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MSc

Dietary protein requirement and intermediary metabolism response to protein / carbohydrate ratio in zebra seabream (*Diplodus cervinus*, Lowe 1838) juveniles

Filipe Fernandes Coutinho

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

O Presidente do Júri,

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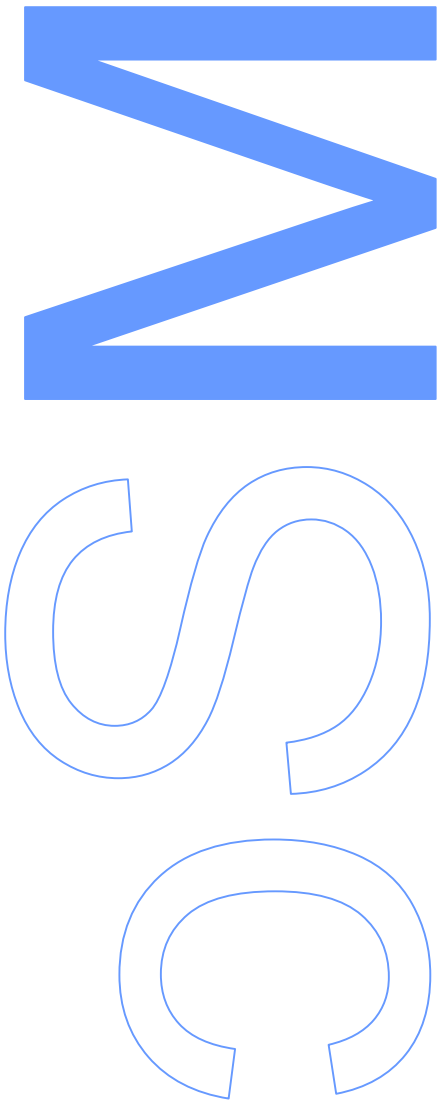

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Abstract

In the last decade the production potential of several sparid species have been investigated with the goal of diversifying European aquaculture and by so maintain profitability. Among the *Diplodus* genus several species have already been considered but no attention was yet given to zebra seabream (*Diplodus cervinus*). In order to evaluate the potential of this relatively unknown omnivorous species to aquaculture production, it is crucial to attaining some basic knowledge of its growth rate, feed utilization and protein requirement. In this scope, a growth trial was conducted to evaluate the effects of nine diets, containing increasing protein levels (5, 15, 25, 30, 35, 40, 45, 50 and 55%) and concomitant decrease of the carbohydrate content, on growth response, nutrient utilization, whole-body and liver composition, ammonia and urea excretion and on the activity of key enzymes of intermediary metabolism. Based on growth and protein retention results, zebra seabream protein requirement was also estimated by fitting the data to a polynomial model.

Final body weight, weight gain, feed efficiency and daily growth index significantly increased with the dietary protein level up to 40% (40P). Feed intake, energy intake (EI) and retention (kJ kg ABW^{-1} day^{-1} or % EI) were statistically equivalent between 25P and 55P diets. Nitrogen intake (NI, g kg ABW^{-1} day^{-1}) and ammonia excretion (mg NH_4–N kg^{-1} day^{-1} or %NI) significantly increased with the dietary protein level. Nitrogen retention (g kg ABW^{-1} day^{-1}) increased with the dietary protein but were statistically equivalent between 35P and 55P diets. The contribution of the urea excretion to total excreted N was higher in fish fed 15P and 25P diets. Fish fed diets 5P and 15P presented the lowest whole-body dry matter, protein, lipid and energy contents, which were also lower than those fish initial whole-body composition. The hepatosomatic index decreased as dietary protein content increased. Fish fed diets 50P and 55P presented lower liver lipid contents than those fed diets 25P and 45P, while no differences were found on hepatic glycogen. With the exception of the protein content, whole-body composition of fish fed 25 to 55% protein diets was statistically equivalent.

Optimum dietary protein requirement was estimated to be 43.8% for maximum weight gain and 46.2% for maximum N retention, corresponding to a protein intake of 7.63 g kg ABW^{-1} day^{-1}. Protein requirement for maintenance was estimated to be 1.01 g kg ABW^{-1} day^{-1}. The activities of aspartate aminotransferase, fructose-1,6-bisphosphatase, fatty acid synthetase and hexokinase were not affected by dietary treatments. Alanine aminotransferase activity was higher in fish fed diet 55P. Glutamate dehydrogenase activity was higher in fish fed diets 45P and 55P. Fish fed

diet 45P also revealed significant higher glucose-6-phosphate dehydrogenase and lower glucokinase activities than fish fed the 25P diet. Malic enzyme activity decreased with the dietary protein increase.

Overall, results revealed that zebra seabream is a slow growing species with a relatively high dietary protein requirement, which makes it not very appealing for aquaculture production. On the other hand, this species ability to adapt protein catabolism to protein intake and efficiently utilize starch as energy source, stand as valuable attributes in the context of increasing the use of plant-based aquafeeds and in semi-intensive aquaculture production.

Keywords: growth performance; intermediary metabolism; nitrogen excretion; nutrient utilization; protein requirement; Zebra seabream.

Resumo

Na última década, o potencial de produção de vários esparídeos tem vindo a ser investigado com o objetivo de diversificar a aquicultura europeia e assim manter a sua rentabilidade económica. Várias espécies pertencentes ao género *Diplodus* foram já consideradas, à excepção do Sargo-veado (*Diplodus cervinus*). A fim de avaliar o potencial para a produção aquícola desta espécie omnívora relativamente desconhecida, é imperativo adquirir conhecimento básico quanto à sua taxa de crescimento, utilização do alimento e necessidade de proteína. Neste âmbito, foi realizado um ensaio de crescimento para avaliar os efeitos de níveis crescentes de proteína na dieta (5, 15, 25, 30, 35, 40, 45, 50 e 55%) e consequente redução dos níveis de hidratos de carbono, no crescimento, utilização dos nutrientes, excreção de amónia e ureia, bem como na actividade de enzimas-chave do metabolismo intermediário. Com base nos resultados de crescimento e retenção proteica, as necessidades proteicas do Sargo-veado foram também estimadas através do ajuste dos dados a um modelo polinomial.

O peso médio final, o ganho de peso, a eficiência de utilização do alimento e o índice de crescimento diário aumentaram significativamente com o nível de proteína da dieta até 40% (40P). A ingestão do alimento, e a ingestão (IE) e retenção (kJ kg peso corporal médio $(PM)^{-1}$ dia^{-1} ou % IE) de energia foram estatisticamente equivalentes entre as dietas 25P e 55P. A ingestão de azoto (IA, g kg PM^{-1} dia^{-1}) e a excreção de amónia (mg NH_4–N kg^{-1} dia^{-1} ou %IA) aumentou significativamente com o nível de proteína da dieta. A retenção azotada (g kg PM^{-1} dia^{-1}) aumentou com a proteína da dieta, mas foi estatisticamente equivalente entre as dietas 35P e 55P. A contribuição da excreção de ureia para a excreção azotada total foi superior nos peixes alimentados com as dietas 15P e 25P. Os peixes alimentados com as dietas 5P e 15P apresentaram o conteúdo corporal em matéria seca, proteína, lípidos e energia mais baixos, tendo sido inclusivamente inferiores à composição corporal inicial. O índice hepatossomático diminuiu com o aumento da proteína. Os peixes alimentados com as dietas 50P e 55P apresentaram um menor conteúdo lipídico hepático do que os alimentados com a dieta 25P. O teor em glicogénio hepático não foi significativamente afectado pelo teor proteico das dietas. Com a excepção do conteúdo proteico, a composição corporal foi estatisticamente equivalente entre os peixes alimentados com os níveis proteicos de 25 a 55%.

A estimativa das necessidades proteicas para um ganho de peso e retenção azotada máximos corresponderam a 43.8 e 46.2%, respectivamente, o que equivale a

uma ingestão proteica de 7.63 g kg PM $^{-1}$ dia $^{-1}$. As necessidades proteicas de manutenção foram estimadas em 1.01 g kg PM $^{-1}$ dia $^{-1}$. A actividade das enzimas aspartato aminotransferase, fructose-1,6-bisfosfatase, complexo sintetase de ácidos gordos e hexocinase não foi afectada pelas dietas testadas. A actividade da alanina aminotransferase foi superior nos peixes alimentados com a dieta 55P. Os peixes alimentados com as dietas 45P e 55P apresentaram uma maior actividade da enzima glutamato desidrogenase. Os peixes alimentados com a dieta 45P também revelaram uma maior actividade da glucose-6-fosfato desidrogenase e uma menor actividade da glucocinase, do que os peixes alimentados com a dieta 25P. A actividade da enzima málica diminuiu com o aumento da proteína da dieta.

