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Dietary taurine requirement of European sea bass (*Dicentrarchus labrax*, L.) juveniles

Nicole Martins Pires

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
Recursos Biológicos Aquáticos

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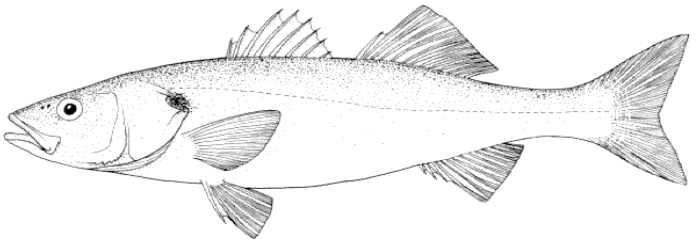
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Recursos Biológicos Aquáticos
Departamento de Biologia
2017

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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The following part I will write in Portuguese since it is to my family.

Obrigada à minha família, sem exceção, pelo apoio incondicional ao longo destes anos.

Aos meus pais, por me proporcionaram a oportunidade de seguir o meu sonho, estando sempre ao meu lado. Aprendi convosco a força de nunca desistir, mesmo quando tudo parece desmoronar. A luta e a persistência são, sem dúvida, a chave para o sucesso. Obrigada!

Abstract

European sea bass (*Dicentrarchus labrax*) is an important carnivorous fish species for Mediterranean aquaculture, whose production heavily relies on fishmeal-rich diets.

Fishmeal (FM) has been the preferential protein source in aquafeeds especially for carnivorous species; however, the increased demand, cost and limited availability of FM led to a reduction of its incorporation in the diets. As alternative to FM, plant feedstuffs have been increasingly used as protein sources in aquafeeds. Nevertheless, plant feedstuffs lack taurine (Tau), a ß-amino acid, which is present in high concentrations in animal feedstuffs and plays important physiological roles. Tau is abundant in FM, and it has been proved to be a conditionally essential amino acid for several marine fish species. Indeed, some fish species fed low-FM based diets, showed growth performance reduction and compromised health, in part associated to Tau deficiency.

In this scope, a 10-week growth trial with European sea bass (*Dicentrarchus labrax*, L.) juveniles (55g) was carried out to establish the optimal dietary Tau level in the diets, and to evaluate the effects of dietary Tau deficiency and excess, on growth performance, whole body composition, hepatosomatic and visceral indexes (Chapter 1); and on plasma biochemical parameters and activity of key enzymes of intermediary metabolism (Chapter 2). To achieve the proposed aims, eight isoproteic (45% crude protein) and isolipidic (18% crude lipid) diets were formulated containing a mixture of plant feedstuffs and fish meal (corresponding to 80% and 20% of total dietary protein, respectively) and increasing Tau levels ranging from 0.2 to 1.7% (diets: 0.2Tau; 0.3Tau; 0.4Tau; 0.5Tau; 0.7Tau; 0.9Tau; 1.2Tau; 1.7Tau).

Dietary Tau up to 0.7% improved growth performance while feed efficiency and protein efficiency ratio reached a maximum with a dietary Tau level of 1.2%. Dietary Tau requirement was estimated based on two models; five-parameter saturation kinetics model (5 SKM) and piecewise linear regression with breakpoint (PLR). Based on these models, the optimal dietary level of Tau for maximum growth, nitrogen retention and feed efficiency was estimated to be 0.6-0.7%.

At the end of the growth trial, protein content, ash, and hepatosomatic and visceral indexes were not affected by dietary Tau level, however total lipid content of fish fed the two higher Tau diets (1.2 and 1.7% Tau) were lower than in the other groups.

Although dietary Tau levels did not affect liver lipid content, hepatic total bile acids production increased with the increase of dietary Tau levels.

In fish fed Tau-deficient diets (0.2 and 0.5% Tau), post-prandial plasma glycaemia, total cholesterol, HDL, and LDL levels were higher than in fish fed diets with Tau level closely to the optimal (0.7% Tau). Excess of dietary Tau lead to slightly increase of glucose, total cholesterol, HDL, and LDL compared to in fish fed the 0.7% Tau diet. The lowest level of plasma total bile acids were observed in fish fed the 0.7% Tau diet, while total and indirect bilirubin increased with dietary Tau increase.

Liver malic and fatty acid synthase activities decreased with the increase of dietary Tau level up to 0.7%, whereas glucose-6-phosphate dehydrogenase activity was not affected by dietary Tau level. Activity of selected ß-oxidation, glycolysis, and gluconeogenesis key enzymes, were higher in fish fed Tau deficient diets (0.2 and 0.5% Tau) compared to the 0.7% Tau diet, and were also slightly increasing with the 1.2% Tau diet. The activities of key enzymes of amino acid catabolism measured decreased as dietary Tau level increased.

Overall, results of this study suggest a dietary Tau requirement for maximum growth and nitrogen accretion around 0.6-0.7%. Moreover, these results highlighted the physiological importance of Tau on cholesterol and bile acid metabolism, and its role on the activity of key enzymes involved in intermediary metabolism.

Keywords: Taurine requirement; Plant feedstuffs; European sea bass; Growth performance, Lipid metabolism, Intermediary metabolism.

Resumo

O Robalo (*Dicentrarchus labrax*) é uma espécie carnívora economicamente importante para a aquacultura Mediterrânica, cuja produção depende fortemente de dietas ricas em farinha de peixe.

A farinha de peixe (FP) tem sido a fonte de proteína preferencial em dietas para animais, especialmente para espécies carnívoras, no entanto, o aumento da procura, custo e disponibilidade limitada da FP, levou a uma redução da sua incorporação nas dietas. Em alternativa à FP, os ingredientes de origem vegetal têm sido cada vez mais utilizados como fontes proteicas nas dietas. Contudo, as matérias primas vegetais têm uma deficiência de taurina (Tau), um ß-amino acido que está presente em elevadas quantidades em ingredientes de origem animal e que possui importantes funções fisiológicas. A Tau é abundante na FP e provou ser um aminoácido condicionalmente essencial para várias espécies de peixes marinhos. De facto, algumas espécies alimentadas com dietas com baixo teor de FP, apresentaram uma redução no crescimento e alterações no sistema imunitário, em parte associados à deficiência de Tau.

Neste âmbito, um ensaio de crescimento de 10 semanas com juvenis de Robalo (*Dicentrarchus labrax*, L.) (55g) foi realizado para determinar o nível ótimo de Tau em dietas ricas em ingredientes de origem vegetal e avaliar os efeitos da deficiência e do excesso de Tau no crescimento, composição corporal, índices hepatossomático e visceral (Capítulo 1); e nos parâmetros bioquímicos do plasma e na atividade das enzimas chaves do metabolismo intermediário (Capítulo 2). Para atingir os objetivos propostos foram formuladas oito dietas isoproteicas (45% proteína) e isolipídicas (18% lípidos) contendo uma mistura de ingredientes de origem vegetal e FP como fontes de proteína (correspondendo a 80% e 20% da proteína total) e níveis crescentes de Tau, entre 0.2% até 1.7%; dietas: 0.2Tau; 0.3Tau; 0.4Tau; 0.5Tau; 0.7Tau; 0.9Tau; 1.2Tau; 1.7Tau.

O crescimento aumentou significativamente com o aumento do nível de Tau na dieta até 0.7%, enquanto que a eficiência alimentar e taxa de eficiência proteica atingiram o máximo com o nível 1.2% de Tau.

A necessidade de Tau foi estimada baseada em dois modelos, o modelo cinético saturação de cinco parâmetros (5 SKM) e regressão linear piecewise com ponto de quebra (PLR). De acordo com estes modelos, o nível ótimo de Tau para máximo índice de crescimento diário, retenção proteica e eficiência alimentar é entre 0.6-0.7%.

No final do ensaio, o conteúdo corporal em proteína, cinzas, e os índices hepatossomático e visceral não foram afetados pelo nível de Tau na dieta. No entanto, o conteúdo lipídico total dos peixes alimentados com as dietas com maior nível de Tau (1.2 e 1.7%Tau) foi menor do que nos outros grupos.

Embora, o conteúdo lipídico do fígado não tenha sido afetado pelo nível de Tau, a produção de ácidos biliares totais aumentou com o aumento de Tau na dieta.

Nos peixes alimentados com dietas deficientes em Tau (0.2 e 0.5% Tau), os níveis plasmáticos de glucose, colesterol total, HDL e LDL, foram mais elevados do que nos peixes alimentados com o nível de Tau próximo do estimado como ideal (0.7% Tau). O excesso de Tau provocou um ligeiro aumento da glucose e colesterol total, HDL e LDL comparando com os valores obtidos na dieta 0.7%Tau. Os níveis plasmáticos dos ácidos biliares totais foram mais baixos nos peixes alimentados com 0.7% Tau, enquanto que a bilirrubina total e indireta diminuíram com o aumento da Tau na dieta.

A atividade das enzimas málica e ácidos gordos sintetase, diminui com o aumento do nível de Tau na dieta até 0.7%, enquanto que a atividade da glucose-6-fosfato desidrogenase não foi afetada. A atividade das enzimas chave da ß-oxidação, glicólise e gliconeogénese foram maiores nos peixes alimentados com as dietas deficientes em Tau (0.2 e 0.5% Tau) comparando com o nível 0.7% Tau, e aumentaram ligeiramente no nível 1.2% Tau. As enzimas chave do catabolismo de amino ácidos diminuíram com o aumento do nível de Tau na dieta.

Em suma, os resultados sugerem um nível ótimo de Tau para máximo crescimento, retenção proteica e eficiência alimentar à volta dos 0.6-0.7%. Além disso, estes resultados enfatizam a importância fisiológica da Tau no metabolismo do colesterol e ácidos bilares, bem como a sua intervenção na atividade das enzimas chaves envolvidas no metabolismo intermediário.

Palavras-chave: Necessidade taurina; Ingredientes de origem vegetal; Robalo; Crescimento; Metabolismo lipídico, Metabolismo intermediário.

Index

Abstract 3
Resumo 5
Table list 8
Abbreviatures List 9

CHAPTER 1 - GENERAL INTRODUCTION 10
1.1. Fish consumption and aquaculture development 11
1.2. European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758) 14
1.2.1. Biology 14
1.2.2. Nutritional requirements 15
1.3. Alternative protein sources for European sea bass 19
1.3.1. Intermediary metabolism in fish 20
1.3.2. Taurine 23

CHAPTER 2 - OPTIMAL DIETARY TAURINE LEVEL FOR GROWTH AND NITROGEN ACCRETION OF EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*, L.) JUVENILES 29
Abstract 30
Introduction 31
Material e Methods 32
Results 36
Discussion 38
References 42

CHAPTER 3 - METABOLIC RESPONSE TO DIETARY TAURINE LEVELS IN EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*, L.) JUVENILES 49
Abstract 50
Introduction 51
Material e Methods 53
Results 58
Discussion 60
References 67

CHAPTER 4 – GENERAL CONCLUSIONS 78

REFERENCES 80

Figure list

Fig. 1- Relative contribution of aquaculture and captures to world fish supply for human consumption. (FAO, 2016).

Fig. 2 - European sea bass (Dicentrarchus labrax) (Source Fishbase, 2017).

Fig. 3 - Schematic representative of the major pathways involved in intermediary metabolism. The key enzymes of the amino acid catabolism, glycolytic, gluconeogenic, fatty acid synthesis and ß-oxidation are represented: alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), hexokinase (HK), glucokinase (GK), pyruvate kinase (PK), fructose-1,6-biphosphatase (FBPase), glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), 3-hydroxyacyl-CoA (HOAD). Glucose-6-phosphate (G6P), phosphoenolpyruvate (PEP), oxaloacetate (OAA) Adapted from Enes et al. (2009), Engelking (2010).

Fig. 4 - Chemical structure of taurine. Adapted from Huxtable (1992).

Fig. 5 - Main pathway of taurine biosynthesis in teleost species. The key enzymes are represented: Cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase (CSD). Adapted from: Engelking (2010); Salze and Davis (2015).

Table list

Table 1 – Essential, Non-essential and Conditionally essential amino acids for fish.

Table 2 – Amino acid requirements (g/16 g N) of European sea bass estimated by dose-response, ideal protein concept and, daily EAA increment.

Table 3 – Minimal dietary vitamin requirements recommended by NRC and established by dose-response studies for European sea bass.

Abbreviatures List

AA – Amino acids

ADC – Apparent digestibility coefficient

Acetyl-CoA – Acetyl-coenzyme A

ALT – Alanine aminotransferase

AST – Aspartate aminotransferase

ATP - Adenosine triphosphate

CDO – Cysteine dioxygenase

CSD – Cysteine sulphinate decarboxylase

DE – Digestible energy

DHA – Docosahexaenoic acid

DM – Dry matter

DP – Digestible protein

EAA – Essential amino acid

EPA – Ecosapentaenoic acid

FAS – Fatty acid synthetase

FBPase – Frutose-1,6-biphosphate

FM – Fishmeal

FP – Farinha de peixe

GDH – Glutamate dehydrogenase

GK – Glucokinase

G6PDH – Glucose-6-phosphate dehydrogenase

IBW – Initial body weight

HDL – High-density lipoprotein

HK – Hexokinase

HOAD – 3-hydroxyacyl-CoA

kg – kilogram

L – Litros

LC-PUFA – Long-chain polyunsaturated fatty acids

LDL – Low-density lipoprotein

ME – Malic enzyme

Mt – Million tonnes

N – Nitrogen

NADH – reduced form of nicotinamide adenine dinucleotide

NADPH - reduced form of nicotinamide adenine dinucleotide phosphate

NEAA – Non-essential amino acids

NRC – National research council

PEP – phosphoenolpyruvate

PF – Plant feedstuffs

PLR – Piecewise linear regression with breakpoint model

PK – Pyruvate kinase

Tau – Taurine

4-SKM – Four parameter saturation kinetic model

5-SKM – Five parameter saturation kinetic model

Chapter 1 - General Introduction

1.1. Fish consumption and aquaculture development

Fish is considered an important food commodity known for its unique flavor and nutritional characteristics. With a high biological value protein, is considered an excellent source of this macronutrient, accounting for circa 17% of total animal protein consumed worldwide (Béné et al., 2015); it is also an important source of lipids, especially long-chain omega-3 polyunsaturated fatty acids (LC-PUFAs), which are known to have beneficial effects in several human pathologies. Fish is also rich in essential micronutrients, such as vitamins (D, A, and B) and minerals (calcium, iodine, zinc, selenium, and iron) (Béné et al. 2015; Troell et al., 2015). Due its high nutritive characteristics and the benefits associated to fish consumption, it is observed an increasing demand for high quality fish products and consequently, in the last five decades world fish supply for human consumption outpaces growth rate of world population (FAO, 2016). Europe (EU-28) is the fifth major consumer of seafood products, with an estimate of 24.9 kg *per capita* per year. Consumption varies greatly across Europe, with Portugal as the major consumer, with 55.3 kg per person, and Hungary as the minor consumer, with only 4.6 Kg per person, in 2014 (Eumofa, 2016).

For a long time, fisheries were the primary source of fish for human consumption. However, the unsustainable exploitation of fishery resources and irresponsible fishing practices have led to the decline of wild fish stocks, and to consequent stabilization of global total captures. Thus, world fish supply for human consumption has been threatened (FAO, 2016).

