also important to be developed especially during long periods of time.

These studies could help to elucidate the optimum methionine:taurine ration for growth promotion, nutrient digestibility and antioxidant defense among others. In addition to the possibility of identifying most of the side effects caused by a long time sulpur amino acid diet deficiency.

The results from these assays could provide knowledge for the development of a diet no longer formulated with low-fishmeal but formulated with all plant ingredients contributing to the development of an environmental friendly diet, through correct use of sustainable plant resources. Consequently, stablishing a real sustainable aquaculture, both from an economic and ecological perspectives.