Em suma, os resultados revelaram que o Sargo-veado é uma espécie de crescimento lento com necessidades proteicas elevadas, o que a torna pouco atraente para a produção aquícola. Por outro lado, o facto de esta espécie ser capaz de adaptar o catabolismo proteico à ingestão proteica e de utilizar o amido como uma eficiente fonte de energia constituem atributos de elevado valor num contexto de utilização de rações à base de matérias-primas vegetais, bem como na produção aquícola semi-intensiva.

Palavras-chave: Excreção azotada; metabolismo intermediário; necessidades proteicas; Sargo-veado; taxa de crescimento; utilização dos nutrientes.

Contents

1. Introduction

Worldwide annual *per capita* fish consumption grew from an average of 17.0 kg in the 2000s to 18.4 kg in 2009, and is expected to reach 18.6 kg in 2010. In Europe *per capita* fish consumption in 2009 exceeded the average world fish consumption reaching 22.0 kg. In 2010, within European Union (EU), Portugal was the major fish consuming country, with an annual *per capita* fish consumption of circa 61.6 kg, which contributed with more than 20% to the total animal protein supply. With the continuous increase in world fish demand and the world capture fisheries production relatively stagnated for the past decades (around 90 million tonnes), major increases in fish food production are forecast to come from aquaculture (European Commission, 2012; FAO, 2012).

In the last decade aquaculture had an average annual growth rate of 5.8%. Although a slowing in aquaculture growth to 2.4% between 2012 and 2021 is anticipated, it is expected that aquaculture will remain one of the fastest-growing food-production sectors. In this sense aquaculture contribution to global fishery production is expected to increase from 40% on average in 2009-2011 to 46% in 2021 (FAO, 2012).

In 2010 marine fish production only represented 3.1% (1.8 million tonnes) of world aquaculture production, while its value ascended to 6.7% (8.0 million dollars) of the total, revealing the high potential value of this still emerging segment of aquaculture production. In 2010, aquaculture production in the EU represented 2.1% in terms of volume and 3.4% in terms of value of global aquaculture production. In the same year, marine fish production represented 12.2% in terms of volume and 23.7% in terms of value of total EU aquaculture production, revealing a much higher share of marine fish production in EU aquaculture when compared to the world average (FAO, 2012; FIGIS, 2012).

Gilthead seabream (*Sparus aurata,* Linnaeus 1758) and European seabass (*Dicentrarchus labrax*, Linnaeus 1758) are the two main marine species produced in the EU, representing alone 89.4% (55.2 and 43.2%, respectively) in terms of quantity and 84.2% (47.3 and 37.0%, respectively) in terms of value of total 2010 marine production. In the specific case of Portugal, turbot (*Scophthalmus maximus*, Linnaeus 1758), gilthead seabream and European seabass were the three major produced marine fish species in 2010, representing 96.2% (45.3, 34.3 and 16.6%, respectively) in terms of quantity and 97.3% (52.7, 29 and 15.6%), in terms of value of total marine aquaculture production (FIGIS, 2012).

The fact that marine aquaculture production in the EU has been restricted to a relatively small number of fish species created the need to further diversify exploited species, in order to decrease concurrence within the sector and to maintain profitability (Hernandez et al., 2001). The *Sparidae* family includes a large number of species with high economic value that are potential candidates for EU aquaculture. Moreover, as these candidate Sparid species may be produced by adapting existing gilthead seabream production technologies, they are of special interest to Mediterranean aquaculture industry (Pavlidis and Mylonas, 2011). Sharpsnout seabream (*Diplodus puntazzo*, Cetti 1777) appears as one of the most promising bream species, presenting growth rates similar to European seabass. White seabream (*Diplodus sargus*, Linnaeus 1758) has also been recognized as good candidate (Guerreiro et al., 2010), despite its slower growth rate than sharpsnout seabream, while annular seabream (*Diplodus annularis*, Linnaeus 1758) and common two-banded seabream (*Diplodus vulgaris*, Geoffroy Saint-Hilaire 1817) present growth rates considered inappropriate for intensive aquaculture production (Divanach et al., 1993; Ozório et al., 2009). Zebra seabream (*Diplodus cervinus,* Lowe 1838) is another species within the *Diplodus* genus that may be of potential value, though little attention was given to it up to now (Figure 1).

Fig. 1 – Zebra seabream (Photo by Filipe Coutinho)

Zebra seabream is a coastal demersal species that inhabits rocky bottoms of warm areas (18 to 24ºC) down to depths of about 300m, but is more frequent up to 80m. This species is found from the Bay of Biscay to Cape Verde Islands, from Angola to South Africa, as well as in the warmer areas of the Mediterranean Sea and around Madeira and Canary Islands (Bauchot and Hureau, 1990). In its natural habitat zebra seabream is known to be a slow growing species. It is a long-lived (lifespan estimated of up to 17 years) protogynous hermaphrodite species which maturity is reached at a

late age (4 and 5 years for females and males, respectively). Males mature later and with a larger total length than females (327 mm and 5 years old or 273 mm and 4 years old, respectively) and the reproductive season extends from spring to summer (Pajuelo et al., 2003). It is an omnivorous species that presents both pelagic and benthic predation. Similarly to gilthead seabream, zebra seabream exhibits a nonspecific predation and its diet consists mainly on crustaceans, and bivalve molluscs (Derbal and Kara, 2006).

Despite not presenting a very promising growth rate, the fact that zebra seabream only reaches maturity at late age and with relatively large size makes it worthwhile studying its potential for aquaculture production, since along the grow-out stage no energy is lost through gonad maturation. Besides, the species omnivorous nature highlight the possible efficient use of lipids and carbohydrates as energy sources, as well as the tolerance of high levels of dietary plant protein in the diet. If this proves to be the case, it could be feasible to develop commercial diets more cost-effective than those used for feeding carnivorous marine species, whose diets still incorporate a high proportion of fishmeal (which increases diets costs). However, as no nutritional information or growth performance under culture conditions is available for this species, some basic nutritional research is still required to evaluate its potential for aquaculture production.

The estimation of protein requirement for a given species provides the basis for practical feeds formulation, particularly when no further nutritional information is available (NRC, 2011). By having lower energy requirement than terrestrial animals, fish require higher protein concentration in the diet to achieve maximum growth rate, although in absolute terms no or little differences in protein requirements are noticed between fish and farm animals (Bowen, 1987; Cowey and Luquet,1983; Wilson, 2002).

However protein role in satisfying fish energy requirements is considered high and protein usually represents the most important energy source particularly in carnivorous fish, which is to great extent attributable to an absence of metabolic regulation of amino acids oxidation (Cowey and Luquet, 1983; Weber and Haman, 1996). This expenditure of amino acids (AA) through inevitable catabolism comprises 20 to 40% of the absorbed AA above maintenance requirements (AA needed to maintain fish protein pool stable), which generally comprise only 5 to 20% of total AA requirements (Abboudi et al., 2007, 2009; Bureau et al., 2002; Richard et al., 2010). Nevertheless, despite the inevitable AA catabolism, the major fate of AA (25 to 55% of total AA consumed) is protein synthesis, including structural and functional molecules (NRC, 2011).

Protein requirement refer to the amount of protein needed to fulfil fish EAA requirements as well as the demand of amino groups, for the synthesis of non-essential AA (NEAA), and AA to meet energy and other metabolic demands. Protein requirement is usually determined by dose-response trials with fishmeal as the main dietary protein source, given its high quality protein content and ideal EAA profile for fish (NRC, 2011; Wilson, 2002). In fact, diets deficiency in one or more EAA may depress feed intake, limit protein deposition or other AA retention, resulting on growth retardation, higher AA catabolism and consequent increase of nitrogen excretion (Roberts, 2002; Tacon, 1992; Tibaldi and Kaushik, 2005; Wilson, 2002). This is of particular importance when total or partial fishmeal replacement by vegetable protein is intended, as AA composition and availability are dependent of the feedstuff used, justifying nowadays trend to adjust diets AA profile by using a mixture of several protein sources (Kaushik and Seiliez, 2010; Peres and Oliva-Teles, 2008).

In dose-response studies more importance is given to the number of dietary protein levels tested rather than to the number of replicates, in order to improve model fitting to the data and to increase the degrees of freedom thus increasing the robustness of the estimate (Hernandez-Llamas, 2009). Besides, tested protein levels must be positioned aiming that ½ of it is around an expected requirement interval, but should also ensure a good representativeness of both ascending and plateau portions of the response curve, in order to ensure accurate requirements estimation (Shearer, 2000).

Several models are available for fitting the dose-response relationships: broken line; exponential; quadratic; five-parameter saturation kinetics (5-SKM, Mercer et al., 1989); four-parameter saturation kinetics (4-SKM, Mercer et al., 1984). From these, 4-SKM, 5-SKM and quadratic (second-order polynomial) models usually provide more accurate estimates. Model choice must be the one responsible for the best data fit and still has a biological meaning (Shearer, 2000). In the case of the 4-SKM, as the maximum response is given by an asymptote, the protein requirement value is arbitrary chosen as corresponding to 95 or 99% of the plateau-value (NRC, 2011; Shearer, 2000). In several studies, using the exponential and polynomial models, protein requirement has also been determines as corresponding to 95 or 99% of the maximum response (Coutinho et al., 2012; Encarnação et al., 2004; Ozório et al., 2009; Sá et al., 2008a).