Aquaculture emerged as a viable complement to fisheries, assuming an important role in fish supply for human consumption. Currently, nearly 50 percent of global fish consumed is provided by the aquaculture industry. Attending the continuous growth of human population, rapid urbanization and increasingly recognition of fish as an important food commodity, it is expected that this share of aquaculture within total fisheries will reach 57 percent by 2025 (Figure 1).

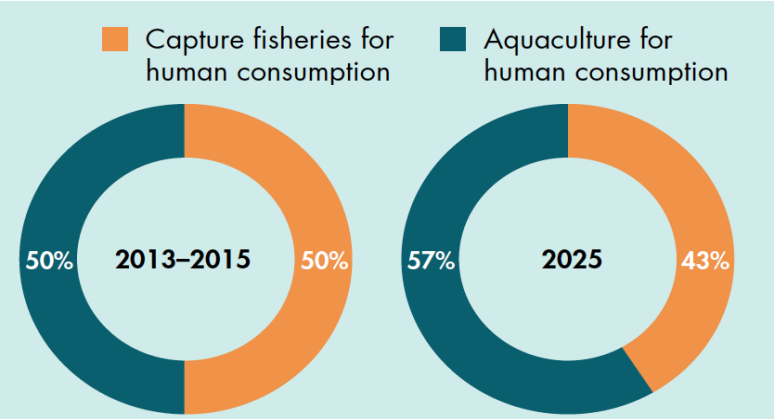


Fig.1 - Relative contribution of aquaculture and captures to world fish supply for human consumption. (FAO, 2016).

World aquaculture production has expressively increased in last 30-40's, growing at an annual rate of 10.8% in the 70's and the 80's, of 9.5% between 1990 and 2000, and of 6.5% between 2000 and 2012 (FAO, 2014). Despite this annual average decreased growth rate along the years, aquaculture production in terms of volume has increased, from 32 million tonnes (Mt) in 2000, up to 66.6 Mt in 2012, and up to 73.8 Mt in 2014 (FAO, 2014, 2016).

For the past two decades, Asia dominates aquaculture production, accounting with 89 percent for total fish production (Bostock, 2010; FAO, 2016). Africa and America have improved their shares, from 1.23 and 4.39 percent in 2000, up to 2.32 and 4.54 percent in 2014, respectively. On the other hand, Europe and Oceania decreased its share from 6.33 and 0.39% in 2000, to 3.97 and 0.26% in 2014, respectively (FAO, 2016). In fact, European aquaculture (excluding non-EU members) remained relatively stable since 1990's, amounting to 1.28 Mt and EUR 4 billion in 2014 (Eumofa, 2016). In global terms, European aquaculture occupied a share of 1.25% in terms of volume, which corresponded to 3.4% in terms of value, in 2013 (EU, 2016).

The rise of aquaculture production involved a gradual adaptation of farming methods, with a gradual replacement of traditional, low-cost methods to more modern production systems, with higher costs. Nowadays, almost 600 species are produced in aquaculture under several systems and conditions (FAO, 2016). Most of these species production depend on nutrient input mainly through aquafeeds. Aquafeeds for carnivorous species, namely for marine fish species, have been dominated by two ingredients, fish meal and fish oil. Fish oil is the primary lipid source being rich in essential fatty acids, and fishmeal (FM) is considered the most adequate protein source for fish, especially for carnivorous species. Nowadays, marine fish aquafeeds contain about 20-65% of

FM and salmonid aquafeeds contain circa 25-40% FM (Tacon et al., 2011). Indeed, FM is a high quality and highly digestible ingredient, with high protein content, with properly amino acid profile, highly digestible nutrients and high palatability, and is a source of vitamins and minerals, and lack antinutrient factors (Oliva-Teles et al., 2015). In 2010, it was estimated that 73% of total FM produced worldwide was used in the aquaculture industry (Shepherd and Jackson, 2013). However, in recent years FM production has decreased, due to overexploitation of marine resources, leading to an increase of FM price and a reduction of market availability; consequently, there has been a decreased incorporation of FM in aquafeeds (Tacon et al., 2011).

Aquaculture faces the challenge of finding alternative ingredients to FM, thus reducing the dependence on FM and fish oil, while maintaining aquafeeds nutritional quality, ensuring the maximum growth potential and well-being of the animals (Hansen, et al., 2007). Nowadays, industry and researchers are exploring possible alternatives to FM, such as terrestrial animal by-products, plant-feedstuffs, single-cell proteins, microbial protein, etc (Tacon et al., 2011). (PF) are for several reasons including the cost of production and food security issues, the preferential alternative protein sources used in aquafeeds (Gatlin III et al., 2007; Olsen and Hasan, 2012). In fact, PF have been successfully used in diets for several marine fish species (Oliva-Teles et al., 2015).

European sea bass (*Dicentrarchus labrax*) is the main marine fish species produced in European aquaculture, particularly in the Mediterranean region (Oliva-Teles, 2000). It was one of the first non-salmonid marine species to be successfully cultivated and commercialized in Europe (Kousolaki et al. 2015). In recent years, sea bass production has increased, reaching 158.4 Mt in 2015, with Turkey, Greece, Spain, and Italy as the major producers (FEAP, 2016).

1.2. European sea bass (*Dicentrarchus labrax,* Linnaeus, 1758)

1.2.1. Biology

European sea bass is a marine teleost belonging to the Moronidae family (Figure 3). It has an elongated body with 8 to 10 dorsal spines, 12 to 13 dorsal soft rays, 3 anal spines, 10 to 12 anal soft rays. The posterior edge of the operculum is finely serrated, and the lower part possesses strong denticles directed forward. It has 2 flat opercula spines and the mouth is moderately protractile. Vomerine teeth are present anteriorly in a crescent band (Fishbase).

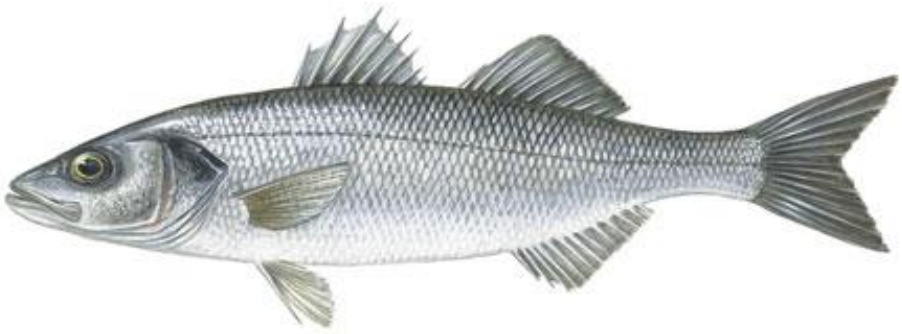


Fig.2 - European sea bass *(Dicentrarchus labrax)* (Source Fishbase, 2017*)*.

European sea bass has a wide geographic distribution, from Norway to Morocco, Canary Islands and Senegal, and the Mediterranean and Black Sea (FAO, 2017). It is a benthopelagic (demersal behavior) species, inhabiting shallow waters as well as several kinds of bottoms of coastal areas up to 100-150 meters depth. Being eurythermic (5-28˚C) and euryhaline (3‰ to full strength sea water) species, it can be found in coastal inshore waters, estuaries, brackish water lagoons and, sporadically, in rivers.

European sea bass is a gonochoric species. In the Mediterranean populations, sexual maturity occurs at an age of 2 to 4 years, while in the Atlantic populations it occurs later (males between 4-7 years of age; females between 5-8 years of age). Breeding occurs only once per year, mostly in winter (December to March) for the Mediterranean population, and up to summer (June) for the Atlantic population (Kousolaki et al. 2015). Belonging to the trophic level 3.8 (according to Fishbase), it is a strict carnivorous species and predatory feeds on small fish, prawns, crabs, and cuttlefish.

1.2.2. Nutritional requirements

Proteins are considered the most important component of fish diets, with carnivorous fish species having high protein requirements (Wilson, 2002). The optimal dietary protein level for sea bass juveniles was estimated to be around 48-50% and it was not affected by water temperature (Hidalgo and Alliot, 1988; Peres and Oliva-Teles, 1999a). Similar results were obtained by Ballestrazi et al. (1994), which observed a higher growth rate with diets including 49 or 54% protein compared to 44% protein. Nevertheless, optimum protein level is still a matter of controversy. Very early studies suggested a dietary protein optimum level for sea bass juveniles between 52-60% (Metailler et al., 1981), while later studies revealed that dietary protein levels could be decreased to 48-54% (Hidalgo and Alliot, 1988; Peres and Oliva-Teles, 1999a) or even lower, around 43% (Dias et al., 1998), whithout compromising growth performance. These discrepant results may be explained, at least in part, by differences in fish size and feedstuff composition of the experimental diets.

Besides, protein requirement is dependent on dietary energy density, hence a balance between digestible protein and digestible energy is important to optimal growth and nutrient utilization. In sea bass juveniles, some studies were carried out to evaluate the best digestible protein (DP) to digestible energy (DE) ratio, demonstrating a best DP/DE ratio for optimal growth performance and nutrient utilization around 21-22 mg kJ^{-1} (Dias et al., 1998a; Lanari et al., 1999; Peres and Oliva-Teles 1999). Neverthless, a higher DP/DE ratio, about 24-27 mg kJ^{-1}, was also reported (Garcia-Alcázar et al., 1994; Pérez et al., 1997).

Fish do not have specific protein requirement, instead, fish requires amino acids, that are the protein components, and play important physiological roles, ensuring growth performance and welfare. Amino acids (AA) requirement depends on capacity of biosynthesis, thereby if an AA is not biosynthesized or it is biosynthesized insufficiently by the organism, it is classified as essential amino acid (EAA), while an AA that is efficiently biosynthetised by the organism is considered a non-essential amino acids (NEAA) (Table 1). Besides, some AA are considered conditionally essential AA, if under certain conditions their metabolic demand can arise above their biosynthesis capacity (Table 1).

Table 1. Essential, Nonessential and Conditionally essential amino acids for fish.

Essential AA	Nonessential AA	Conditionally essential AA
Arginine	Alanine	Cysteine
Histidine	Asparagine	Glutamine
Isoleucine	Aspartate	Hydroxyproline
Leucine	Glutamate	Proline
Lysine	Glycine	Taurine
Methionine	Serine	Tyrosine
Phenylalanine		
Threonine		
Tryptophan		
Valine		

Adapted from Li et al. (2009).

European sea bass requires the same 10 EAA, as other fish species (Oliva-Teles, 2000). So far, the quantitative requirements of EAA based on dose-response method estimated for sea bass includes, arginine (Tibaldi et al., 1994), lysine (Tibaldi and Lanari, 1991), methionine and cysteine (Thebault et al., 1985; Tulli et al., 2010), tryptophan (Tibaldi et al., 1993), threonine (Tibaldi and Tulli, 1999) (Table 2). Moreover, the EAA requirements of sea bass were also estimated based on the ideal protein concept (Tibaldi et al. 1996; Kaushik, 1998), and daily EAA increment (Tibaldi et al. 1996) method (Table 2).

Table 2. Amino acid requirements (g/16 g N) of European sea bass estimated by dose-response, ideal protein concept and daily EAA increment. .

	Methods		
Amino acids	**Dose-response**	**Ideal protein concept**	**Daily EAA increment**
Arginine	3.9	3.8	3.7
Lysine	4.8	4.8	4.7
Histidine	-	1.6	1.3
Isoleucine	-	2.6	2.5
Leucine	-	4.3	4.1
Methionine + Cystine	4	2.2	2.2
Phenylalanine+ Tyrosine	-	4.3	4.1
Threonine	2.3	2.6	2.4
Tryptophan	0.5	0.5	0.5
Valine	-	2.9	2.8

Thebault et al. (1985); Tibaldi and Lanari (1991); Tibaldi et al. (1993, 1994, 1996); Kaushik (1998); Tibaldi and Tulli (1999); Tulli et al. (2010).

Lipids are also an important component of fish diets. For sea bass juveniles, some studies indicated that optimal dietary lipid level must be around 12% without beneficial effects on growth being observed above this level (Alliot et al., 1974; Peres and Oliva-Teles, 1999a). In contrast, Dias et al. (1998) and Lanari et al. (1999), revealed that increasing dietary lipid content up to 18-19% improved growth performance.

Similar to proteins, fish do not require lipids but fatty acids. As many carnivorous marine fish, sea bass lacks the capacity of synthetize long chain polyunsaturated fatty acids (LC-PUFAs), namely ecosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); therefore, these fatty acids must be supplemented in the diet (NRC, 2011). Available data regarding essential fatty acids requirements of sea bass are scarce; however, the reported quantitative essential fatty acid requirements of juvenile sea bass are estimated to be around 0.7-1% of the diet (Coutteau et al., 1996; Skalli and Robin, 2004).

Like other vertebrates, fish do not have specific carbohydrate requirements, even so incorporation of carbohydrates in the diet is recommended, as it constitutes an inexpensive source of energy and its supply spares the use of proteins and lipids for energy purposes, and improves protein utilization. Nevertheless, European sea bass (E. sea bass) does not seem to use carbohydrates efficiently, as it has limited capability to digest and metabolize carbohydrates

(Oliva-Teles, 2000). Data available about carbohydrates utilization by sea bass appear to be contradictory. For instance, it was reported that sea bass did not digest efficiently raw starch, its digestibility decreasing with the increase of dietary starch level (Moreira et al., 2008). However, dietary incorporation of gelatinized starch improved growth performance and feed utilization (Dias et al., 1998). In fact, several studies indicate that E. sea bass digest efficiently pregelatinized starch, with an apparent digestibility coefficient (ADC) above 90% (Dias et al., 1998; Peres and Oliva-Teles, 2002; Moreira et al., 2008; Alton and Yildirim, 2010). On the other hand, Enes et al. (2006) reported high ADC of both raw and waxy starch, regardless of incorporation level.

Moreover, Lanari et al. (1999) recommended that dietary incorporation of digestible carbohydrates such as dextrin or gelatinized starch should not exceed 20% of diet. In contrast, Gouveia et al. (1995) showed that either raw or gelatinized starch could be included in the diets at 25% without significant effecting growth performance. As reviewed by Enes et al. (2011), incorporation of digestible carbohydrates in diets for sea bass is suggested to be limited to 20%, as high levels of starch (>30%) appear to reduce growth and feed utilization of sea bass.

Despite the reported ability of sea bass to digest dietary carbohydrates, their protein sparing effect is not unequivocally confirmed. Hidalgo and Alliot (1988), reported a protein-sparing effect of pregelatinized starch incorporated in diets at 15% and similar results were obtained for this species by Gouveia et al., (1995) and Enes et al., (2006). Contrary, no protein sparing effect by dietary carbohydrates was observed by Peres and Oliva-Teles (2002) and Alton and Yildirim (2010).

Up now, available data on vitamins requirements of E. sea bass are scarce. NRC (1993; 2011) present the minimal amount requirement levels to support an adequate growth performance of salmonids and those values were used as a reference for different fish species. Moreover, it was confirmed that the NRC recommendations for dietary vitamins requirements were adequate in practical diets for E. sea bass, but not in semi-purified diets (Woodward, 1994; Kaushik et al. 1998b). To avoid surplus of vitamins, several studies were performed to establish the minimal vitamin dietary level for sea bass (Table 3). Thereby, NRC (2011) estimated dietary allowances of vitamin A and C for E. sea bass (Table 3).

Vitamin A is known to negatively influence morphogenesis of E. sea bass when present at high levels in the diets (Villeneuve et al., 2005). Mazurais et al. (2009) evaluated the effects of excess or absence of vitamin A in ossification process in sea bass larvae, reporting an optimal level of 5-31, (mg kg/ diet) (Table 3). Vitamin D is a fat-soluble pro-hormone that is crucial for maintaining

calcium and phosphate homeostasis and protecting skeletal integrity. Darias, et al. (2010), evaluated the effect of vitamin D_3 in ontogenesis systems and ossification of sea bass reporting an optimal dietary level of 27600 (min IU/kg) (Table 3). Ascorbic acid is also an important vitamin in skeletogenesis, and its deficiency is often associated with the appearance of skeletal abnormalities. Darias, et al. (2011) reported an increase in survival and growth of sea bass larvae with the increase of dietary ascorbic acid up to 15 mg ascorbic acid /kg, without statistical differences in growth above that level (Table 3).

Regarding minerals requirements, data is only available for phosphorous, with estimated dietary requirements of 0.65% for sea bass (Oliva-Teles and Pimental-Rodrigues, 2004).

Table 3. Minimal dietary vitamin requirements recommended by NRC and established by dose-response studies for European sea bass.

	European sea bass	
	NRC (2011)	*Dose-response*
Vitamin A	31 mg/kg	5-31 mg/kg
Vitamin D_3	-	27600 IU/kg
Ascorbic acid	20 mg/kg	15 mg/kg

Mazurais et al. (2009) ; Darias et al. (2010, 2011); NRC (1993, 2011).

1.3. Alternative protein sources for European sea bass

Sustainable aquaculture production of E. sea bass depends of reducing dietary FM incorporation. Many studies showed that this species can cope with a high PF incorporation (>60%), in the diets (Ballestrazzi et al., 1994; Bonaldo et al., 2008; Messina et al., 2005; Tibaldi et al., 1999, 2005), or even an almost total (95%) (Kaushik et al., 2004), and total (100%) (Torrecillas et al., 2017), FM replacement, without compromising growth performance and nutrient utilization.

However, high replacement levels of FM in the diets still usually leads to poor growth performance, lower feed efficiency and reduced feed intake. Indeed, several studies performed in E. sea bass described growth depression, lower feed efficiency and feed intake of this species fed PF-rich diets (Dias et al., 1997, 2003; Lanari and D'Agaro, 2005; Tulli et al., 2007; Geay et al., 2011; Le Boucher et al., 2011).

This may be related with PF nutritional drawbacks. Comparatively to FM, PF have an unbalanced amino acid profile, being limiting in one or more EAA, with lysine and methionine usually being the first limiting EAA (Gatlin III et al., 2007; Hardy, 2010).

It was already seen that PF may lead to physiological alterations in fish, due to lack of certain amino acids that play important roles in several metabolic processes, such as gluconeogenesis and lipogenesis (Cowey, 1980), or overall in lipid metabolism and fat deposition (De Francesco et al., 2007). In sea bass, few data are available regarding these aspects, although it has been reported that FM replacement by PF lead to adjustment in gluconeogenesis, and lipid metabolism (Dias et al., 2005; Messina et al., 2013). Thus, dietary composition may affect the main metabolic pathways, in the organisms.

1.3.1. Intermediary metabolism

Amino acids catabolism begins with removal of the α-amino group of amino acids, involving keto acid acceptors such as pyruvate (forming alanine), oxaloacetate (forming aspartate) or to α-ketoglutarate (forming glutamate), leaving a α-keto acid hydrocarbon skeleton, which is ready to be used for energy purposes or for carbohydrate synthesis (glucogenic amino acids) and lipid (ketogenic amino acids) synthesis (Figure 3) (Engelking, 2010). Glutamate dehydrogenase (GDH, EC 1.4.1.2) is one of the key enzymes involved in amino acid catabolism, and catalyses the deamination of glutamate resulting in α-Keto acid and in the direct release of ammonium ion (NH_4^+) (Figure 3). Alanine aminotransferase (ALT, EC 2.6.1.2) and Aspartate aminotransferase (AST, EC 2.6.1.1) are also considered key enzymes of amino acid catabolism. ALT catalyses transfer of amino group of alanine to α-keto acid, thus forming glutamate and pyruvate; AST catalyses the aspartate amino group transfer to α-keto acid, thus producing glutamate and oxaloacetate (Figure 3).

Glycolysis is the only route by which glucose is metabolized to pyruvate. All enzymes involved in glycolytic pathway were reported to exist in fish (Enes et al., 2009). The regulation of glycolytic pathway is dependent on the activities of key enzymes hexokinases (HK-I, HK-II, HK-III and HK-IV), 6-phosphofructokinase-1 (PFK1, EC 2.7.1.11) and pyruvate kinase (PK) (Figure 3). Hexokinases (HK, EC 2.7.1.1) catalyse glycolysis first reaction, which consists on the phosphorylation of glucose to glucose-6-phosphate (G6P), a molecule that may be used in other metabolic pathways, such as glycogenesis and the pentose phosphate pathway (Figure 3) (Enes et al., 2009; Engelking, 2010). HK is characterized by high glucose affinity and it is inhibited by

G6P, but not by insulin (Engelking, 2010). Contrarily to the other HK, HK-IV or glucokinase (GK, EC 2.7.1.2), is characterized by low glucose affinity and lack of inhibition by G6P, yet GK is stimulated by insulin. Moreover, it has been described that GK is under nutritional regulation and many studies indicated that dietary carbohydrates promote changes in both GK activity and gene expression (Enes et al., 2006a, 2008a; Moreira et al., 2008), while HK are not under nutritional regulation (Panserat et al., 2000b; Enes et al., 2006a, b,2008 a, b; Moreira et al., 2008). Furthermore, there are evidences that in some fish species, including sea bass, GK is the main regulatory enzyme involved in glycolysis (Panserat et al., 2000; Enes et al., 2006a, 2008a, b).

PK (EC 2.7.1.40) is another key glycolytic enzyme that catalyses the last step in glycolysis, the conversion of phosphoenolpyruvate (PEP) to pyruvate. Then pyruvate is subsequently converted into acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex. Acetyl-CoA can then enter the mitochondrial citric acid cycle (also called the Krebs or tricarboxylic acid – TCA – cycle) for complete oxidation to CO_2 and water, with liberation of free energy as ATP (Engelking, 2010) (Figure 3).

Gluconeogenesis consists in de *novo* synthesis of glucose from non-glucose substrates, such as lactate, glycerol and α-keto acids and ensure the glucose requirements for metabolic purposes (Enes et al., 2009). Gluconeogenesis is regulated by the activity of key enzymes; the phosphoenolpyruvate carboxylase (PEPCK, EC 4.1.1.32) catalyse the conversion of oxaloacetate to phosphoenolpyruvate; fructose- 1,6 – bisphosphate (FBPase, EC 3.1.3.11) (Figure 3), catalyse the hydrolysis of fructose-1,6-biphosphate to fructose 6-phosphate, and glucose-6-phosphatase (G6Pase, EC 3.1.3.9) catalyse the dephosphorylation of glucose-6-phosphate to glucose (Engelking, 2010).

The pentose phosphate pathway constitutes an alternative catabolic via for glucose that leads to the production of reducing equivalents under the form of NADPH, which is required for lipid biosynthesis, and of ribose-5-phosphate, which is required for nucleotides formation. One of the key enzymes involved in this pathway is glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) (Figure 3) (Enes et al., 2009).

Catabolism of lipids, also known as ß-oxidation, involves a sequential cleavage of two-carbon units of fatty acids, released as acetyl-CoA, through a cycle series of reactions catalysed by several distinct enzyme activities. Activated fatty acids are transported into the mitochondrion in the form of fatty acylcarnitine esters formed through the action of carnitine palmitolytransferase- 1

and 2 (CPT1 and CPT2, EC 2.3.1.21) and then converted back into fatty acyl-CoA derivatives and inserted in the ß-oxidation spiral. The ß-oxidation spiral is characterized by four reactions catalysed by oxidases, the acyl-CoA dehydrogenase (ACAD, EC 1.3.1.8), the enoyl-CoA hydratase (ECHS1, EC 4.2.1.74), 3-hydroxyacyl-CoA (HOAD, EC 1.1.1.35), and 3-ketoacyl-CoA thiolase (ACAA, EC 2.3.1.16). 3-hydroxyacyl-CoA (HOAD) catalyse the third step of ß-oxidation spiral, which consists in the oxidation of L- 3-hydroxyacyl-CoA by NAD^+, converting the hydroxyl group into a keto group (Figure 3) and that is used for accessing rate of ß-oxidation catabolism (Schulz, 1991).

The biosynthesis of new endogenous lipid up to palmitate (C_{16}) is lipogenesis and takes place in cytoplasm (Tocher, 2003). Acetyl-CoA derived mostly from glucose, amino acids and, citrate is the primary substrate donor in lipogenesis, however Acetyl-CoA must first be converted into malonyl-CoA, obtained by carboxylation of the acetyl-CoA by the acetyl-CoA carboxylase (ACC EC 6.4.1.2) (Figure 3). Malonyl-CoA has two main functions: 1) as donor of acetyl groups, and 2) help to coordinate ß-oxidation by inhibition of enzyme carnitine palmitolytransferase 1 (CPT-1). The remaining steps of lipogenesis are regulated by a multienzymes complex, named fatty acid synthetases (FAS, EC 2.3.1.38) (Tocher, 2003; Engelking, 2010). This process requires high amount of NADPH. Thus, other key pathways in lipogenesis are those that generate the NADPH equivalents. In fish, NADPH is mainly supplied by the pentose phosphate pathway, with the action of dehydrogenases of the pentose phosphate pathway (mainly G6PDH) or malic enzyme (ME, EC 1.1.1.40) (Figure 3), with the main NADPH-generating enzyme in marine fish, including sea bass, possibly being G6PDH (Dias et al., 1998; Dias et al., 2004; Menoyo et al., 2004; Castro et al., 2015).

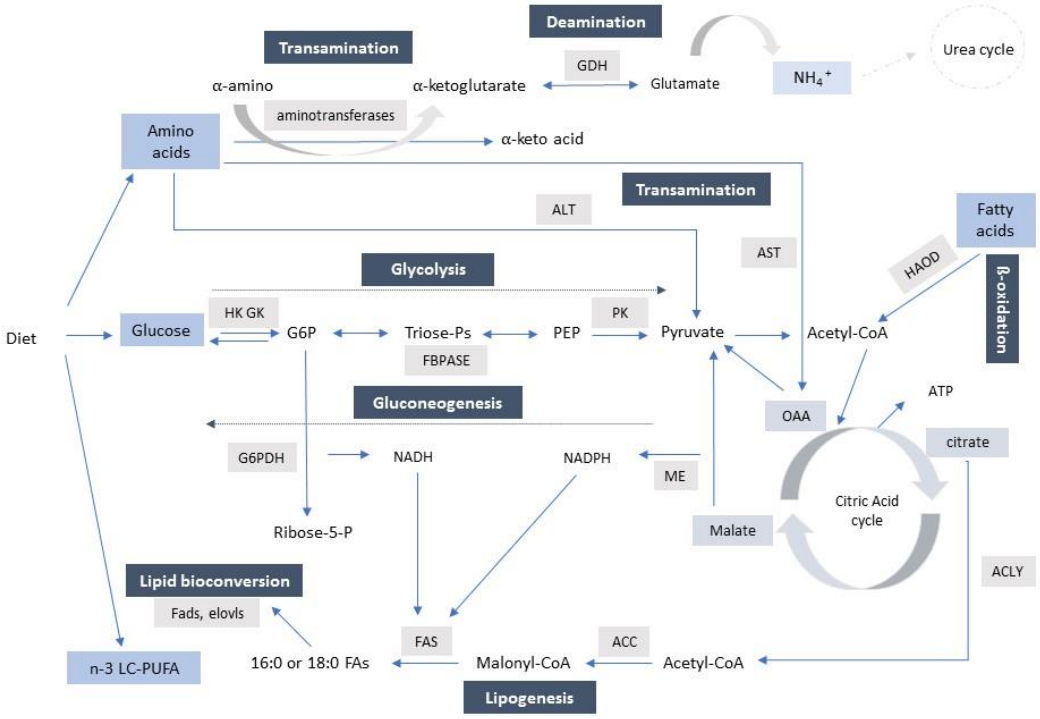


Fig. 3 - Schematic representative of the major pathways involved in intermediary metabolism. The key enzymes of the amino acid catabolism, glycolytic, gluconeogenic, fatty acid synthesis and ß-oxidation are represented: alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GDH), hexokinase (HK), glucokinase (GK), pyruvate kinase (PK), fructose-1,6-biphosphatase (FBPase), glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), 3-hydroxyacyl-CoA (HOAD). Glucose-6-phosphate (G6P), phosphoenolpyruvate (PEP), oxaloacetate (OAA) Adapted from Enes et al. (2009), Engelking (2010).

1.3.2. Taurine

Taurine (2-aminoethanesulfonic acid) is a neutral ß-amino acid which is present in high concentrations in algae and in the animal kingdom, including insects and arthropods, but that is generally absent or present in trace amounts in the bacteria and plant kingdoms (Huxtable, 1992). Taurine (Tau) differs from the more amino acids in being a sulfonic amino acid, since it has a sulfuric group instead of a carboxyl group, as in the remaining amino acids, and for being a ß-amino acid and not an α-amino acid because the amino group ($-H_2N$) is attached to secondary carbon rather than an α-carbon (Figure 4).

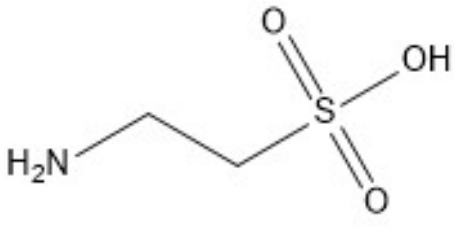


Fig.4 - Chemical structure of taurine. Adapted from Huxtable (1992).

Tau can be endogenously synthetized from methionine/cysteine. The possible Tau biosynthesis pathways have been studied over the years (Salze and Davis, 2015), however, the results pointed the cysteinesulfinate-dependent (Pathway 1, Figure 5) and cysteamine (Pathway 2, Figure 5) pathways as the most biologically valuable. In cysteinesulfinate-dependent pathway, cysteine is oxidized to cysteine sulfonic acid with action of cysteine dioxygenase (CDO). CDO is a regulator enzyme in the flux of cysteine to Tau and its activity is highly responsive to substrate availability, thus playing an important role in maintenance of intracellular cysteine levels. In turn, cysteine sulfonic acid is decarboxylated into hypotaurine by the enzyme cysteinesulfinate decarboxylase (CSD). Hypotaurine is spontaneously oxidized to Tau (Salze and Davis, 2015; Andersen et al., 2016). In the cysteamine pathway, cysteamine is produced via coenzyme A turnover and pantetheine oxidation by cysteamine dioxygenase. Cysteamine is then converted into hypotaurine through intervention of enzyme 2-aminoethanethiol dioxygenase (ADO) (Salze and Davis, 2015). In fish, Tau biosynthesis pathways are poorly understood however, some early studies reveal that some species can synthesize taurine through cysteine (Yokoyama et al., 1997). Assuming that in fish cysteine is the precursor of Tau biosynthesis, Yokoyama et al. (2001) evaluated the activity of the enzyme CSD in several fish species reporting low or absence activity of CSD.