The protein-to-energy ratio of experimental diets is also a relevant issue that needs to be taken into account. Because fish regulate intake to meet energy requirement, excess dietary energy may limit feed intake, independently of the dietary

protein level, while a limited non-protein dietary energy level may increase the use of AA to meet energy needs, thus increasing its catabolism (NRC, 2011; Wilson, 2002). The duration of the trial is also important as it needs to be long enough to assure representative differences in the response variables. Although both weight gain and protein retention have been used as response variables for protein requirements estimates (Coutinho et al., 2012; Encarnação et al., 2004; Glencross et al., 2011; Ozório et al., 2009; Sá et al., 2008a; Trung et al., 2011; Zhang et al., 2011), protein retention is preferred, since using weight gain as criterion, possible changes in whole body composition during growth would be ignored (Cowey, 1992).

Protein quantification is also an inherent question in this type of studies, as crude protein is generally calculated based on total nitrogen content (assuming that N content of an average protein is 16%), with the associated error of not taking into account the amount of nonprotein N containing compounds of feed ingredients, which in some cases may ascend to 10 or even 20% of assumed protein content (Helland et al., 2010; Mariotti et al., 2008).

However, when correctly applied protein requirements estimates are very useful tools for the formulation of practical or commercial diets, with special relevance in the case of new species for aquaculture production. Protein, by representing one of the major diet constituents and being also one of the most expensive, is in great part responsible for the final feed price. Moreover, following amino acid catabolism carbon dioxide, bicarbonate and ammonia, which is highly toxic, are excreted by fish, having a very negative impact on the environment (Enes et al., 2009; NRC, 2011; Peres and Oliva-Teles, 2001, 2002). Both reasons demonstrate the importance of determining the exact amount of protein inclusion in feeds that assure maximal fish growth, at the lowest feed price and environmental impact. In this sense, it is also essential to gain further understanding of fish protein metabolism in order to maximise protein use for plastic rather than for energy purposes (Enes et al., 2009; Kumar et al., 2010).

If dietary protein is supplied above requirements only a part of it will be used for the synthesis of new proteins, while most part of the excess AA will be catabolized and converted to energy (Wilson, 2002). This and other previously referred imbalances between AA supply and utilization for protein synthesis all lead to AA oxidation in the liver (NRC, 2011). Amino acids breakdown starts by a deamination, in most cases by transdeamination, with removal of an amino group that can rather be converted in ammonia or transferred to α-ketoglutarate to form glutamic acid (involved in ammonia excretion). The carbon skeletons (α-keto acids) produced by deamination can then be further metabolized in the tricarboxylic acid cycle (TCA), following different oxidative

pathways depending of the type of carbon skeleton (depends of the original AA) and yielding energy (net biological efficiency of converting protein to energy is of about 40%) or being converted to other compounds, such as fatty acids, glucose and/or glycogen. (Wilson, 2002; NRC, 2011).

In order to prevent ammonia high toxicity, higher vertebrates spend energy converting ammonia to urea or uric acid, for excretion in the urine. On the contrary most teleost fish are ammoniotelic, presenting an extremely efficient transfer mechanism for ammonia across the gills (by direct diffusion of NH_3 from the blood to water and/or through Na^+/NH_4^+ exchange) and having only a limited portion of N excretion in the form of urea (5-15% of total N excretion) (Wilson, 2002; NRC, 2011). This high efficiency of using AA as energy sources may help to explain the important role of protein on satisfying fish energy requirements, as well as the lower maximum feed protein retention achievable in fish (up to 55%) when compared to other animals. The plasma ammonia concentration is dependent of the fish species and protein intake and begins to rise 3-8 hours following a meal. While fresh water fish present higher levels of plasma nitrogen after a meal than marine fish (6.5 vs 3.5mg/L respectively), the last have much higher plasma urea concentrations (6.5-7 vs 44-59 mg/L, respectively) that do not seem to be related to dietary protein intake (Halver and Hardy, 2002).

It is known that AA utilization is affected by numerous factors, including diet composition, and that metabolic utilization of absorbed glucose as well as the amount of net energy that can be derived from digestible carbohydrate are both limited and species dependent (Bureau et al., 1997; Kaushik and Seiliez, 2010). In this sense, alterations on fish intermediary metabolism induced by an increase of dietary protein with the expense of carbohydrate are to be expected and deserve to be investigated. Thus, dietary effects in the activities of key enzymes involved in the main metabolic pathways, which are AA catabolism (alanine aminotransferase (ALAT); aspartate aminotransferase (ASAT); glutamate dehydrogenase (GDH)), gluconeogenesis (fructose1,6-bisphosphatase (FBPase)), glycolysis (glucokinase (GK); hexokinase (HK); pyruvate kinase (PK)), glycogenesis, pentose-phosphate pathway (glucose-6 phosphate dehydrogenase (G6PDH)) and lipogenesis (malic enzyme (ME); fatty acid synthetase (FAS)), deserve to be evaluated.

Depending on the fish species, some general metabolic responses to major dietary nutrients have already been identified. For instance, it is known that dietary carbohydrates stimulate glycolysis, glycogenesis and lipogenesis, while reducing protein catabolism and gluconeogenesis (Pérez-Jiménez et al., 2009).

On the other hand the effect of dietary protein on the hepatic activity of key enzymes involved in amino acid catabolism is relatively contradictory (Peres and Oliva-Teles, 2007). Evidence suggest that protein-rich diets stimulate gluconeogenesis (Pérez-Jiménez 2009) and AA catabolism, by increasing the hepatic activity of ALAT, ASAT and GDH (Bibiano et al., 2006; Gallagher, 1999; Gaye-Siessegger et al., 2006; Sánchez-Muros et al., 1998; Stone et al., 2003; Suárez et al., 1995). In the same way, low-protein diets or AA imbalances have also been reported to affect the activity of specific AA deamination and transamination enzymes (Fournier et al., 2003; Kim et al., 1992; Suarez et al., 1995). However, other authors reported an absence of regulation of those enzymes by dietary protein or AA (Gouillou-Coustans et al., 2002; Kirchner et al., 2003; Moyano et al., 1991; Peres and Oliva-Teles, 2006, 2007). Moreover, and despite the wide use of AA deaminating and transaminating enzymes activity as indicators of dietary protein and AA utilization, Cowey (1995) defended a non-adaptive character of deaminating enzymes, while Moyano et al. (1991) reported for GDH and transaminase enzymes activities being highly susceptibility to different nutritional factors.

Despite the inconstancies on the AA catabolism response to dietary protein levels, if part of the dietary protein energy is substituted by carbohydrates, a decrease of AA-catabolizing enzymes activity is to be expected, especially in the case of herbivorous and omnivorous fish species (Fernández et al., 2007; Metón et al., 1999; Shimeno et al., 1995; Walton, 1986). When ammonia excretion data is available, a good correlation of this data with glutamate dehydrogenase (GDH) activity, which is considered a key enzyme for ammonia production through its role in transdeamination (Cowey and Walton, 1989), may also be found (Peres and Oliva- Teles, 2006, 2008).

An increase of dietary carbohydrate levels with the expense of protein has also been shown to result on a decreased gluconeogenic pathway activity (Bonamusa et al., 1992; Borrebaek et al., 1993; Cowey et al., 1977, 1981; Lupiáñez et al., 1989; Panserat et al., 2002; Shikata et al., 1994; Shimeno et al., 1979, 1995; Suárez et al., 1995, 2002; Walton, 1986), while stimulating glycolytic enzymes activity (Caseras et al., 2002; Fernández et al., 2007; Kirchner et al., 2005; Lupiáñez et al., 1989; Metón et al., 1999, 2000; Panserat et al., 2000; Suárez et al., 2002; Walton, 1986).

A clear response pattern of lipogenesis to the substitution of dietary protein by carbohydrates has still not been found. In some cases it was shown to up regulate G6PDH activity (Bonamusa et al., 1992; Borrebaek et al., 1993; Fernandez et al., 2007; Méton et al., 1999; Shimeno et al., 1995) and ME (Borrebaek et al., 1993; Shimeno et al., 1995), which are responsible for the generation of reducing power (in the form of

NADPH) required for fatty acids synthesis, while an absence of regulation of G6PDH (Lupiáñez et al., 1989; Sá et al., 2007, 2008b; Shikata et al., 1994; Shimeno, 1979; Suarez et al., 2002), ME (Sá et al., 2007, 2008b; Walton, 1986) and FAS (Sá et al., 2008b) activities, or even a decrease of G6PDH (Borrebaek et al., 1993; Suárez et al., 2002) and FAS (Sá et al., 2007) activities were also reported. However both high levels of carbohydrate in the diets and a lower EAA/NEAA have been shown to increase lipogenic enzymes activity (Barroso et al., 2001; Fynn-Aikins et al., 1992; Likimani and Wilson, 1982; Peres and Oliva-Teles, 2006; Suárez et al., 1995), which is also affected by the dietary protein quality (Dias et al., 1999).