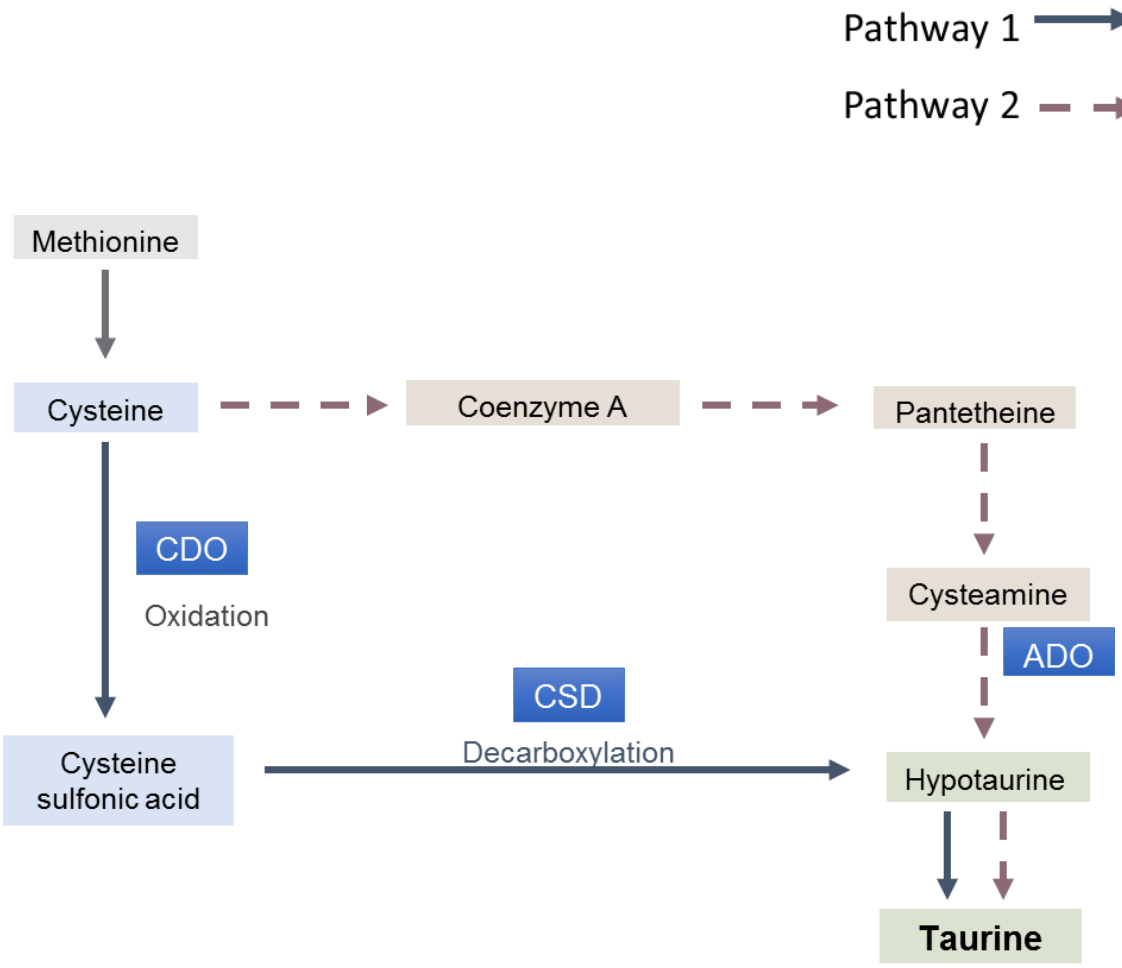


Fig. 5 - Main pathways of taurine biosynthesis. The key enzymes are represented: Cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase (CSD), 2-aminoethanethiol dioxygenase (ADO). Adapted from: Salze and Davis (2015).

The capacity to synthesize Tau seems to be species dependent, CSD activity/expression, feeding habits, and development stage (Chatzifotis et al., 2008; El-Sayed, 2014; Salze and Davis, 2015). It has been described that some marine fish species, such as red sea bream, Japanese flounder and yellowtail have low or negligible ability to synthesize Tau, particularly during rapid growth stages, due to the low or absence of CSD activity in the liver. Indeed, Tau synthesis capability seems to be dependent of fish development stage, with Tau requirement being usually higher for larvae (1-3%) than for juveniles (0.1-0.5%) (Salze and Davis, 2015). For these species, a dietary Tau inclusion is required to avoid impaired growth performance and physiological functions. Thus, Tau is considered an essential nutrient for these species (Kim et al., 2005; El-

Sayed, 2014; Salze and Davis, 2015). On the other hand, some studies reported that freshwater fish species, such as rainbow trout, Atlantic salmon, channel catfish, or common carp are able to synthetize Tau from cysteine (El-Sayed, 2014). A dietary Tau supplementation did not improve growth performance of common carp or channel catfish, but enhanced growth performance of rainbow trout (El-Sayed, 2014). Thus, although rainbow trout is able to synthetize Tau, it does not seem to produce it at sufficient rates to meet requirements for maximum growth when fed low-FM based diets (Gaylord et al., 2006). Thereby, the need of Tau in diets for freshwater fish is still unclear and requires further elucidation (Salze and Davis, 2015).

Tau quantitative requirements has been estimated for juveniles of marine fish carnivorous species such as cobia (Lunger et al., 2007) and, Japanese flounder (Kim, et al., 2005; Kim, et al., 2007). These studies have shown a positive effect of dietary Tau supplementation on growth, feed efficiency and fish health. For larval stages of cobia (Salze et al., 2011, 2012), Japanese flounder (Chen et al., 2005) and, red sea bream (Chen et al., 2004) enrichment of the live prey with Tau improved growth performance, survival rates, the activity of digestive enzymes and, morphological aspects, proving the essentiality of Tau in early life stages. Nutrient requirements of fish are classically determined by dose-response studies, where graded level of a target nutrient in the diets and growth response are usually the parameters considered. Quantitative nutrient requirement may however be indirectly determined based on parameter, other than growth response, such as nitrogen retention, survival, body composition, enzymatic activities (Brotons Martinez et al., 2004; Peres and Oliva-Teles, 2008). Several models are availabe for dose-response relationship. Broken line, exponential, quadratic, four-parameter saturation kinetics (4-SKM; Mercer et al., 1984) and five-parameter saturation kinetics (5-SKM; Mercer et al., 1989) being the most used. 5-SKM and 4-SKM usually provide more accurate estimates of requirements, once they are based on the concept of kinetic reactions and therefore more arguably biologically than other models (Pesti et al., 2009).

Tau is not incorporated into proteins but it comprises 30-50% of the blood free amino acid pool and it is involved in several physiological functions in vertebrates. In fish, Tau functions are associated to osmoregulation; anti-oxidation, cell membrane stabilization, modulation of calcium levels, immunomodulation, and bile acids conjugation (Huxtable, 1992; Salze and Davis, 2015). Bile acids conjugation is well-documented in fish (Kim et al., 2007). Bile acids are synthetized in the liver from cholesterol, then they may be conjugated with Tau or glycine; however, there are evidences that in vertebrates, except in mammals, Tau is the only amino acid conjugated with

cholesterol derivatives to form bile salts (Goto et al., 2001). Bile salts are then stored in the gall bladder and released into the intestinal lumen in response to the presence of food in the intestine to emulsify lipids and to facilitate absorption of lipids and fat-soluble vitamins (Kim et al., 2007; El-Sayed, 2014). Therefore, Tau may enhance lipid metabolism, by increasing the activity of the bile salt-activated lipase in the liver, as reported by Chatzifotis et al. (2008) in common dentex. In turn, Tau deficiency is associated to green liver syndrome in red sea bream (Goto et al., 2001; Takagi et al., 2006) and yellowtail (Takagi et al., 2005, 2006). Both species, when fed diets with low or non-FM content revealed lower growth rates and feed efficiency, and high incidence of green liver syndrome comparatively with fish fed the control FM-based diet. Green liver syndrome has been attributed to decrease in the excretion of bile pigments from the liver into the bile and increased production of haemolytic biliverdin (Takagi, et al., 2005; 2006).

1.4. Aims

Considering there is a trend for carnivorous fish diets to include higher levels of PF, which are devoid of Tau, it is necessary to know if there is a Tau requirement for the species of concern.

In sea bass, previous studies reported that Tau supplementation to PF-rich diets improved growth performance and feed conversation ratio of fry (Brotons-Martinez et al., 2004) and juveniles (Kanashiro et al., 2014). Therefore, Tau seems to be a conditionally essential amino acid or an essential amino acid for sea bass.

Thus, the overall aim of this thesis was to evaluate the optimal dietary Tau level for sea bass juveniles and, to further understand the effects of a Tau deficiency and excess on growth, body composition, plasma metabolites, bile acids production, intermediary metabolism and hepatic composition of sea bass juveniles fed PF-rich diets.

Chapter 2 - Optimal dietary taurine level for growth and nitrogen accretion of European sea bass (*Dicentrarchus labrax*, L.) juveniles

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(Submitted to Aquaculture)

OPTIMAL DIETARY TAURINE LEVEL FOR GROWTH AND NITROGEN ACCRETION OF EUROPEAN SEA BASS (*Dicentrarchus labrax*, L.) JUVENILES

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Abstract

A growth trial was conducted to determine the dietary taurine requirement of European sea bass juveniles fed plant feedstuffs-based diets. Eight isoproteic (45% crude protein) and isolipidic (18% crude lipid) diets were formulated containing a mixture of plant feedstuffs and fish meal (corresponding to 80% and 20% of total dietary protein, respectively) and taurine (Tau) levels ranging from 0 to 1.5%. The basal diet (0% Tau) contains 0.2% of Tau, and therefore, the real Tau levels studied varies from 0.2 and 1.7% (diets 0.2Tau; 0.3Tau; 0.4Tau; 0.5Tau; 0.7Tau; 0.9Tau; 1.2Tau; 1.7Tau). Triplicate groups of 12 fish (IBW=55g) were fed each diet for 10 weeks. Dietary Tau level up to 0.7% improved growth performance while maximum feed efficiency and protein efficiency ratios were obtained with a dietary Tau level of 1.2%. Whole-body lipid content of fish fed the 1.2 and 1.7% Tau diets was lower than in the other groups, while whole-body protein and ash contents, and hepatosomatic and visceral indexes were not affected by diet composition.
Based on a five-parameter saturation kinetics model, dietary Tau requirement for maximum growth and nitrogen accretion was estimated to be 0.63% DM and 0.59% DM, respectively.

Keywords: Taurine requirement; Plant feedstuffs; European sea bass; Growth performance

Introduction

Fishmeal (FM) is considered the most adequate protein source for fish diets and its removal from aquafeeds usually results in reduced growth performance and feed utilization (Gaylord et al., 2006; Jirsa et al., 2010; Rossi and Davis, 2012). Thus, although there is a decreasing incorporation of FM in aquafeeds, it is still included at significant levels in diets for carnivorous marine fish (Bendiksen 2011; Tacon, 2011). However, FM is a finite, expensive, and environmentally unsustainable resource, and to ensure an economic and sustainable development of aquaculture it is crucial to reduce aquafeeds dependency of this commodity (Gatlin III et al., 2007).

Over the years, numerous efforts have been made to find economically and environmentally sustainable alternatives to FM (Oliva-Teles et al., 2015). Plant feedstuffs (PF) are the most obvious alternatives, as they are abundant and readily available worldwide, have relatively constant composition, and competitive cost (Hardy, 2010). However, comparatively to FM, PF usually have lower protein content and unbalanced amino acid profiles, high fiber content and present anti-nutrient factors (Gatlin III et al., 2007; Kader et al., 2012b). For carnivorous fish, data shows that partial FM replacement by PF is possible without compromising growth performance (Hansen et al., 2007; Kaushik et al., 2004; Mente et al., 2003). However, total FM replacement usually has negative effects for most carnivorous species, leading to reduced growth performance, morphological intestinal alterations, and impaired health status (Gaylord et al., 2006; Le Boucher et al., 2011).

PF lack taurine (Tau) a non-proteinaceous beta-amino acid that is abundant in FM and other animal feedstuffs (El-Sayed, 2014). Some studies associate Tau deficiency to reduced growth performance and feed efficiency and the pathological green liver syndrome observed in some fish fed PF-based diets (Goto et al., 2001b; Takagi et al., 2005, 2006b, 2010, 2011). Thus, Tau has been considered a conditionally essential amino acid, required by several carnivorous fish species fed diets including low or none FM (NRC, 2011; Salze and Davis, 2015).

A dietary Tau requirement for a given species or growth stanza is dependent on the capacity of the organism to synthetize this amino acid at adequate levels to meet requirements (El-Sayed, 2014; Salze and Davis, 2015). In fish, Tau is mainly synthesized from cysteine through a hepatic transsulphuration pathway (Andersen et al., 2016; El-Sayed, 2014; Salze and Davis 2015). One of the key enzymes involved in Tau synthesis is cysteinesulphinate decarboxylase (CSD) and the ability to synthesize Tau depends on the gene expression and metabolic activity of this rate-limiting

enzyme (Jirsa et al., 2014; Yokoyama et al., 2001). Some studies with marine fish reported a lack or low capacity to synthetize Tau, particularly during rapid growth stages, due to low or absence of CSD activity in the liver (Goto et al., 2001b; Park et al., 2002; Takagi et al., 2005). Thereby, for these species a dietary Tau inclusion is required to avoid impaired growth performance, physiological functions and pathological signs. Recognizing Tau as a conditionally essential amino acid for several marine fish species (NRC, 2011), makes it a priority to establish the dietary Tau requirement to assure that plant-based diets are not unbalanced for the species and life stanza considered (Salze and Davis, 2015).

European sea bass (*Dicentrarchus labrax*, L.) is one of the main species produced in Mediterranean aquaculture. It is a carnivorous marine species with high protein requirements (Oliva-Teles, 2000) but known to accept well diets including high levels of PF (<60%) (Ballestrazzi et al., 1994; Bonaldo et al., 2008; Messina et al., 2005, 2013; Tibaldi et al., 1999, 2005). In fact, almost total FM replacement was already achieved without negative effects on growth performance (Kaushik et al., 2004). In the study by Kaushik et al. (2004) diets were not supplemented with Tau and even so growth performance was not impaired. However, some studies with sea bass evidenced reduced growth performance with diets including high levels of PF (Dias et al., 1997, 2003; Lanari and D'Agaro, 2005; Tulli et al., 2007; Geay et al., 2011; Le Boucher et al., 2011). Further, it was recently demonstrated that supplementing dietary Tau to low FM diets improved growth performance of sea bass (Kanashiro et al., 2014). These contradictory results suggest that high inclusion of PF in diets for this species still needs to be improved to ensure production success of sea bass.

Considering the trend for reducing FM level in sea bass aquafeeds it is priority to establish the Tau requirement that guarantee an adequate performance and feed utilization of the animals. Thus, the aim of this study was to establish the dietary requirement of Tau for European sea bass juveniles fed plant feedstuffs-based diets.