In view of the above considerations, this thesis aims to:

1. Evaluate the growth response, feed utilization and body composition of zebra seabream under experimental conditions and fed graded dietary protein levels;
2. Estimate zebra seabream protein requirements for maintenance, maximum growth and maximum protein retention;
3. Assess the effect of feeding increasing protein levels on the main intermediary metabolism pathways and on urea and ammonia excretion.

2. Material and Methods

2.1. Experimental diets

Nine diets were formulated to contain different protein levels (namely 5; 15; 25; 30; 35; 40; 45; 50 and 55%) and 18% lipid level, with fishmeal and fish oil as protein and lipid sources. All dietary ingredients were finely ground, well mixed and dry-pelleted in a laboratory pellet mill (CPM, California Pellet Mill) through a 2-mm die. Dibasic calcium phosphate was added to adjust dietary phosphorus level to the one estimated for the 55% protein diet. Diets were dried at 50 ºC for 24 hours and stored in plastic bags until used. Ingredients and proximate composition of the experimental diets are presented in Table 1.

Table 1 **-** Ingredients composition and proximate analysis of experimental diets.

Diets	P5	P15	P25	P30	P35	P40	P45	P50	P55
Ingredients (% dry weight)									
Fish meal[1]	1.8	15.9	29.9	37.0	44.0	51.0	58.1	65.1	72.1
CPSP[2]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cod liver oil	16.8	15.5	14.2	13.5	12.8	12.2	11.5	10.8	10.2
Gelatinized starch[3]	65.0	54.0	43.0	37.5	32.0	26.5	21.0	15.5	9.2
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix[4]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Mineral premix[5]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Binder[6]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Dibasic calcium phosphate	7.8	6.1	4.4	3.5	2.7	1.8	1.0	0.1	0.0
Proximate analysis (% dry weight)									
Dry matter (%)	93.7	94.4	93.6	92.0	91.0	94.3	94.8	91.0	92.1
Crude protein	6.6	15.8	25.8	30.7	35.7	40.2	45.3	49.6	54.0
Gross lipid	18.0	17.9	18.3	18.0	17.6	17.3	17.3	17.7	17.3
Starch	65.9	56.8	43.7	36.5	32.8	24.3	19.1	14.3	8.6
Ash	10.1	11.1	12.1	12.6	13.1	13.7	14.1	14.6	16.2
Gross energy (kJ g^{-1})	19.2	19.7	20.2	20.1	20.3	20.4	20.4	20.5	21.1
P/E (g MJ^{-1})	3.5	8.0	12.8	15.3	17.6	19.7	22.2	24.2	25.7

[1] Pesquera Diamante, Steam Dried LT. Austral Group, S.A. Peru (CP: 71.1% DM; CF: 9.5% DM).
[2] Soluble fish protein concentrate. Sopropèche, France (CP: 74.1% DM; CF: 20.1% DM).
[3] Pregelatinized maize starch. C-Gel Instant – 12016, Cerestar, Mechelen, Belgium.
[4] Vitamins (mg kg^{-1} diet): retinol, 18.000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); α-tocopherol, 35; menadion, 10; thiamine, 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.
[5] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 078; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.44 (g kg^{-1} diet).
[6] Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate) Agil, UK.

2.2. Growth trial

The trial was carried out at the experimental facilities of the Marine Zoology Station, Faculty of Sciences, Porto University, in a thermo-regulated water semi-recirculation system equipped with 18 cylindrical fibreglass tanks of 100 l water capacity, supplied with a continuous flow of filtered seawater.

Zebra seabream (*Diplodus cervinus*) juveniles were provided by IPIMAR/CRIPSul, at Olhão. After transportation to the experimental facilities the animals were submitted to a quarantine period of two weeks and then acclimated for a month to the rearing system. During this period the fish were fed a commercial diet. Then, 18 homogenous groups of 20 fish with a mean body weight of 7.7 g were established and randomly distributed to each tank. Experimental diets were randomly assigned to duplicates of these groups. During the trial, fish were fed to apparent visual satiety twice a day, 6 days a week. Utmost care was taken to assure that all feed supplied was consumed. The trial lasted 14 weeks and during this period, water temperature was maintained at 22 ± 1 ºC, salinity averaged 33 ± 2‰ and nitrogenous compounds levels were maintained near 0.00 mg/l. A natural photoperiod was adopted.

2.3. Sampling

During the trial, fish of each tank were bulk-weighted every three weeks under slight anaesthesia (ethylene glycol monophenyl ether, 0.3 ml l^{-1}), after one day of feed deprivation.

Measurements of total ammonia and urea-nitrogen excretions were performed during the growth trial, on week 13, for 2 consecutive days in each tank. For that purpose, water flow rate in the tanks was reduced to 0.2-0.5 l/min the day before and during the sampling days. Water samples were collected in the outlet of each tank at 0, 2, 4, 6, 8, 10 and 12 h after the first meal. Water collected in the outlet of a tank without fish was used as blank.

A random sample of 25 fish from the initial batch were taken, killed by lethal anaesthesia (ethylene glycol monophenyl ether) and pooled for whole-body composition analysis. At the end of the trial and following one day of food deprivation, 5 fish per tank were killed by lethal anaesthesia, weighted and whole viscera and liver extracted and weighted, for visceral index (VI) and hepatossomatic index (HIS) calculation. Liver samples were then stored in a freezer at -20 ºC until analysis, while individual carcasses and respective viscera were joined together for whole-body (without liver) composition analysis. The remaining fish from each tank were also

slightly anaesthetised, bulk weighted, put back into the respective tank and continued to be fed their respective diets for two more days. Then, from treatments receiving 25, 35, 45 and 55% protein diets, 3 h after the first meal, liver from 9 fish per tank were sampled, distributed into 3 pools of 3 livers each, immediately frozen in liquid nitrogen and then stored at -80º C until measurement of soluble protein and enzymatic activities. Based on whole-body composition analysis, nitrogen retention (g N kg ABW^{-1} day^{-1} and % N intake) and energy retention (kJ kg ABW^{-1} day^{-1} and % E intake) were estimated.

2.4. Definition of the terms used:

2.4.1. Average body weight (g)

ABW = (FBW-IBW) / 2

IBW: initial body weight (g)
FBW: final body weight (g)

2.4.2. Weight gain (g kg ABW^{-1} day^{-1})

WG = ((FBW-IBW) x 1000) / (ABW x nb days)

2.4.3. Feed intake (g kg ABW^{-1} day^{-1})

FI = ((feed intake (g dry matter / fish) x 1000) / (ABW x nb days)

2.4.4. Feed efficiency

FE = wet weight gain / dry feed intake

2.4.5. Daily growth index

DGI = (($FBW^{1/3}$-$IBW^{1/3}$) x 100) / nb days

2.4.6. Protein efficiency ratio

PER = (FBW-IBW) / Protein intake (g dry matter)

2.4.7. Nitrogen balance

2.4.7.1. Nitrogen intake (g N kg ABW^{-1} day^{-1})

NI = (N intake x 1000) / (ABW x nb day)

N intake: g dry matter

2.4.7.2. Nitrogen retention (g N kg ABW^{-1} day^{-1})

NR = (FBW×FBN) – (IBW×IBN) / (ABW × nb days)

IBN: initial body nitrogen content

FBN: final body nitrogen content

2.4.7.3. Nitrogen retention (% NI)

NR = ((FBW×FBN – IBW×IBN) / NI) × 100

2.4.8. Energy balance

2.4.8.1. Energy intake (kJ kg ABW^{-1} day^{-1})

EI = (E intake x 1000) / (ABW x nb day)

E ingestion: energy (kJ) of the feed (g dry matter) ingested by each fish

2.4.8.2. Energy retention (kJ E kg ABW^{-1} day^{-1})

ER = (FBW×FBE – IBW×IBE) / (ABW × nb days)

IBE: initial body energy content

FBE: final body energy content

2.4.8.3. Energy retention (% EI)

ER (% EI) = ((FBW×FBE – IBW×IBE) / EI) × 100

2.4.9. Hepatosomatic index (%)

HSI = (Liver wet weight (g) / whole-body wet weight (g)) x 100

2.4.10. Visceral index (%)

VI = (Viscera wet weight (g) / whole-body wet weight (g)) x 100

2.5. Analytical methods

2.5.1. Proximate analysis

Chemical analysis of the experimental diets and whole fish were conducted as follows: water content, by drying samples in an oven at 105 ºC until constant weight; ash, by incineration in a muffle furnace at 450 ºC for 16 h; protein (N × 6.25), according to the Kjeldahl method after acid digestion using a Kjeltec system; lipid, by petroleum ether extraction in a SoxTec System HT apparatus; starch according to Beutler (1984); energy, by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, model 1261). Total ammonia nitrogen was measured by the indophenol blue method (Koroleff, 1983a) and urea nitrogen by the diacetyl monoxime method (Koroleff, 1983b). Whole-fish was dried and homogenized before analysis.