Material e Methods

Experimental diets

Eight isoproteic (45% crude protein) and isolipidic (18% crude lipid) diets were formulated to contain a mixture of plant feedstuffs and fish meal as protein sources (corresponding to 80% and 20% of total dietary protein, respectively) and fish oil as the sole lipid source. Taurine (Tau) was

included in the diets at levels ranging from 0 to 1.5%. The basal diet (0% Tau) contains 0.2% of Tau, and therefore, the real Tau levels evaluated varies from 0.2 and 1.7% (diets 0.2Tau; 0.3Tau; 0.4Tau; 0.5Tau; 0.7Tau; 0.9Tau; 1.2Tau; 1.7Tau). Diets were formulated to have an essential amino acid profile corresponding to that of European sea bass whole-body amino acid profile (Kaushik et al., 1998). Tau was coated with agar before mixing with dietary ingredients. For that purpose, agar was dissolved in boiled water (10% of the diet) and cooled at 40˚C before adding Tau. All dietary ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (CPM) through a 3-mm die. The pellets were then dried in an oven at 65˚C for 24h, placed in plastic bags and stored in the refrigerator until used.

For amino acids analysis, the samples were hydrolyzed with 6 N hydrochloric acid at 112 ˚C under an atmosphere of N_2 for 23h. Samples were then derivatized with phenylisothiocyanate (PITC) before separation by gradient exchange chromatography (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), according to the Pico – Tag method, as described by Cohen *et al* (1989). Chromatographic peaks were identified, integrated and quantified with a Waters Breeze software package by comparing to a known amino acid standard (Pierce NC10180). Cysteine and tryptophan were not determined.

Dietary composition and proximate analysis is presented in Table 1, and dietary amino acid composition is present in Table 2.

Table 1. Ingredient composition and proximate analysis (% dry weight) of the experimental diets.

Tau level (%DM)	**0.2**	**0.3**	**0.4**	**0.5**	**0.7**	**0.9**	**1.2**	**1.7**
Ingredients								
Fish meal[1]	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Soybean meal[2]	20	20	20	20	20	20	20	20
Corn gluten[3]	20	20	20	20	20	20	20	20
Wheat gluten[4]	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
Wheat[5]	10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8
Pea protein concentrate[6]	4.7	4.7	4.7	4.7	4.7	4.7	4.7	4.7
Cod liver oil	15.3	15.3	15.3	15.3	15.3	15.3	15.3	15.3
Vitamin[7]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Coline	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral[8]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Agar	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Blinder[9]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Dicalcium phosphate	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Cellulose	1.5	1.4	1.3	1.2	1.0	0.8	0.5	0.0
Taurine	0.0	0.1	0.2	0.3	0.5	0.7	1.0	1.5
Proximate analysis								
Dry matter (%)	87.0	88.0	90.8	94.5	94.8	95.4	86.6	88.3
Ash	10.2	9.7	9.5	9.6	9.2	9.3	9.9	8.7
Protein	44.6	45.3	45.0	45.2	45.7	45.5	45.7	44.7
Lipid	18.0	17.9	18.1	18.2	18.2	18.0	18.2	18.1

[1] Pesquera Centinela, Steam Dried LT, Chile (CP: 69.6%; CL: 7.7%), Sorgal, S.A. Ovar, Portugal
[2] Non-GMO ; Cargill France SAS, St. Germain-en-Laye, France. (CP : 47.9% CL : 2.1%)
[3] Sorgal, S.A Ovar, Portugal (CP : 71.2% CL : 2.9%)
[4] Sorgal, S.A Ovar, Portugal (CP: 83.2% CL: 3.9%)
[5] Sorgal, S.A Ovar, Portugal (CP: 14.9% CL: 2.2%)
[6] Sorgal, S.A. Ovar, Portugal (CP: 51.6; CL 2.5%)
[7] 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.
[8] 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).
[9] Aquacube. Agil, UK

Table 2. Amino acid composition (as percentage of protein) of the experimental diets.

Tau level (%DM)	0.2	0.3	0.4	0.5	0.7	0.9	1.2	1.7
Arginine	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3
Histidine	2.6	2.6	2.7	2.7	2.6	2.6	2.6	2.6
Isoleucine	5.0	4.9	5.0	5.0	4.9	4.9	4.9	4.9
Leucine	9.5	9.6	9.5	9.6	9.5	9.5	9.5	9.5
Lysine	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3
Methionine	2.4	2.4	2.3	2.4	2.4	2.3	2.3	2.3
Phenylalanine	6.0	6.0	5.9	6.0	6.0	5.9	5.9	5.9
Threonine	4.1	4.0	4.1	3.9	4.0	4.0	4.0	4.0
Valine	5.9	5.9	5.9	5.9	5.9	5.9	5.8	5.8
Taurine	9.0	13.5	18.1	22.6	31.7	40.9	54.8	78.1
Tyrosine	4.4	4.5	4.5	4.5	4.4	4.4	4.4	4.4
Alanine	7.8	7.8	7.8	7.9	7.8	7.9	7.8	7.8
Aspartic acid	9.0	8.9	8.9	8.9	8.8	8.9	8.9	8.9
Glutamic acid	17.1	16.9	16.8	16.9	17.0	16.9	16.8	16.9
Glycine	2.8	2.8	2.8	2.7	2.8	2.9	2.7	2.7
Serine	4.8	4.9	4.9	4.8	4.9	4.8	4.9	4.8
Proline	6.8	6.7	6.9	6.7	6.8	6.8	6.8	6.7

Growth trial

The growth trial was carried out at the Marine Zoology Station, University of Porto. The experiment was conducted by trained scientists (following FELASA category C recommendations) according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes.

The trial was performed in a thermo-regulated recirculation water system equipped with 24 cylindrical fiberglass tanks of 100 L water capacity, supplied with continuous flow of filtered seawater and aerification provided by diffusion through air stones. During the trial, the water temperature was maintained at ± 24˚C, salinity at 34‰, ammonia below 0.05 mg l^{-1}, and photoperiod controlled for 12 hours light and 12 hours dark. European sea bass (*Dicentrarchus labrax*) juveniles were provided by Instituto Português do Mar e da Atmosfera pilot aquaculture facility at Olhão, Algarve. After transportation to the experimental facilities fish were maintained in quarantine for 2 months. Fish were then acclimatized to the experimental facilities and water temperature for 2 weeks. At the end of the acclimatization period, 24 homogenous groups of 12 European sea bass (IBW=55g) were randomly distributed to the experimental tanks. Each diet was randomly assigned to triplicate groups of these fish. During the trial fish were fed by hand,

twice a day, 6 days a week, until visual apparent satiety (established after the first three pellets reach the bottom of the tank). The trial lasted 10 weeks and at the end of the trial fish were bulk weighed following one day of feed deprivation. Ten fish from the initial stock population and three fish from each tank at the end of the trial were randomly sampled for whole-body composition analysis. Wet weight, liver and viscera weight of these fish were individually recorded for determination of the hepatosomatic and visceral indexes.

Chemical analysis

Chemical analysis of feedstuffs, diets and whole-body composition were performed according to standard methods (AOAC, 1980). Briefly: dry matter, by drying the samples at 105 ˚C until constant weight; ash by incineration in a muffle furnace at 450 ˚C for 16 h; protein content (N x 6.25) by the Kjeldahl method following acid digestion, using Kjeltec digestion and distillation units (Tecator systems, Höganäs, Sweden, models 1015 and 1026, respectively); lipid content by extraction with petroleum ether using a SoxTec (Tecator systems, Höganäs, Sweden, extraction unit model 1043 and service unit model 1046).

Data analysis

Data is presented as means and pooled standard error of the mean (SEM). Statistical evaluation of the data was done by one-way analysis of variance after checking for normality and homogeneity of variances, and when appropriate data was normalized. The probability level for rejection of the null hypotheses was 0.05. Significant differences between means ($P< 0.05$) were determined by the Tukey multiple range test.

For Tau requirement estimation, several regression models were tested. The Five-parameter saturation kinetics model (Mercer *et al.,* 1989) and the piecewise linear regression model provided the best fit to the daily growth index data and N retention. In both models, Tau requirement was estimated from the level that produced maximum response (Shearer, 2000).

All statistical analysis was performed using the SPSS 24.0 for Windows software package.

Results

Data on growth performance and feed utilization of sea bass fed the experimental diets is presented in Table 3. Fish promptly accepted the experimental diets, and mortality during the trial was low and unaffected by diet composition. Final body weight, weight gain and daily growth index increased with dietary taurine level up to 0.7%. Feed intake was not much affected by diet

composition though it was lower for fish fed diet 1.2Tau than diets 0.2Tau, 0.4Tau, 0.5Tau and 0.7Tau. Feed efficiency and protein efficiency ratio tended to improve as dietary Tau increased up to 1.2%, but were only statistically different between diets 0.2Tau and1.2Tau. N retention, expressed either as g/kg/day or as %NI, was not affected by diet composition.

Table 3. Growth performance of European sea bass fed the experimental diets.

Tau level (%DM)	0.2	0.3	0.4	0.5	0.7	0.9	1.2	1.7	SEM
IBW (g)[1]	55.0	55.0	55.0	55.1	54.9	54.9	55.0	55.0	0.03
FBW (g) [1]	96.2^{a}	98.7^{ab}	103.4^{abc}	115.1^{bc}	116.8^{c}	108.8^{abc}	105.4^{abc}	106.3^{abc}	1.75
WG (g/kg/day)	8.6^{a}	9.0^{ab}	9.7^{abc}	11.2^{bc}	11.4^{c}	10.4^{abc}	9.9^{abc}	10.1^{abc}	0.24
DGI[2]	1.23^{a}	1.29^{ab}	1.40^{abc}	1.68^{bc}	1.72^{c}	1.54^{abc}	1.45^{abc}	1.48^{abc}	0.04
FI (g/kg/day)	12.2^{b}	11.3^{ab}	12.3^{b}	12.2^{b}	11.9^{b}	11.8^{ab}	9.7^{a}	11.3^{ab}	0.21
FE[3]	0.70^{a}	0.79^{ab}	0.78^{ab}	0.92^{ab}	0.96^{ab}	0.88^{ab}	1.02^{b}	0.89^{ab}	0.03
PER[4]	1.6^{a}	1.7^{ab}	1.7^{ab}	2.0^{ab}	2.1^{ab}	2.0^{ab}	2.3^{b}	2.0^{ab}	0.06
NR (g/kg/day)[5]	0.25	0.27	0.33	0.31	0.34	0.30	0.29	0.30	0.01
NR (%NI)[6]	28.7	32.6	37.4	35.5	38.9	35.2	40.5	37.2	1.14
Mortality (%)	0.0	9.7	3.0	6.0	6.0	6.6	12.7	6.7	1.53

Values are presented as mean (n=3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (Tukey test; P<0.05).
[1]Initial and final body weight;
[2]Daily growth index = ($FBW^{1/3}$−$IBW^{1/3}$)/time in days) ×100;
[3]Feed efficiency = wet weight gain/dry feed intake;
[4]Protein efficiency ratio = wet weight gain/crude protein intake;
[5]Nitrogen retention (g/kg/day) = (FBW×FBN−IBW×IBN)/ (ABW × time in days).
[6]Nitrogen retention (% nitrogen intake) = (FBW×FBN−IBW×IBN)/(NI)) ×100; IBN and FBN: initial and final body nitrogen content; ABW: average body weight (IBW+FBW)/2; NI: nitrogen intake.

At the end of the trial, whole-body dry matter, protein and lipid content were higher than the initial values while ash content was lower (Table 4). At the end of the trial, whole-body dry matter and lipid content of fish fed diets 1.2Tau and 1.7Tau were lower than in fish fed the 0.4Tau diet. Whole-body protein and ash content, and hepatosomatic and visceral indexes were not affected by diet composition.

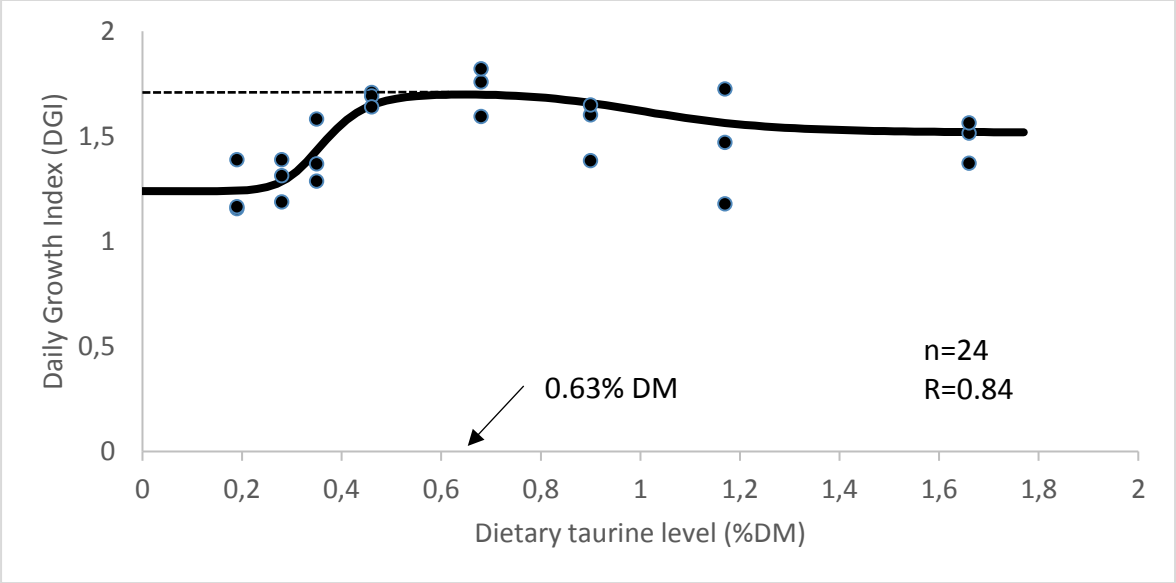
Table 4. Whole body composition (% wet weight) and liver index of European sea bass fed experimental diets.

		Tau level (%DM)								
	Initial	0.2	0.3	0.4	0.5	0.7	0.9	1.2	1.7	SEM
Dry matter	27.9	33.4^{ab}	32.6^{ab}	37.4^{b}	32.2^{a}	33.2^{ab}	34.2^{ab}	32.1^{a}	32.1^{a}	0.46
Protein	14.1	17.6	17.9	19.3	17.3	17.9	17.7	17.6	17.8	0.23
Lipid	4.1	10.2^{ab}	9.4^{ab}	12.3^{b}	9.4^{ab}	10.3^{ab}	11.5^{ab}	8.9^{a}	9.0^{a}	0.29
Ash	6.4	5.2	5.4	5.8	5.0	5.3	4.8	5.1	5.2	0.10
HSI[1]	-	1.3	1.2	1.2	1.1	1.2	1.1	1.2	1.3	0.02
VI[2]	-	10.0	9.4	9.1	10.0	9.2	9.4	8.5	9.3	0.20

Values are presented as mean (n=3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (Tukey test; P < 0.05).
[1] Hepatosomatic index = (liver weight/ whole body weight) × 100.
[2] Visceral index = (visceral weight/ whole body weight) × 100.

Daily growth index was well fitted to both five-parameter saturation kinetics model (5 SKM) and to piecewise linear regression with breakpoint model (PLR), with dietary Tau requirement being estimated to be 0.63% (r=0.84, Figure 1) and 0.64% (r=0.91), respectively. The two models also adjusted to N retention (expressed as g/kg/day). Based on N retention, Tau requirement was estimated to be 0.59% (5SKM model) or 0.72 (PLR). Dietary Tau for maximum FE only fitted with the PLR, with Tau requirement estimated to be 0.71%.

Figure 1. Dose-response relationship between dietary taurine content (%DM) and daily growth index (DGI). Each graphic point represents the average value of each tank. Model parameter estimates are summarized in Table 5.



Discussion

Kaushik et al. (2004) showed that European sea bass could be fed a diet almost devoid of FM without negative effects on growth performance. That study was performed with on-growing seabass with 190g and performance of fish fed a diet including only 5% FM and without Tau supplementation was identical to that of fish fed a FM-based diet. Recently, it was also shown that 1% Tau supplementation to a diet containing just 8% FM did not affect growth performance of European sea bass juveniles with 6.9g (Coutinho et al., 2017). On the contrary, dietary Tau supplementation improved growth performance and feed conversion of European sea bass fry

with 0.8g fed a FM-based diet (Brotons-Martinez et al., 2004) or of 9.4g juveniles fed diets including either 12.5% or 25% FM (Kanashiro et al. 2014).

A dietary Tau requirement is related to the capacity of synthesizing this amino acid by the animals, mainly through the cysteine-sulfinate pathway, which involves cysteinesulfinate decarboxylase (CSD) (Salze and Davis 2015). It has already been proved that some species, particularly marine carnivorous species such as japanese flounder (Park et al., 2002), red sea bream (Matsunari et al., 2008; Takagi et al., 2010) and yellowtail (Matsunari et al., 2005), have low ability to synthesize Tau due to the absence or low activity of CSD and therefore have a dietary Tau requirement.

Tau requirement may also change with fish development stage, due to changes in CSD activity or expression, which may modify fish ability to synthesize Tau endogenously (Kim et al., 2005; Yokoyama et al., 2001). For instance, dietary Tau requirement of 6.3g turbot was estimated to be 1.15% while that of 166g turbot was estimated to be 0.64% (Qi et al., 2012). In 0.5g yellowtail, dietary Tau inclusion up to 1.2% improved performance of fish during the first 3-weeks (weight of 10g) but not at the end of 6-weehs of feeding (weight of 42g) (Matsunari et al. 2005). Further, Tau requirement of 0.18g Japanese flounder is 1.5% (Kim et al., 2005) while Tau requirement of 0.7 and 9.6g fish is 0.5% (Kim et al. 2007). This may contribute to partially explain the apparent contradictory data on Tau requirement in the above mentioned studies with European sea bass.

The present study was performed with 55g European sea bass, fed diets with 12.5% FM, and showed a dietary Tau requirement of 0.6-0.7%. This Tau requirement estimation is slightly higher than the value estimated for 0.8g sea bass fry by Brotons-Martinez et al. (2004), which reported a dietary Tau requirement of 0.45-0.54%. In the study by Brotons-Martinez et al. (2004) the control diet included 60% FM, and even though fish performance improved with the addition of 0.2-0.3% Tau to the diet, to provide a total dietary Tau level of 0.4-0.5%. This is in line with the results of Kanashiro et al. (2014) who showed that growth performance of European sea bass fed 12.5% or 25% FM diets improved when diets were supplemented with 1% Tau. As current commercial diets for European sea bass usually include 25% or lower FM levels, it is important to take into consideration the Tau provided by the diets, as growth performance may be jeopardized due to dietary Tau levels below requirement for maximum fish performance.

Present results and those of Brotons-Martinez et al. (2004) suggest that European sea bass has similar dietary Tau requirement or red sea bream (0.50%; Matsunari et al. 2008), but lower than

requirements estimated for parrot fish (0.88%; Lim et al., 2013), white sea bass (0.99%; Jirsa et al. 2014), sablefish (1.5%; Johnson et al., 2015).

In this study, dietary Tau requirement was well adjusted to a 5SKM regression model, indicating that after an optimum dietary inclusion level there is a reduction on growth performance, possibly due to toxic effect of dietary Tau excess (Shearer 2000). Also in Japanese flounder (Park e al. 2002), large turbot (Qi et al. 2012) and sablefish (Johnson et al. 2015) growth performance depression was observed in fish fed diet with excess Tau. On the contrary, in white sea bass no growth depression occurs even at high dietary Tau levels (1.7%; Jirsa et al. 2014).

Growth depression due to excess dietary Tau was also described in higher vertebrates, namely in pigs (Liu et al., 2014), and were related to biochemical lesions provoked by the excess Tau consumption. In fact, Tau was indicated as toxic to human and rats neural cell lines (Pean et al., 1995). The negative effects of high Tau levels observed by Liu et al. (2014) occurred in piglets but not in pigs, and the authors suggest that an increasing tolerance to high Tau levels in older pigs could be due to improvements on the digestive system.

In the present study, FI did not improve with dietary Tau supplementation suggesting that it was ineffective as feed attractant for European sea bass. Indeed, effective feed attractants for this species were established to be mixtures of free-amino acids (Mackie and Mitchel, 1985). Nevertheless, for some fish species higher feed intake with Tau supplemented diets was associated to its feed attractant properties (Chatzifotis et al., 2008; Kim et al., 2005).

As consequence of an almost constant FI and increased growth performance, FE also improved with dietary Tau supplementation up to the requirement level. Also for sea bass fry (0.8g) an improvement in FE was reported when Tau was added to the diets (Brotons-Martinez et al., 2004). Contrarily, Kanashiro et al. (2014) and Coutinho et al. (2017) observed no significant effects on feed utilization in sea bass juveniles fed diets supplemented with 1% Tau. An improvement of feed efficiency as dietary Tau increased was also described in other fish species (Lunger et al., 2007; Park et al*.,* 2002; Jirsa et al., 2014; Johnson et al., 2015) due to similar FI and growth responses as observed in the present study.

As in this study, other studies either in European sea bass (Kanashiro et al., 2014; Coutinho et al. 2017) or in other species (Johnson et al., 2015) found that dietary Tau supplementation had no significant effects on whole body-composition, HSI and VI indexes. Nevertheless, an increase of whole-body protein and lipid content in turbot (Qi et al., 2012), of whole-body protein content in

cobia (Watson et al., 2013), or a decrease of visceral and hepatosomatic indexes in cobia (Lunger et al., 2007) and yellow catfish (Li et al., 2015) were observed with the increase of dietary Tau level.

The lack of a dietary Tau effect on whole body composition, hepatosomatic and visceral indexes observed in the present study was somehow unexpected, as Tau availability is often associated to improvement of nutrient digestion and absorption, particularly of lipids, as Tau is required for bile acids production. Therefore, Tau is required for increasing lipids availability and absorption (Goto et al., 2001; Kim et al., 2007, 2008b) and its lack in the diets promotes the green liver syndrome (Takagi 2005; 2006; 2010).

Overall, results of this study indicate that for maximum growth performance and N retention European sea bass juveniles require a dietary Tau level of 0.6-0.7%DM. The data generated in the present study will be useful in developing balanced practical plant-based diets for this species.

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Chapter 3 - Metabolic response to dietary taurine levels in European sea bass (*Dicentrarchus labrax*, L.) juveniles

METABOLIC RESPONSE TO DIETARY TAURINE LEVELS IN EUROPEAN SEA BASS (*Dicentrarchus labrax*, L.) JUVENILES

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Abstract

A study was undertaken to evaluate the metabolic responses to four dietary Tau levels in European sea bass. For that purpose, four isoproteic (45% crude protein) and isolipidic (18% crude lipid) diets were formulated, containing a mixture of plant feedstuffs and fish meal (corresponding to 80% and 20% of total dietary protein, respectively), and increasing levels of Tau (0.2, 0.5, 0.7 and 1.2%). Triplicate groups of 12 European sea bass juveniles (average body mass = 55g) were fed with these diets for 10 weeks. Tau supplementation increased hepatic total bile acids content, while the opposite occurred for plasma total bile acids. Plasma total cholesterol, HDL and LDL cholesterol also decreased whereas plasma triglycerides were not affected with the increase of Tau level up to 0.7%, activity of lipogenesis key enzymes, FAS and ME, but not G6PDH, decreased with the increase of dietary Tau up to 0.7%. Post-prandial plasma glycaemia and the activity of hepatic key enzymes of glycolysis and gluconeogenesis decreased with increase of Tau supplementation. Overall, present results indicate that Tau appear to be involved in different pathways of both lipid and glucose metabolism having a hypocholesterolaemia and hypoglycaemia action.

Keywords: Taurine, Plant feedstuffs, European sea bass, Bile acids, Lipid metabolism

Introduction

World fish consumption has expressively increased in last decades (Klinger and Naylor, 2015). With fisheries stabilized in the past 20-30's years, aquaculture has been the major contributor for the growth in fish consumption *per capita* and nowadays nearly half of fish consumed worldwide is provided by aquaculture (FAO, 2016; Troell et al., 2015).

Aquaculture production of carnivorous fish species still relies in fishmeal (FM) and fish oil, as the main ingredients in the diets (Tacon and Hasan, 2011; Tacon and Metian, 2015). However, FM utilization faces several challenges due its high price and world limited availability. Currently, the demand of FM for aquafeeds outpaces FM global production threatening aquaculture sustainability (Gatlin et al., 2007; Tacon and Metian, 2015). It is now recognized the need to identify economic, and environmental sustainable alternatives to FM, reducing aquafeeds dependency of this commodity and ensuring the nutritional quality of diets (Oliva-Teles et al., 2015).

Due to the past restrictions to the use of terrestrial animal by-products in aquafeeds, plant feedstuffs (PF) are currently the most used alternative ingredients to FM in the European Union (Olsen and Hasan, 2012).

Collateral effects of FM replacement by PF are often associated with nutritional drawbacks of PF, such as low protein content, imbalances of one or more essential amino acids, presence of antinutritional factors, low digestibility and reduced palatability (Oliva-Teles et al., 2015). Moreover, increased evidences have been pointed out to the biological importance of molecules that are present in FM but not in PF, such as nucleotides, hydroxyproline and taurine (Aksnes, 2005, 2008; Zang et al., 2013; Kanashiro et al., 2014).

Taurine (Tau), is a neutral ß-amino acid derived from the metabolism of sulphur-containing amino acids, as cysteine (Salze and Davis, 2015). Several studies have shown that many fish species are unable to endogenously synthesize enough Tau to meet requirements (Yokoyama *et al.,* 2001; Lunger et al., 2007; Kim *et al.,* 2008; Salze et al., 2015, 2017). Thus, Tau has been considered to be a conditionally essential amino acid for certain fish species, and its inclusion in PF-based diets leading to improvements of growth performance and feed utilization (Brotons-Martinez et al., 2004; Matsunari *et al.,* 2005; Takagi *et al.,* 2006b; Lunger *et al.,* 2007; Chatzifotis *et al.,* 2008; Qi *et al.,* 2012; Díaz-Rosales et al. 2014; Jirsa *et al.,* 2014; Johnson *et al.,* 2015).

Insufficient dietary Tau level, is often associated to reduced growth (Park et al., 2002; Matsunari et al., 2005, 2008a,b; Gaylord et al., 2006; Lunger et al., 2007; Chatzifotis et al., 2008; Takagi, et al., 2010; 2011; Qi et al., 2012; Bañuelos-Vargas et al., 2014; Jirsa et al., 2014; Watson et al., 2014; Johnson et al., 2015; Li et al., 2013; Salze et al., 2016, 2017), anaemia (Takagi et al., 2008), and green liver syndrome (Goto et al., 2001; Takagi et al., 2006). Moreover, Tau has been reported to affect other physiological functions, such as osmoregulation (Huxtable, 1992; Takagi et al., 2006), antioxidant status (Espe and Holen, 2013; Bañuelos-Vargas et al., 2014; Feidantsis et al., 2014; Coutinho et al., 2017) and development of the visual and neural systems (Sturman, 1988).

In mammals, Tau has been reported to have hypoglycaemia effects, increasing the sensitivity of tissue receptors, and hypocholesterolaemia properties, regulating plasma cholesterol levels (Huxtable, 1992; Lourenço and Camilo, 2002). In fish, these effects are still poorly studied. Tau hypoglycaemia effect in fish may be associated to increased glucose up-take (Bañuelos-Vargas et al., 2014; Han et al., 2014; Watson et al., 2014a) and Tau hypocholesterolaemia may be related to an increase of cholesterol 7a-hydroxylase activity, a rate-limiting enzyme in the conversion of cholesterol to bile acids in liver (Espe et al., 2012; Han et al., 2014).

One well documented function of Tau is it intervention in bile salt conjugation (Boouckenooghe *et al.,* 2006). Bile acids are derived from cholesterol, synthesized in the liver, stored in the gall bladder and released into the intestinal lumen to emulsify lipids, facilitating lipids and fat-soluble vitamins absorption (El-Sayed, 2014; Kim et al., 2007). Bile acids may be conjugated with Tau or glycine; however, there are evidences that for the majority of fish species, Tau is the only amino acid that conjugates with cholesterol derivatives to form bile salts (Goto et al., 1996; Kim et al., 2007; Salze et al., 2015). Therefore, Tau may interact with lipid metabolism, and studies in common dentex (*Dentex dentex*) showed that it increased the activity of bile salt-activated lipase (Chatzifotis et al., 2008) and in Atlantic salmon it reduced whole-body lipid content (Espe et al., 2012).

European sea bass (*Dicentrarchus labrax*) is a carnivorous marine fish species with high demand and economic value, particularly important in Mediterranean aquaculture (Oliva-Teles, 2000). The incorporation of high PF levels in diets for European sea bass is still problematic. While some studies reported that sea bass cope well with low FM diets if duly supplemented with limiting amino acids without compromising growth performance (Tulli et al., 1999; Kaushik et al., 2004; Messina, et al., 2005; Magalhães et al., 2017), other studies showed adverse effects of PF-based

diets on growth performance, feed utilization efficiency, gut function and morphology, and lipid metabolism (Dias et al., 2005; Geay et al., 2011; Torrecillas et al., 2017). Moreover, for European sea bass it has been reported that dietary FM replacement by PF lead to adjustment in gluconeogenesis, lipogenesis and lipid metabolism (Dias et al., 2005; Messina et al., 2013), and total replacement of FM and fish oil by PF and vegetable oils induced several metabolic and physiological alterations (Geay et al., 2011).

For European sea bass, Tau essentiality was recently confirmed, and established to be 0.6-0.7% DM (Martins et al., 2017). However, the physiological roles of Tau are poorly studied. Thus, the aim of this study was to evaluate the metabolic response of European sea bass fed low FM diets supplemented with Tau bellow and above requirements.

Material e Methods

Experimental diets

Four isoproteic (45% crude protein) and isolipidic (18% crude lipid) diets were formulated, containing a mixture of plant feedstuffs and fish meal (corresponding to 80% and 20% of total dietary protein, respectively), and Tau levels below (0.2; 0.5 Tau) and above (0.7 1.2 Tau) requirements, as determined by Martins et al., (2017). Diets were formulated to have an essential amino acid profile corresponding to the whole-body profile of European sea bass (Kaushik et al., 1998; Peres and Oliva-Teles, 2006, 2007).

All dietary ingredients were finely ground, thoroughly mixed and dry pelleted in a laboratory pellet mill (CPM) through a 3-mm die. The pellets were dried in an oven at 65˚C for 24h and stored in the refrigerator. Ingredient composition and proximate analysis are present in Table 1 and amino acid composition in Table 2.

Proximate analysis of ingredients and, diets were performed according to standard methods (AOAC, 1980). For amino acids analysis, the samples were hydrolysed with 6 N hydrochloric acid at 112 ˚C under an atmosphere of N_2 for 23h. Samples were then derivatized with phenylisothiocyanate (PITC) reagent before separation by gradient exchange chromatography (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), according to the Pico – Tag method as described by Cohen et al (1989). Chromatographic peaks were identified, integrated and quantified with a Waters Breeze software package by comparing to a known amino acid standard (Pierce NC10180).