To determine the hepatic glycogen content, a portion of liver was homogenized in five volumes of iced-cold distilled water and stored at − 80 °C until analysis. The glycogen content was determined by amyloglucosidase hydrolysis following the method described by Roehrig and Allred (1974). Hepatic lipids were determined gravimetrically according to Folch et al. (1957). Hepatic soluble protein concentration was determined according to Bradford (1976), using a commercial kit (Sigma protein Kit, cod. B6916) and bovine serum albumin as standard.

2.5.2. Enzymes of intermediary metabolism

Activity of key enzymes of the main metabolic pathways was determined in the liver. Tissue samples were homogenized in nine volumes of ice-cold 100 mM Tris–HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-10 0, pH 7.8. All procedures were performed on ice. Homogenates were centrifuged at 30,000 × g for 30 min at 4 °C and the resultant supernatants were kept in aliquots and stored at − 80 °C for further enzyme assays.

All enzyme activities were measured at 340 nm in an absorbance microplate reader (model ELx808™, Bio-Tek Instruments, USA), monitoring the changes in absorbance of NADH or NADP at 37ºC. The optimal substrate and protein concentrations for measurement of maximal activity for each enzyme were established by preliminary assays. The enzymatic reactions were initiated by the addition of tissue extract.

Hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activities were measured as previously described by Vijayan et al. (1990). The reaction mixture

contained 50 mM imidazole–HCl buffer (pH 7.4), 2.5mM ATP, 5mM $MgCl_2$, 0.4mM NADP, 2 units mL^{-1} G6PDH, and 1 mM (HK) or 100 mM (GK) glucose.

Fructose 1,6-bisphosphatase (FBPase; EC 3.1.3.11) activity was performed with a reaction mixture consisting of 42,84 mM imidazole–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 0.5 mM NADP, 2 units mL^{-1} G6PDH, 2 units mL^{-1} PGI and 0.5mM fructose 1,6- bisphosphate (Morales et al., 1990).

Glutamate dehydrogenase (GDH; EC 1.4.1.2) activity was performed using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 0.2 mM NADH, 1 mM ADP, 100 mM ammonium acetate, 2 units mL^{-1} LDH and 10 mM α-ketoglutarate (Morales et al., 1990).

Alanine aminotransferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT, EC 2.6.1.1) activities were measured using commercial kits from Spinreact (ALAT/GPT, ref. 41283; ASAT/GOT, ref. 41273).

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was assayed as previously described by Morales et al. (1990), using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 5 mM MgCl2, 2 mM NADP and 1 mM glucose-6-phosphate.

Malic enzyme (ME; EC 1.1.1.40) activity was performed using a reaction mixture containing 50 mM imidazole–HCl buffer (pH 7.4), 5 mM MgCl2, 0.4 mM NADP and 2 mM L-malate (Singer et al., 1990).

Fatty acid synthetase (FAS; EC 2.3.1.38) activity was quantified as described by Chang et al. (1967) and modified by Chakrabarty and Leveille (1969). Samples were incubated with solution A (100 mM potassium phosphate buffer pH 6.5, 0.1 Mm NADPH and 25 µM acetyl-CoA) for 10 min. Then solution B (100 mM potassium phosphate buffer pH 6.5 and 600 mM malonyl-CoA) was added to this mixture.

All enzyme activities were expressed as milliunits per milligram of hepatic soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate per minute under the above assay conditions.

2.6 Statistical analysis

As at the end of the trial 100% mortality was attained for one of the replicate tanks of the 5% protein diet, data from this diet was only used for protein requirements estimations and for the determination of Pearson correlation coefficients. Data were analysed by one-way ANOVA. Before analysis data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test) and

$\log_{10}(x+1)$ or arcsen($\sqrt{(x/100)}$) transformed when required. Significant differences ($p < 0.05$) among means were determined by the Tukey multiple range test. Statistical analysis was performed using a SPSS for Windows version 20 software package.

For protein requirement estimations several regression models were tested but only the second-order polynomial model provided adequate fitting to the data. This model was used to fit weight gain (g kg ABW^{-1} day^{-1}) and nitrogen retention (g N kg ABW^{-1} day^{-1}) to dietary protein levels and to fit protein retention to protein intake. The dietary protein requirement was defined as corresponding to 95% of the maximum ordinate value. Model parameters were estimated by the least-square principle using Statistica for Windows version 10 software package.

3. Results

Despite the fact that no pathological signs were observed during the trial some mortality occurred, especially for the 5 and 15% protein diets, which were not well accepted by the fish. Growth performance and feed utilization of fish fed the experimental diets are presented in Table 2. Final body weight, weight gain, daily growth index and feed efficiency significantly increased with the dietary protein level up to 40%. Fish fed the 15% protein diet presented a significant higher protein efficiency ratio than fish fed diets with 45 to 55% protein.

Nitrogen and energy balances and ammonia and urea excretion data are presented in Table 3. Feed intake and both energy intake (EI) and energy retention (expressed as kJ kg ABW^{-1} day^{-1} or as % EI) were significantly lower for the 15% protein diet. Nitrogen intake (NI, g kg ABW^{-1} day^{-1}) and retention (g kg ABW^{-1} day^{-1}) and ammonia excretion (mg NH_4–N kg^{-1} day^{-1}) significantly increased with the dietary protein level, revealing very good linear correlation coefficients to dietary protein: R^2 = 0.99, p < 0.000, n = 17; R^2 = 0.95, p < 0.000, n = 17; R^2 = 0.96, p < 0.000, n = 16, respectively. Nevertheless, nitrogen retention (g kg ABW^{-1} day^{-1}) was statistically equivalent between 35 and 55% dietary protein levels, while nitrogen retention (% NI) was higher for 15% protein diet when compared to 55% protein. Ammonia excretion (% NI) increased with the dietary protein increase up to 40%. Fish fed the 15% protein diet presented the lowest urea excretion rate (mg NH_4–N kg^{-1} day^{-1}). However, urea excretion expressed as percentage of NI was statistically higher for fish fed 15% protein diet. The contribution of urea excretion to the total N excretion was higher for 15% and 25% protein diets.

At the end of the trial whole-body protein was higher in fish fed the three higher protein levels (Table 4). The 5 and 15% dietary protein levels resulted in the lower dry matter, protein, lipid and energy and higher ash whole-body content when compared to the other protein levels tested, or fish initial whole-body composition. The lower liver lipid contents were obtained with the 50 and 55% protein diets, though differences were only significant between these diets and the 25 and 45% protein diet. No statistical differences among diets were found on hepatic glycogen. The hepatosomatic index decreased as dietary protein content increased (excluding the 5% protein diet), while no differences in visceral index were observed among groups.

Weight gain (g kg ABW^{-1} day^{-1}) and N retention (g kg ABW^{-1} day^{-1}) were fitted to dietary protein level by a second-order polynomial model (Figures 2 and 3, respectively). Based on this model, the dietary protein level for 95% maximal weight gain and N retention was estimated to be 43.8% and 46.2%, respectively. By fitting

protein retention (g kg ABW^{-1} day^{-1}) to protein intake (g kg ABW^{-1} day^{-1}) (Figure 4), protein requirements for maintenance and for maximal protein retention were estimated to be 1.0 and 7.6 g kg ABW^{-1} day^{-1}, respectively.

Table 2 **-** Growth performance and feed utilization efficiency of zebra seabream fed the experimental diets[1].

Diets	P5	P15	P25	P30	P35	P40	P45	P50	P55	SEM
Initial body weight (g)	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	0.004
Final body weight (g)	6.5	10.2 ^{a}	13.5 ^{ab}	14.6 ^{b}	16.5 ^{bc}	19.2 ^{c}	19.3 ^{c}	20.0 ^{c}	20.4 ^{c}	0.91
Mortality (%)	87.5	40.0	12.5	2.5	12.5	12.5	15.0	7.5	5.0	3.22
Weight gain (g kg ABW^{-1} day^{-1})	-1.81	2.94 ^{a}	5.73 ^{b}	6.50 ^{b}	7.60 ^{bc}	8.98 ^{c}	9.04 ^{c}	9.33 ^{c}	9.49 ^{c}	0.76
Feed intake (g kg ABW^{-1} day^{-1})	4.2	9.9^{a}	15.7^{b}	15.0 ^{b}	15.2 ^{b}	14.7 ^{b}	14.7 ^{b}	15.8 ^{b}	16.1 ^{b}	0.52
Feed efficiency	-0.44	0.30 ^{a}	0.36 ^{ab}	0.43 ^{bc}	0.50 ^{cd}	0.61 ^{de}	0.62 ^{e}	0.59 ^{de}	0.59 ^{de}	0.06
Daily growth index	-0.12	0.21 ^{a}	0.43 ^{b}	0.49 ^{b}	0.60^{bc}	0.74 ^{c}	0.75 ^{c}	0.78 ^{c}	0.80 ^{c}	0.05
Protein efficiency ratio	-6.57	1.90 ^{b}	1.41 ^{ab}	1.40 ^{ab}	1.40 ^{ab}	1.52 ^{ab}	1.36 ^{a}	1.19 ^{a}	1.09 ^{a}	0.47

[1] Means (n = 2) in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean.