Table 1. Ingredient composition and proximate analysis (% dry weight) of experimental diets.

	Tau level (% DM)			
	0.2	0.5	0.7	1.2
Ingredients				
Fish meal[1]	12.5	12.5	12.5	12.5
Soybean meal[2]	20	20	20	20
Corn gluten[3]	20	20	20	20
Wheat gluten[4]	7.2	7.2	7.2	7.2
Wheat[5]	10.8	10.8	10.8	10.8
Pea protein concentrate[6]	4.7	4.7	4.7	4.7
Cod liver oil	15.3	15.3	15.3	15.3
Vitamin[7]	1.0	1.0	1.0	1.0
Coline	0.5	0.5	0.5	0.5
Mineral[8]	1.0	1.0	1.0	1.0
Agar	1.0	1.0	1.0	1.0
Blinder[9]	1.0	1.0	1.0	1.0
Dicalcium phosphate	3.5	3.5	3.5	3.5
Cellulose	1.5	1.2	1.0	0.5
Taurine	0.2	0.5	0.7	1.2
Proximate analysis				
Dry matter (%)	87.0	94.5	94.8	86.6
Ash	10.2	9.6	9.2	9.9
Protein	44.6	45.2	45.7	45.7
Lipid	18.0	18.2	18.2	18.2

[1] Pesquera Centinela, Steam Dried LT, Chile (CP: 69.6%; CL: 7.7%), Sorgal, S.A. Ovar, Portugal
[2] Non-GMO; Cargill France SAS, St. Germain-en-Laye, France. (CP: 47.9% CL: 2.1%)
[3] Sorgal, S.A Ovar, Portugal (CP: 71.2% CL: 2.9%)
[4] Sorgal, S.A Ovar, Portugal (CP: 83.2% CL:3.9%)
[5] Sorgal, S.A Ovar, Portugal (CP: 14.9% CL: 2.2%)
[6] Sorgal, S.A. Ovar, Portugal (CP: 51.6; CL 2.5%)
[7] 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.
[8] 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).
[9] Aquacube. Agil,

Table 2. Determined amino acid composition (% Protein) of the experimental diets.

	Tau level (%DM)			
	0.2	**0.5**	**0.7**	**1.2**
Arginine	5.3	5.3	5.3	5.3
Histidine	2.6	2.7	2.6	2.6
Isoleucine	5.0	5.0	4.9	4.9
Leucine	9.5	9.6	9.5	9.5
Lysine	5.3	5.3	5.3	5.3
Methionine	2.4	2.4	2.4	2.3
Phenylalanine	6.0	6.0	6.0	5.9
Threonine	4.1	3.9	4.0	4.0
Valine	5.9	5.9	5.9	5.8
Taurine	9.0	22.6	31.7	78.1
Tyrosine	4.4	4.5	4.4	4.4
Alanine	7.8	7.9	7.8	7.8
Aspartic acid	9.0	8.9	8.8	8.9
Glutamic acid	17.1	16.9	17.0	16.8
Glycine	2.8	2.7	2.8	2.7
Serine	4.8	4.8	4.9	4.9
Proline	6.8	6.7	6.8	6.8

Experimental conditions and sampling

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

The trial was carried out at the Marine Zoology Station, University of Porto, in a thermo-regulated recirculating water system equipped with 12 cylindrical fiberglass tanks of 100 L water capacity, supplied with a continuous flow of filtered seawater and aerification provided by diffusion through air stones. European sea bass (*Dicentrarchus labrax*) juveniles were provided by a commercial fish aquaculture. Prior to the trial, fish were acclimated to the experimental facilities for two weeks. Then, 12 homogenous groups of 12 fish with a mean body weight of 55g were established and diets were randomly assigned to triplicate of these groups. During the trial fish were fed by hand, twice a day, 6 days a week, until visual apparent satiety (established after the first three pellets reach the bottom of the tank). The trial lasted 10 weeks and during this period, water temperature was maintained at 24˚C ±1˚C, salinity average at 34‰, ammonia below 0.05 mg l^{-1}, and photoperiod controlled for 12:12 hours light: dark.

At the end of the feeding period, fish were anaesthetised with 0.3 ml/l methylethanol, and bulk weighed, following one day of feed deprivation. Fish continued to be fed for 3 more days and then blood and liver from 3 fish per tank were randomly sampled, 4h after the morning meal. Blood was

collected from the caudal vein, immediately centrifuged and the plasma frozen at -80˚C until analysis. Liver samples were immediately frozen in liquid nitrogen and then stored at -80˚C until measurement of enzymatic activities. For liver composition analysis, livers of another 3 fish per tank were collected and stored at -20˚C.

Liver composition

Hepatic lipid and total bile acids content were determined in lyophilized liver samples. Hepatic lipid content was determined gravimetrically according to Folch et al. (1957).

For total bile acids measurement, a solvent extraction procedure was done followed by the quantification of total bile acids using a commercial kit from Spinreact, S.A. (Gerona, Spain) (Kit. cod. 1001030). All measurements were done a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

Plasma analysis

Plasma metabolites were analysed using commercial kits from Spinreact, S.A. (Gerona, Spain) glucose (Kit cod. 1001191), triglycerides (Kit cod. 1001312), lactate (Kit cod. 1001330), albumin (Kit cod. 1001020), total protein (Kit cod. 1001291), bilirubin total and direct (Kit cod. 1002250) and, total bile acids (Kit cod. 1001030). All measurements were taken using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

Enzymatic activity

Liver samples were homogenized (dilution 1:7) in ice-cold buffer (100 mM – Tris-HCl, 0.1 mM– EDTA and 0.1 % triton X-100 (v/v), pH 7.8). All procedures were performed on ice. Homogenates were centrifuged at 20 000 **g** for 30 min at 4˚C, the resultant supernatant was collected and aliquots stored at - 80˚C until analysis. All enzymes activities were measured at 37˚C and changes in absorbance of NADPH at 340 nm were monitored to determine the enzyme activity in a microplate reader (ELx808^{TM}; BioTek Instruments), using 6.22 mM/cm as the millimolar extinction coefficient for NADPH. The optimal substrate and protein concentrations for the measurement of maximal activity of each enzyme were established by preliminary assays.

The specific assay conditions for each enzyme is described in (Pérez-Jimenez, et al., 2012, 2013). Briefly:

Malic enzyme (ME, EC 1.1.1.40) activity was performed using a reaction mixture containing 71.4 mM imidazole-HCl buffer (pH 7.4), 100mM $MgCl_2$, 8 mM NADP, and 40 mM L-malate.

Fatty acid synthase (FAS, EC 2.3.1.38) activity was measured using a reaction mixture consisteding of 0.01 mM NADPH, 0.025 mM Acetyl-CoA, 100 mM phosphate buffer and 0.6 mM malonyl CoA.

Glucose-6-phosphate-dehydrogenase (G6PDH, EC 1.1.1.49) activity was assayed using a reaction mixture containing 71.4 mM imidazole-HCl buffer (pH 7.4), 100 mM $MgCl_2$, 20 mM NADP, 10 mM glucose-6-phosphate.

3-Hydroxyacyl CoA dehydrogenase (HOAD, EC 1.1.1.35) activity was performed using a reaction mixture containing 71.4 mM imidazole-HCl buffer (pH 8), 2 mM NADH, 2 mM Acetoacetil CoA.

Hexokinase (HK*,* EC 2.7.1.1) *and glucokinase* (HK-IV*,* EC 2.7.1.2) activities were determined using a reaction mixture containing 71.4 mM imidazole – HCl (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 performed using a reaction mixture containing 71.4 mM imidazole-HCl buffer (pH 7.4), 100 mM $MgCl_2$, 2 M ClK, 3 mM NADH, 20 mM ADP, 2 units mL^{-1} LDH and 40 mM PEP.

Fructose 1,6-bisphosphatse (FBPase, EC 3.1.3.11) activity was determined with a reaction mixture containing 71.4 mM imidazole-HCl buffer (pH 7.4), 100 mM $MgCl_2$, 10 mM NADP, 240 mM 2-mercaptoetanol, 2 units mL^{-1} G6PD, 2 units mL^{-1} PGI and 0.5 mM fructose 1,6 bisphosphate.

Aspartate aminotransferase (ASAT/GOT, EC 2.6.1.1.) and *alanine aminotransferase* (ALAT/GPT, EC 2.6.1.2.) activities were assayed with kits from Spinreact (ASAT/GOT, ref 41273; ALAT/GPT, ref 41283).

Glutamate dehydrogenase (GDH, EC 1.4.1.2) activity was assayed using a reaction mixture containing 71.4 mM imidazole-HCl buffer (pH 7.4), 2.9 mM NADH, 14.3 mM ADP, 3.3 M ammonium acetate and 2 units mL^{-1} LDH.

Enzyme activities were expressed as milliunits per milligram of hepatic soluble protein (specific activity). Protein concentration was determined according to Bradford using the Bio-Rad protein assay kit and bovine serum albumin as standard. One unit of enzyme activity was defined as the amount of enzyme required to transform 1µmol of substrate per minute under the above assay conditions.

Statistical analysis

Data were checked for normal distribution and homogeneity of variances and when appropriate normalized. Statistical evaluation of the data was carried out by one-way analysis of variance and regression analysis. Significant differences among means ($P< 0.05$) were determined by the Tukey multiple range test. Statistical analysis was conducted using the SPSS 24.0 for Windows software package.

Results

Fish accepted well all experimental diets and mortality was low and not affected by dietary Tau level. Detailed data on growth performance and feed utilization of fish fed the experimental diets is presented elsewhere (Martins et al., 2017). Briefly, dietary Tau supplementation improved growth performance up to a dietary Tau level of 0.7%, while feed efficiency and N retention improved as dietary Tau level increased (Table 3). Whole-body composition was not affected by dietary Tau levels (see Martins et al., 2017).

Table 3. Growth performance of European sea bass fed with experimental diets.

	Tau level (% DM)				
	0.2	**0.5**	**0.7**	**1.2**	**SEM**
IBW (g)[1]	55.0	55.1	54.9	55.0	0.05
FBW (g)[1]	96.2^{a}	115.1^{b}	116.8^{b}	105.4^{ab}	3.00
WG (%)[2]	74.9^{a}	108.7^{b}	112.6^{b}	91.8^{ab}	5.47
DGI[3]	1.23^{a}	1.68^{b}	1.72^{b}	1.45^{ab}	0.07
FE[4]	0.70	0.92	0.96	1.02	0.05
NR (%NI)[5]	28.7^{a}	35.5^{ab}	38.9^{ab}	40.5^{b}	1.76
Mortality (%)	0.0	6.0^{ab}	6.0^{ab}	12.7^{b}	1.81

Values are presented as mean (n=3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (Tukey test; $P<0.05$).
[1]IBW and FWB: initial and final body weight;
[2]WG (%): weight gain (%) (100 x (FBW-IBW))/FBW;
[3]DGI: daily growth index (FBW1/3-IBW1/3)/time in days) ×100;
[4]FE: feed efficiency (wet weight gain/dry feed intake);
[5]NR: nitrogen retention (FBW ×IBW)/ (ABW × time in days); ABW: average body weight (IBW+FBW)/2.

Hepatic lipid content was not affected by dietary Tau supplementation, though a trend was observed for an increase of hepatic lipids until Tau requirement level was met (0.7% Tau) and, total hepatic bile acids content increased with the increase of dietary Tau levels (Table 4).

Post-prandial plasma glycaemia and total protein were considerably higher in fish fed the 0.2 Tau diet than the other diets (Table 4). On the contrary, plasma lactate was lower in fish fed the 0.2 Tau diet than the other diets. Dietary Tau supplementation did not affect plasma albumin and triglycerides (Table 4).

Plasma total cholesterol, significantly decreased as dietary Tau increased up to 0.7% (Table 4). Similarly, cholesterol HDL and LDL were also significantly lower in fish fed diets 0.7 and 1.2 Tau than the diets with lower Tau levels.

Plasma total bile acids were observed higher in fish fed the 0.5% Tau than diet 0.7% Tau, and it was not different among groups fed diets with 0.2 and 1.2% Tau. Total and indirect bilirubin were higher in fish fed diet 1.2 Tau than the other diets, while direct bilirubin was not affected by dietary Tau level (Table 4).

Table 4. Liver lipid and total bile acid level, and plasma selected parameters of European sea bass fed the experimental diets.

	Tau level (% DM)				
	0.2	**0.5**	**0.7**	**1.2**	**SEM**
Liver					
Lipids (% wet weight)	12.6	13.4	16.2	15.6	0.92
Total bile acids (µmol L^{-1})	0.7^{a}	1.0^{ab}	1.0^{ab}	1.4^{b}	0.09
Plasma					
Glucose (mg dl^{-1})	100.9^{b}	47.9^{a}	40.2^{a}	69.1^{ab}	5.85
Lactate (mg dl^{-1})	26.8^{a}	46.6^{b}	42.5^{ab}	56.5^{b}	1.29
Total protein (mg dl^{-1})	2.04^{b}	1.24^{a}	1.13^{a}	1.59^{ab}	0.08
Albumin (mg dl^{-1})	1.31	1.48	1.37	1.40	0.03
Cholesterol total (mg dl^{-1})	276.5^{c}	218.8^{b}	182.7^{a}	193.3^{ab}	8.47
Triglycerides (mg dl^{-1})	247.8	230.5	226.6	235.2	8.00
Cholesterol HDL (mg dl^{-1})	17.0^{b}	17.6^{b}	9.50^{a}	10.5^{a}	1.10
Cholesterol LDL (mg dl^{-1})	96.5^{b}	97.8^{b}	69.0^{a}	72.0^{a}	3.33
Total bile acids (µmol L^{-1})	92.8^{ab}	102.5^{b}	79.0^{a}	85.4^{ab}	2.91
Bilirubin Total (mg dl^{-1})	19.8^{a}	26.4^{a}	23.3^{a}	39.2^{b}	1.87
Bilirubin Indirect (mg dl^{-1})	5.5^{a}	12.3^{bc}	10.1^{ab}	22.9^{c}	1.83
Bilirubin Direct (mg dl^{-1})	14.3	14.0	13.2	16.3	0.68

Values are presented as mean (n=9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (Tukey test; $P < 0.05$).

Hepatic malic enzyme activity also decreased as dietary Tau increased up to 0.7% but it then increased as dietary Tau further increased (Table 5). Glucose-6-phosphate dehydrogenase activity also trended to decrease as dietary Tau level increased, but differences between groups were not statistically significant. The activity of all other enzymes measured decreased with the increase of Tau up to 0.7%, no further differences being observed, except for ASAT and GDH (Table 5). For these two enzymes, activity further decreased up to the highest dietary Tau level tested.

Table 5. Enzymatic activity (mU mg^{-1} protein) of European sea bass fed experimental diets.

	Tau level (% DM)				
	0.2	**0.5**	**0.7**	**1.2**	**SEM**
Lipogenesis					
ME	3.9^{c}	2.9^{b}	2.0^{a}	3.0^{b}	0.15
FAS	0.6^{b}	0.3^{ab}	0.2^{a}	0.2^{a}	0.04
G6PDH	239.6	152.1	183.0	150.9	14.1
β-Oxidation					
HOAD	29.2^{c}	23.1^{b}	17.0^{a}	18.6^{ab}	1.09
Glycolysis					
HK	4.3^{b}	3.6^{ab}	2.8^{a}	3.7^{ab}	0.17
GK	5.3^{b}	4.2^{ab}	3.1^{a}	4.2^{ab}	0.19
PK	39.3^{c}	30.1^{b}	19.5^{a}	23.1^{ab}	1.56
Gluconeogenesis					
FBPASE	30.3^{b}	21.1^{a}	17.5^{a}	19.9^{a}	1.04
Amino acid catabolism					
ALAT	377.5^{b}	264.0^{a}	191.8^{a}	192.0^{a}	16.5
ASAT	1371.9^{c}	822.8^{b}	709.0^{ab}	498.6^{a}	66.0
GDH	72.5^{b}	55.9^{ab}	50.3^{ab}	46.9^{a}	19.1

Values are presented as mean (n=9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters are significantly different (Tukey test; $P < 0.05$). ME, malic enzyme; FAS, fatty acid synthase; HOAD, 3-hidroxiacil Co-A dehydrogenase; G6PDH, glucose 6-fosfato dehydrogenase; HK, hexokinase; GK, glucokinase; PK; pyruvate kinase; FBPASE, fructose biphosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GDH, glutamate dehydrogenase.

Discussion

The essentially of Tau for European sea bass fed low-FM diets was confirmed in the previous study, being a dietary Tau supplementation of 0.6-0.7% required for optimum growth performance and feed efficiency (Martins et al., 2017).

The slight decrease, but not statistically significant decrease in growth performance of fish fed the highest Tau level may be explained by a decrease in feed intake (see Martins et al., 2017). The lower performance and feed intake, in fish fed excess dietary Tau level could be related with the acid properties of Tau (Carr, 1982). On the contrary, the lower growth, feed intake and N retention of fish fed the lowest dietary Tau level (0.2%) cannot be attributed to a feed intake reduction, but to a physiological mal-junction due to severe Tau deficiency.

In the present study, Tau supplementation increased protein utilization, which is in line with the observed reduction of hepatic key enzymes activity involved in amino acid (AA) catabolism. Albeit the specific role of Tau in AA metabolism is not clear, it has been demonstrated in humans (Baek et al., 2012) and mouse (Zhang et al., 2011) that Tau may improve AA metabolism through

signalling properties. Moreover, it has already been documented, that AA and their metabolites as Tau, are regulators of the protein phosphorylation cascade (Wu et al., 2009).

In fish, the activity of hepatic key enzymes of AA catabolism has been used as indicators of the metabolic utilization of dietary AA (Cowey, 1980). GDH is considered the key enzyme in ammonia production though its role in transdeamination and, therefore, high GDH activity is in accordance with high nitrogen excretion and, consequently, higher protein catabolism (Karl and Bastrop, 1995). Enzymes ALAT and ASAT are involved in transamination reactions and decreasing liver ASAT and ALAT activities, as observed in fish fed diets with increasing dietary Tau levels, indicate a reduction in substrates available for transamination as consequence of reduced dietary AA catabolism (Hansen et al., 2007).

As GDH and ASAT activities were lower and N retention was higher at the highest dietary Tau level tested, this seems to indicate that although maximum dietary Tau requirement for growth was estimated at 0.7%, the requirement for maximum protein utilization is higher. Similarly, an improvement of protein efficiency ratio and, nitrogen retention associated with a decrease in ammonia excretion was also described in California Yellowtail fed optimal dietary Tau level required for the species (Salze et al., 2017).

In sea bass, PF inclusion in the diets influence lipid metabolism and fat deposition (Dias et al., 2005; Messina et al., 2007, 2013). Likewise, it has already been proved that Tau may interfere with lipid metabolism, exhibiting a hypolipidemic action (Chen et al., 2012). In the present study, Tau supplementation to the diets led to plasma total cholesterol, HDL and LDL decrease compared to fish fed Tau deficiency diet, indicating that Tau had a plasma lowering-cholesterol effect in this species. The hypocholesterolaemia effect of Tau has been described in fish (Espe et al., 2012; Yun et al., 2012), mammals, including rats (Park et al., 1998), and humans (Lourenço and Camilo, 2002). In hypercholesterolemic rats, Tau promotes mRNA expression and remarkably increased the activity of cholesterol 7 alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in the conversion of cholesterol to bile acids (Yokogoshi et al., 1999). Moreover, Yokogoshi and co-authors described a correlation between dietary Tau level, CYP7A1 gene expression and plasma cholesterol reduction, suggesting that Tau hypocholesterolaemia effect is related to an improvement of cholesterol degradation.

In fish, the mechanisms involving in lowering-cholesterol effect remain unclear. For instance, Yun et al. (2012) reported that in turbot fed 1% Tau supplemented diet had the highest CYP7A1

and the lowest plasma total cholesterol, HDL and LDL. However, in fish fed a diet supplemented with a combination of Tau (1%) and cholesterol (1%), it was observed a decrease in plasma total cholesterol, HDL and LDL levels, but no significant differences in CYP7A1 activity were observed in comparison, to that of fish fed 1% cholesterol supplemented diets. Thus, the authors concluded that plasma cholesterol-lowering effect was not be correlated with CYP7A1 activity. The authors also concluded that hypolipidemic action of Tau was due a decrease ability of cholesterol synthesis due to decreased expression of cholesterol biosynthesis key enzymes.

In the present study, neither CYP7A1 gene expression nor CYP7A1 activity were determined, but a significant and positive correlation between liver total bile acids content and dietary Tau levels ($R=0.94$; $p<0.05$) was observed, suggesting that dietary Tau promoted an increase of bile acids biosynthesis. A positive correlation between hepatic total bile acids and the increase of dietary Tau levels was also described in red sea bream fed a casein-based diet (Matsunari et al. 2008).

Bile acids have a surfactant function, solubilizing and emulsifying fats to make them more accessible for absorption. Goto et al. (1996) evaluated the bile salts composition of several fish species and concluded that Tau is the only amino acid that conjugates with bile acids. Therefore, absence of Tau may lead to an increase of unconjugated bile acids. Unconjugated bile acids are not present in bile, however they can be reabsorbed from the intestine and returned to the liver via portal venous circulation. Thereby, Tau deficiency may result in more unconjugated bile acids available to be reabsorbed in intestine, thus the high plasma total bile acids levels observed in fish fed Tau deficient diets may be related to disturbances of bile acids absorption, possibly due to an injury of the intestinal mucosa (Mekhjian et al., 1979). Indeed, it has been reported that Tau deficiency could lead to damage in intestine and liver of fish (Yamamoto et al., 2007; López et al., 2015; Salze et al., 2016). Also in sea bass, alterations in intestinal mucosa of fish fed a Tau-deficient diet (0.2% Tau) were reported by Torrecillas et al. (2017).

The lowest plasma total bile acids detected in fish fed optimal dietary Tau level (0.7 Tau), could indicate that more bile acids are conjugated with Tau, being stored in the gallbladder and posteriorly excreted into small intestine, improving lipid digestion. Present results are in accordance to those observed in red sea bream fed non-FM diets, where Tau deficiency lead to an increase in plasma total bile acids, and higher dietary Tau content lead to lower plasma total bile acids (Goto et al., 2001; Matsunari et al., 2008). Moreover, a positive correlation between increasing dietary Tau levels and total bile salts stored in gallbladder was described in some fish

species, in which Tau supplementation was required to improve bile salts action (Goto et al., 2001; Kim et al., 2007, 2008; Matsunaris et al., 2008).

With bile acids solubility increased, its biliary secretion is also enhanced (Espe et al., 2011). In fish, bilirubin is the major constituent of biliary pigments and, conjugated with Tau, is secreted into bile (Goto et al., 2001). Bilirubin is a product of the haemoglobin breakdown, in which the heme group is converted to biliverdin that is transformed into bilirubin. Bilirubin is conjugated with albumin – indirect bilirubin - and then transported to the liver, where it is conjugated with glucuronic acid – direct bilirubin - being then transported to the biliary channels. The lower plasma direct and indirect bilirubin levels observed in fish fed optimal dietary Tau levels, evidence the reduction of haemolysis, possibly due to an antioxidant protection of membranes by Tau. The highest bilirubin levels were detected in fish fed both Tau-deficient and excess diets, suggesting that in both cases there is an increase in haemolysis. These results corroborate those observed in other marine species, such as red sea bream that when fed non-supplemented Tau diet have higher biliverdin levels than in fish fed Tau-supplemented diets (Goto et al., 2001; Takagi et al., 2006).

Tau deficiency is often related with haematocrit decrease and green liver syndrome appearance. These effects have been attributed to an increase in bile pigments excretion from the liver into bile and a decreased production of haemolytic biliverdin (Takagi et al., 2005, 2006). Green liver syndrome was described in red sea bream (Goto et al., 2001; Takagi et al., 2006), yellowtail (Takagi et al., 2005) fed Tau-deficient diets. However, in our study, green liver syndrome was not detected suggesting that appearance of this syndrome could be species-specific.

In the present study, Tau addition to the diets affected the activity of key lipogenic enzymes. The highest ME, FAS and G6PDH activities were verified in fish fed Tau-deficient diet. It was reported in sea bass that lipogenesis enzymes may respond to plasma cholesterol levels (Dias et al., 1998, 2005). As aforementioned, fish fed Tau-deficient diets, presented the highest plasma cholesterol levels. Therefore, the higher lipogenesis key enzymes activities may be related to the hypercholesteremia observed in that group and that is possibly due to Tau deficiency. Contrarily, fish fed optimal dietary Tau levels presented the lowest FAS, EM, and G6PDH activities, suggesting a reduced lipogenesis in fish fed adequate Tau levels. Additionally, it was observed an inverse correlation between hepatic lipid content and lipogenesis enzymes activity. The present results are partially contradictory to those described in totoaba, where Tau supplementation enhanced G6PDH activity but not FAS and ME activities (Bañuelos-Vargas et al., 2014).

Lipogenesis is affected by LXR (hepatic X receptor), a nuclear receptor presents in liver, which in addition to other functions, induces lipogenic gene expression (Schultz et al., 2000). In rats, it was described that Tau directly binds to LXR ligand, repressing cholesterol accumulation, without inducing the expression of FAS (Hoang et al., 2012). So, a possible binding of Tau to LXR in sea bass could explain the decreased on activity of key lipogenesis enzymes while decreasing plasma cholesterol levels. Nevertheless, further studies are necessary to better elucidate the exact mechanism by which Tau is involved in lipid metabolism.

The activity of lipogenesis key enzymes can also be influenced by an excess glucose and protein catabolism. Many studies performed in sea bass suggests that in the liver excess glucose is converted to glycogen, lipids or burned for energy purposes (Dias et al., 1998; Enes et al., 2006a, b, Castro et al., 2015). Thus, the excess of plasma glucose levels and protein catabolism could also induce an increase of lipogenesis key enzymes activity, as it was observed in this study in fish fed Tau-deficient diets.

Moreover, fish fed diets deficient an excess of Tau had higher activity of a key enzyme of β-oxidation (3-hidroxiacil Co-A dehydrogenase) than fish fed optimal dietary Tau level. In rats, it was shown that Tau is involved in β-oxidation by increasing the activity of the rate limiting enzyme carnitine palmitolytransferase, thus promoting the influx of unbound fatty acids through mitochondrial membrane to be oxidized through β-oxidation (Fukuda et al., 2010). This may explain the increased lipid catabolism in fish fed high Tau diets, but further studies are necessary to clarify increased β-oxidation observed in fish fed Tau-deficient diets.

In terrestrial animals, several effects of Tau on glucose metabolism have been reported, including a hypoglycaemic and antidiabetic action, increased glucose uptake by the cells, potentiation of insulin action, and enhanced glycogenesis, glycolysis, and glucose oxidation (Huxtable, 1992). Also in fish, Tau has been recognized to have a hypoglycaemia action, not directly connected to an increase of insulin release but by improving insulin action, facilitating the interaction of insulin with its receptor, and by improving glucose transport (Bañuelos-Vargas et al., 2014; Han et al., 2014). Data on the effect of Tau supplementation on glucose metabolism are scarce, however it is of interest to study, especially for carnivorous fish species, which not tolerate high dietary carbohydrate levels (Enes et al., 2011).

In the present study, both glycolysis and gluconeogenesis were increased in fish fed Tau-deficient diets, suggesting a dysregulation of glucose metabolism due to a lack of available Tau.

This was also reflected on plasma glucose levels, which were also higher in fish fed Tau-deficient diets than in fish fed the diets above optimum dietary Tau level. These results are in accordance with those observed in totoaba juveniles (Bañuelos-Vargas et al., 2014), cobia (Watson et al., 2014), and Japanese flounder (Han et al., 2014).

Previous studies assessing dietary carbohydrate utilization in sea bass (Enes et al., 2006a, b), indicated that HK enzyme is not under nutritional regulation, while GK is affected by dietary composition. Results of our study, corroborating the observations, with HK activity being constant while GK activity was affected by diet composition, being higher in fish having higher plasma glucose levels.

GK and PK activities were higher in fish fed Tau deficient diets, which also had highest plasma glucose levels. In fact, Enes et al. (2006b) reported a similar relationship between high plasma glucose levels and glycolytic enzymes. The authors proposed that elevated plasma glucose levels may promote glycolytic enzymes inducing liver to store excess glucose. This pattern was also described in other marine fish as rainbow trout (Panserat et al., 2001), and gilthead sea bream (Enes et al., 2008b). In the present study we did not measured glycogen content in the liver, but liver lipids and hepatosomatic indices (see Martins et al., 2017) were not affected by dietary Tau level, suggesting that such increased store of glycogen in the liver might not be occurred.

In the present study, the activity of FBPase a key enzyme of gluconeogenesis was also higher in fish fed Tau-deficient and excess diets. This indicates a regulation of gluconeogenesis due to nutritional factors. The regulation of FBPase and gluconeogenesis in European sea bass is still controversial, as some studies indicate that such regulation does occur while other studies confirmed a down-regulation of FBPase activity in relation to nutritional stimulus (Enes et al., 2011).

Overall, these results highlight the physiological importance of Tau on cholesterol and bile acid metabolism. Moreover, it was evident that dietary Tau affected overall metabolism, with protein, carbohydrate and lipid metabolism being dysregulated in fish fed Tau deficient diets. Further studies are still needed to better elucidate the overall role of Tau on metabolism.

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Chapter 4 – General conclusions

The results presented in this thesis allow us to conclude that:

Dietary Tau supplementation improve growth performance and feed utilization of European sea bass juveniles fed low-FM based diets (12.5%).

Optimal Tau inclusion level for maximum daily growth index, nitrogen accretion, and feed utilization varies between 0.6-0.7% depending on the fit model used.

High dietary Tau levels (above 0.7%) seem to negatively affect performance of sea bass.

Dietary Tau level did not affect either HIS and VI or whole-body composition, except for lipid content which was lower in fish fed highest Tau levels.

Total cholesterol, HDL and LDL decreased with the increase of dietary Tau supplementation, which seems to indicate that Tau has a hypocholesterolaemia effect.

Dietary Tau supplementation decreased post-prandial glycaemia suggesting that Tau may have a hypoglycaemia effect in sea bass.

Total bile acids production increased as dietary Tau level increased even above dietary Tau requirement estimated for maximum growth performance.

Further studies are necessary to better understand the effect of Tau on lipogenesis, glycolysis and protein metabolism.

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