Table 3 - Nitrogen (N) and energy (E) balances and ammonia and urea excretion of zebra seabream fed the experimental diets[1].

Diets	P5	P15	P25	P30	P35	P40	P45	P50	P55	SEM
Nitrogen balance										
Intake (g kg ABW^{-1} day^{-1})	0.04	0.25 ^{a}	0.65 ^{b}	0.74^{bc}	0.87^{bcd}	0.95^{cd}	1.07^{de}	1.26^{ef}	1.39^{f}	0.09
Retention (g kg ABW^{-1} day^{-1})	-0.08	0.06 ^{a}	0.14 ^{b}	0.17^{bc}	0.20^{bcd}	0.23^{cd}	0.25^{d}	0.26 ^{d}	0.26 ^{d}	0.02
Retention (% N intake)	-175.9	25.6 ^{b}	21.1 ^{ab}	23.6 ^{ab}	23.3 ^{ab}	23.8 ^{ab}	23.8 ^{ab}	20.6 ^{ab}	18.8 ^{a}	0.59
Energy balance										
Intake (kJ kg ABW^{-1} day^{-1})	79.7	194.5 ^{a}	317.2 ^{b}	301.4 ^{b}	307.9 ^{b}	301.0 ^{b}	300.1 ^{b}	324.8 ^{b}	338.4 ^{b}	11.3
Retention (g kg ABW^{-1} day^{-1})	-33.8	12.6 ^{a}	55.0 ^{b}	62.5 ^{b}	69.4 ^{b}	76.0 ^{b}	75.8 ^{b}	82.0 ^{b}	81.2 ^{b}	5.79
Retention (% E intake)	-42.4	6.7 ^{a}	17.4 ^{b}	20.7 ^{b}	22.5 ^{b}	25.0 ^{b}	25.3 ^{b}	25.2 ^{b}	24.0 ^{b}	1.59
Ammonia excretion										
(mg NH_4–N kg^{-1} day^{-1})	—	53.0 ^{a}	75.9 ^{a}	149.4 ^{ab}	202.6 ^{b}	339.6 ^{c}	406.6 ^{cd}	433.8 ^{cd}	517.1^{d}	42.7
(% N intake)	—	12.8^{ab}	8.8^{a}	18.5^{abc}	22.0^{abc}	31.3 ^{c}	37.2 ^{c}	29.7 ^{bc}	33.2^{c}	2.57
Urea excretion										
(mg Urea–N kg^{-1} day^{-1})	—	37.4^{a}	141.9 ^{c}	112.3 ^{bc}	79.6 ^{b}	70.4 ^{b}	73.6 ^{b}	94.5 ^{bc}	76.4 ^{b}	7.74
(% N intake)	—	53.86 ^{b}	11.63 ^{a}	12.88 ^{a}	8.68 ^{a}	5.94 ^{a}	6.79 ^{a}	6.12 ^{a}	4.82 ^{a}	4.12
(% total N excretion)	—	44.9^{bc}	65.0^{c}	43.2^{b}	28.2^{ab}	17.2^{a}	15.0^{a}	17.9^{a}	12.9^{a}	4.62

[1] Means (n = 2) in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean.

Table 4 **-** Whole-body and liver composition (wet-weight basis), hepatosomatic and visceral indexes of zebra seabream fed experimental diets[1].

Diets	Initial	P5	P15	P25	P30	P35	P40	P45	P50	P55	SEM
Whole-body composition											
Dry matter (%)	29.8	25.2	29.1 ^{a}	32.6 ^{ab}	33.1 ^{b}	33.4 ^{b}	32.2 ^{ab}	32.3 ^{ab}	32.1 ^{ab}	32.6 ^{ab}	0.36
Protein (%)	16.2	14.2	15.6 ^{a}	15.7 ^{a}	16.5 ^{bc}	16.4 ^{bc}	15.9 ^{ab}	17.0 ^{c}	16.9 ^{c}	16.8 ^{c}	0.14
Lipid (%)	6.8	1.8	5.6 ^{a}	10.3 ^{ab}	10.6 ^{b}	10.2 ^{ab}	10.4 ^{ab}	9.3 ^{ab}	9.5 ^{ab}	10.5 ^{b}	0.46
Ash (%)	6.4	8.2	7.2 ^{b}	6.2 ^{ab}	5.9 ^{ab}	5.9 ^{ab}	5.8 ^{ab}	5.6 ^{ab}	5.2 ^{a}	5.4 ^{a}	0.17
Energy (kJ g^{-1})	6.3	4.0	5.8 ^{a}	7.7 ^{ab}	7.9 ^{b}	7.8 ^{b}	7.6 ^{ab}	7.5 ^{ab}	7.8 ^{b}	7.7 ^{ab}	0.19
Liver composition (g $100g^{-1}$)											
Glycogen	–	–	8.13	6.05	7.41	7.04	7.50	4.86	4.56	5.01	0.44
Lipid	–	–	23.1 ^{ab}	31.2 ^{b}	23.5 ^{ab}	20.0 ^{ab}	21.6 ^{ab}	29.1 ^{b}	11.6 ^{a}	14.8 ^{a}	1.69
Indexes (%)											
Hepatosomatic	–	1.1	2.8 ^{b}	2.8 ^{b}	2.7 ^{ab}	2.3 ^{ab}	2.5 ^{ab}	2.2 ^{ab}	1.8 ^{ab}	1.6 ^{a}	0.13
Visceral	–	5.2	7.8	8.7	8.1	8.2	8.3	7.0	7.2	7.2	0.20

[1] Means (n = 2) in the same row with different superscript letters are significantly different ($p < 0.05$). SEM: pooled standard error of the mean.

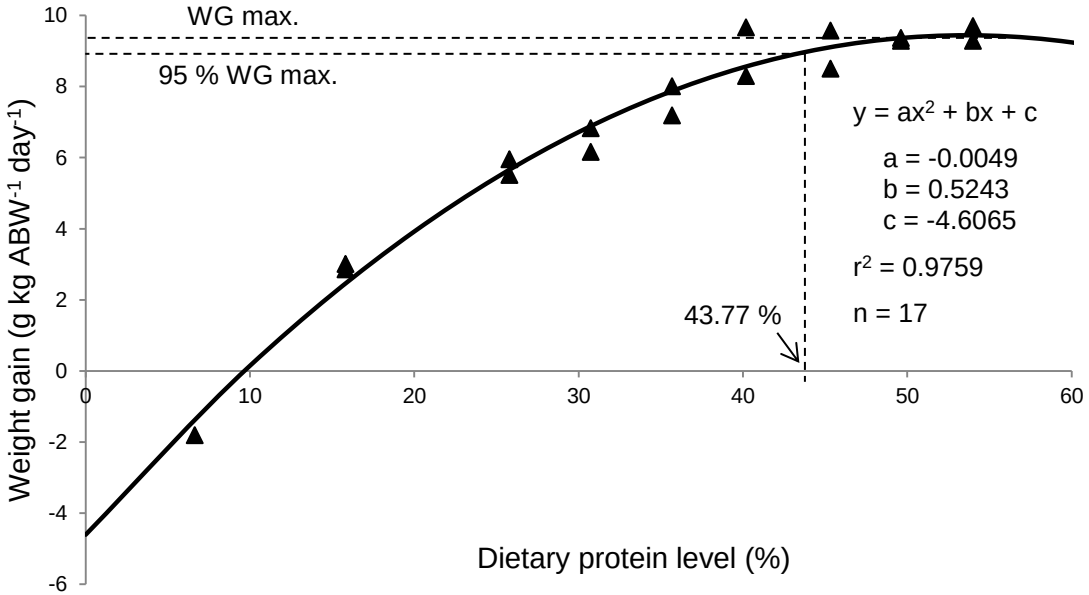

Fig.2 - Second-order polynomial model fitting weight gain (g kg ABW^{-1} day^{-1}) to dietary protein levels in zebra seabream fed the experimental diets. Dietary protein requirement value is indicated by the abscissa value corresponding to 95% of the maximum response. Each graphic point represents the average value of each tank.

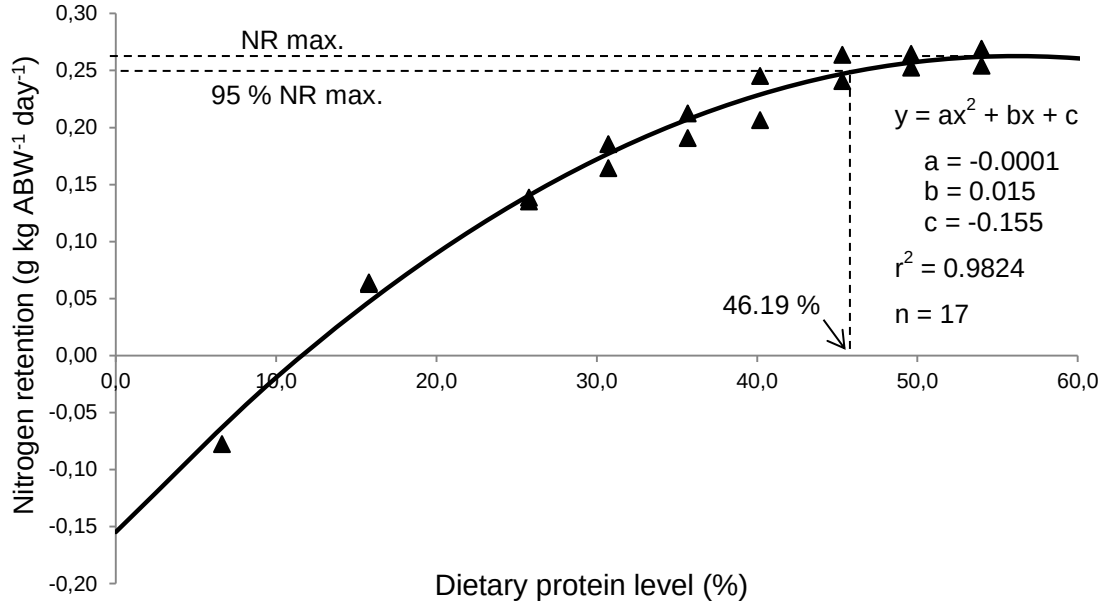

Fig.3 - Second-order polynomial model fitting nitrogen retention (g kg ABW^{-1} day^{-1}) to dietary protein levels in zebra seabream fed the experimental diets. Dietary protein requirement value is indicated by the abscissa value corresponding to 95% of the maximum response. Each graphic point represents the average value of each tank.

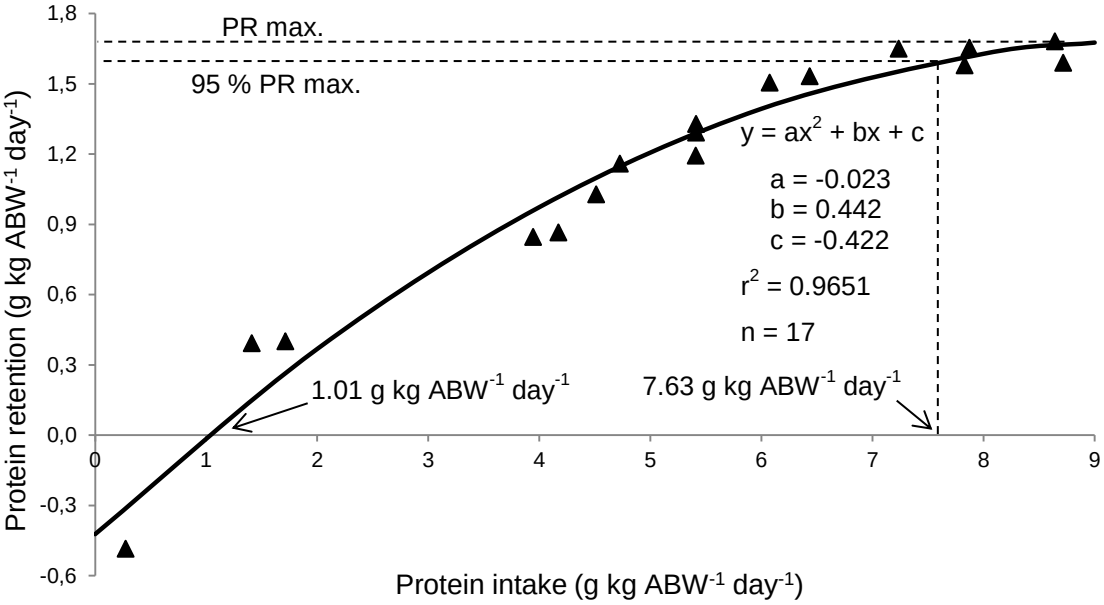

Fig.4 - Second-order polynomial model fitting protein retention (g kg ABW^{-1} day^{-1}) to protein intake (g kg^{-1} day^{-1}) in zebra seabream fed the experimental diets. Protein requirement value is indicated by the abscissa value corresponding to 95% of the maximum response. Maintenance protein requirement value is indicated by the abscissa value for y = 0. Each graphic point represents the average value of each tank.

Activities of aspartate aminotransferase (ASAT), fructose-1,6-bisphosphatase (FBPase), fatty acid synthetase (FAS) and hexokinase (HK) were not affected by dietary treatments (Table 5). Significant higher alanine aminotransferase (ALAT) activity was found in fish fed the 55% protein diet than in other groups. Glutamate dehydrogenase (GDH) activity was higher for the 45 and 55% protein diets than the lower protein diets. Fish fed the 45% protein diet also revealed significant higher glucose-6-phosphate dehydrogenase (G6PDH) and lower glucokinase (GK) activities than fish fed the 25% protein diet. Contrary to ALAT activity, which revealed a positive correlation to the dietary protein level (R^2 = 0.76, p < 0.000, n = 24), malic enzyme activity was negatively correlated to the dietary protein (R^2 = -0.74, p < 0.000, n = 24), showing a significant decreased with the dietary protein increase.

Table 5 - Liver soluble protein content and selected hepatic glycolytic, gluconeogenic, lipogenic and amino acid catabolic enzyme activities (mU mg protein^{-1}) in zebra seabream fed 25, 35, 45 and 55% protein diets[1].

Diets	P25	P35	P45	P55	SEM
Liver soluble protein (mg g^{-1})	14.71	15.88	18.11	23.02	1.54
Amino acid catabolism					
Alanine aminotransferase	298.7^{a}	300.1^{a}	337.1^{a}	479.1^{b}	18.4
Aspartate aminotransferase	943.2	925.9	930.1	1066.6	26.6
Glutamate dehydrogenase	62.8^{a}	58.9^{a}	141.4^{b}	162.5^{b}	10.9
Glycolysis					
Hexokinase	5.69	4.76	4.30	4.63	0.21
Glucokinase	7.30^{b}	5.98^{ab}	5.35^{a}	5.50^{ab}	0.28
Gluconeogenesis					
Fructose-1,6-bisphosphatase	37.8	45.7	43.6	38.2	1.42
Lipogenesis					
Glucose-6-phosphate dehydrogenase	91.0^{a}	102.9^{ab}	128.7^{b}	120.4^{ab}	5.00
Malic enzyme	88.3^{c}	82.2^{bc}	69.2^{ab}	57.5^{a}	3.31
Fatty acid synthetase	1.56	1.34	1.79	1.45	0.15

[1] Means (n = 6) in the same row with different superscript letters are significantly different (p < 0.05). SEM: pooled standard error of the mean.

4. Discussion

Mortality of zebra seabream observed in the present trial may be related to the novelty of the species in aquaculture, and therefore still not being well domesticated. Maximum daily growth index (0.8%) obtained in this trial proven that zebra seabream is a slow growing species, as had been reported by Pajuelo et al. (2003). In fact this maximum daily growth index is lower than values previously reported for juveniles of other slow growing *Diplodus* species, namely white sea bream (around 0.89%; Sá et al., 2008a) and two-banded sea bream (1.22%; Ozório et al., 2009), and almost half that of sharpsnout seabream (1.54%; Coutinho et al., 2012).

Dietary protein requirements estimated based on weight gain (g kg $ABW^{-1}day^{-1}$) or nitrogen retention (g kg ABW^{-1} day^{-1}) corresponded to 43.8 and 46.2%, respectively. This dietary protein requirement is considerably higher than those estimated for juveniles of white sea bream (27% protein; Sá et al., 2008a) and two-banded sea bream (36% protein; Ozório et al., 2009), but is however identical to that of sharpsnout seabream (43% protein; Coutinho et al., 2012). Moreover, when expressed in absolute values, protein requirement for maximum protein retention (7.6 g kg ABW^{-1} day^{-1}) is very similar to that of sharpsnout seabream (7.7 g kg ABW^{-1} day^{-1}; Coutinho et al., 2012), but relatively higher than that estimated for two-banded seabream (6.5 g kg ABW^{-1} day^{-1}; Ozório et al., 2009). This high protein requirement of zebra seabream was in part unexpected, since it is generally accepted that fast growing species present higher protein requirements than slow growing species (Tacon and Cowey, 1985).

Maintenance protein requirement of zebra seabream juveniles was estimated to be 1.0 g kg ABW^{-1} day^{-1}, which corresponds to circa 13.2% of the total protein requirement. This protein requirement for maintenance is lower than values reported for white sea bream (1.4-1.5 g kg ABW^{-1} day^{-1}; Sá et al., 2008a) or two-banded sea bream (2.3 g kg ABW^{-1} day^{-1}; Ozório et al., 2009), but higher than that of sharpsnout seabream (0.7 g kg ABW^{-1} day^{-1}, corresponding to 9.2% of total protein requirements; Coutinho et al., 2012) and is within the range of values reported for marine fish species (from 0.5 to 2.6 g kg BW^{-1} day^{-1}; Alliot and Pastoureaud, 1984; Dias et al., 2003; Gatlin et al., 1986; Kaushik and Gomes, 1988; Kaushik et al., 1995; McGoogan and Gatlin, 1998; Peres and Oliva-Teles, 2005).

As expected, protein intake below maintenance requirement of fish fed 5% protein diet resulted in depressed nutrient utilization and weight loss, suggesting a mobilization of body energy reserves for maintenance purposes, as has also been reported for white seabream and two-banded seabream (Ozório et al., 2009; Sá et al., 2008a). Similarly to present results, Ozório (2009) also reported a higher mortality of

two-banded seabream fed a diet with protein level below maintenance demand, although such high mortality was not observed in white seabream under similar conditions (Sá et al., 2008a).

It is generally accepted that fish, as other animals, regulate feed intake to meet energy demands (Kaushik and Médale, 1994). Regulation of feed intake to meet energy needs was also previously reported in white seabream (Sá et al., 2008a) and sharpsnout seabream (Coutinho et al., 2012; Vivas et al., 2006) and is in accordance with results of the present trial for fish fed diets with 25 to 55% protein. On the other hand, lower feed intake of zebra seabream fed 5 and 15% protein diets is in agreement with fish preference for fasting when offered nutrient-deficient diets, with special emphasis to protein restriction (Vivas et al. 2003, 2006). As consequence of the lower feed intake, and despite presenting the highest protein efficiency ratio and nitrogen retention (% N intake) value, fish fed the 15% protein diet presented the lowest growth rate and a reduction of all body constituents, with the exception of ash and moisture, when compared to the initial whole-body composition.

Feed efficiency of zebra seabream increased with the dietary protein level up to 40%, while in similar studies with white seabream (Sá et al., 2008a) and sharpsnout seabream (Coutinho et al., 2012) feed efficiency was shown to increase linearly with the dietary protein level. These differences seem to result from the progressive reduction of feed intake as dietary protein levels rise, seen in both white seabream and sharpsnout seabream, but not in the present trial.

The reduction of the dietary protein level and consequent decrease of protein to energy ratio, usually leads to an improvement of protein retention, as result of a reduction in the ammonia excretion (Peres and Oliva-Teles, 2001). Thus, a good correlation between ammonia excretion and amino acids catabolism-related enzymes activities and especially GDH, which is considered the key enzyme involved in ammonia production (Cowey and Walton, 1989), is to be expected. Similarly to what was reported for European sea bass (Peres and Oliva-Teles, 2006, 2007), European eel (Suarez et al., 1995) and white seabream (Sá et al., 2008a, 2008b), both ammonia excretion and GDH activity were higher for the higher dietary protein levels. A higher ALAT activity was also obtained for the highest dietary protein level tested, revealing that zebra seabream was to some extent capable of adapting protein catabolism to protein intake, as was also the case of white seabream (Sá et al., 2008a). However no significant effect of dietary protein levels was found in the ASAT activity. Contrary to present results, Sá et al. (2007, 2008a) reported a good correlation between dietary protein levels and ASAT activity in white seabream, while such correlation was not observed by Sá te al. (2008b) in another study in the same species.

Excepting fish fed 25% protein diet, ammonia excretion was more abundant than that of urea. However, urea excretion represented a significant proportion of the total excreted nitrogen, being significantly higher in fish fed the 15 and 25% protein diets. In teleosts, ornithine-urea cycle pathway seems to be limited or absent, with urea being mostly formed through arginolysis and uricolysis (Anderson, 2001; Fournier et al., 2002; Peres and Oliva-Teles, 2006; Walsh, 1998; Wood, 1993). When fish are fed low protein diets most of dietary protein is used for maintenance and liver protein degradation rates tend to increase (Peragon et al., 1994). In the same way, purine nucleotide degradation rates (from DNA and RNA) may also increase, leading to an increase of uricolysis, which in some cases may represent the main pathway for urea synthesis (Anderson, 2001; Terjesen et al., 2001; Vellas and Serfaty, 1974).

As expected, a metabolic adaptation of glycolysis to dietary carbohydrate levels was revealed by the increase of hepatic GK activity with the increase of dietary starch level, although differences were only significant between fish fed 25 and 45% protein diets. Unsurprisingly, no significant difference was observed in HK activity, which is known not to be under nutritional regulation (Enes et al., 2011). However, HK activity recorded for zebra seabream is much higher than reported for European sea bass or gilthead seabream (Couto et al., 2008; Enes et al., 2006a,b; 2008a,b; Moreira et al., 2008; Panserat et al., 2000), probably reflecting this species capacity for utilizing high dietary carbohydrate levels. In the same way, the few differences found in the whole-body composition and the relatively unchanged energy retention in fish fed 25 to 55% protein diets also seem to suggest a high ability to use carbohydrate as energy source, which is in agreement to this species omnivorous habits, and it was also observed in white seabream (Enes et al., 2012; Sá et al., 2007, 2008b) and sharpsnout seabream (Hernandez et al.,2001; Tramati et al., 2005).

Evidence exist that dietary carbohydrate increment stimulates the activity of lipogenic enzymes in the liver of different species (Barroso et al., 2001; Dias et al., 1998, 2004; Fynn-Aikins et al., 1992; Hilton and Atkinson, 1982; Likimani and Wilson, 1982; Suárez et al., 1995). Similarly, in the present trial ME activity was also correlated to dietary starch levels. By contrast, the 25% dietary protein level promoted the lowest G6PDH activity, although differences were only statistically significant between fish fed the 25 and 45% protein diets. Despite the statistical differences observed in ME and G6PDH activities, two of the main enzymes responsible for the reductive power supply needed for lipogenesis, no significant effect on FAS activity was observed. In a similar study with white seabream Sá et al. (2008a) also did not observe any significant effect on FAS activity. The lower liver lipid content of fish fed the 50 and 55% protein diets when compared to fish fed the 25 and 45% protein diets was unexpected, since no

significant differences were observed for both lipid intake or FAS activity (and so on the novo lipid synthesis) of fish fed diets within the range of 25 and 55% protein levels. Thus, further studies will be required to clarify zebra seabream lipid metabolism and utilization.

In several fish species an increase of protein catabolism, resulting from a higher dietary protein level, has been reported to promote an increase of gluconeogenesis (Caseras et al., 2002; Kirchner et al., 2005; Pérez-Jiménez et al., 2009;Suárez et al., 2002). In contrast, higher dietary carbohydrate levels may lead to a reduction of gluconeogenesis pathway (Couto et al., 2008; Guerreiro et al., 2012; Panserat et al., 2002; Suárez et al., 1995, 2002), although an absence of regulation of both FBPase activity and/or gene expression have generally been reported for both carnivorous and omnivorous fish species (Borrebaek and Christophersen, 2000; Enes et al., 2009, 2006a, 2008a; Moreira et al., 2008; Panserat et al., 2002, 2007; Panserat and Kaushik, 2010; Tranulis et al., 1996). In European sea bass and gilthead seabream, dietary protein level rather than carbohydrate level was suggested as the major factor regulating gluconeogenic enzymes activity, as no effect of carbohydrate level on FBPase activity were observed in several studies (Caseras et al., 2002; Enes et al., 2006a, b, 2008a,b,c; 2009; 2011; Fernandez et al., 2007; Moreira et al., 2008). In the present trial, despite the high range of protein and starch levels tested, no significant effect on FBPase activity was observed.

Overall, present results indicate that protein requirement for maximum growth and N retention of zebra seabream is between 43.8 and 46%, corresponding to a protein intake of 7.6 g kg ABW^{-1} day^{-1}, with 13.2% of this value resulting from maintenance protein requirement (1.0 g kg ABW^{-1} day^{-1}). This high protein requirement and the slow growth do not favour zebra seabream as good species for intensive aquaculture production. However, zebra seabream seems capable of efficiently utilizing starch as energy source and adapting protein catabolism to high dietary protein intakes and this may be exploited in the context of increasing the use of plant-based aquafeeds and in semi-intensive aquaculture production.

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