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Nutritional Regulation of Lipid Deposition in Blackspot Seabream (*Pagellus bogaraveo*)

Amélia Cláudia Figueiredo Silva

Tese de Doutoramente em Ciências do Meio Aquático

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Tese de candidatura ao grau de Doutor em Ciências do Meio Aquático submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Thesis for applying to a Doctor degree in Aquatic Enviroment Sciences submitted to the Institute of Biomedical Sciences Abel Salazar of University of Porto

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Directivas Legais

No cumprimento do disposto no Decreto-Lei nº 216/92 de 13 de Outubro, declara-se que a autora desta Tese participou na concepção e na execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redacção dos respectivos manuscritos.

Nesta tese inclui-se ainda dois artigos científicos publicados em revistas internacionais resultantes de uma parte dos resultados obtidos no trabalho experimental, referenciado como:

Figueiredo-Silva, A.C., Corraze, G., Borges, P., Valente, L.M.P., 2009a. Dietary protein/lipid level and protein source effects on growth, tissue composition and lipid metabolism of blackspot seabream (*Pagellus bogaraveo*). *Aquac. Nutr.* (*doi: 10.1111/j.1365-2095.2009.00649.x*)

Figueiredo-Silva, A.C., Corraze, G., Rema, P., Sánchez-Gurmaches, J., Gutiérrez, J., Valente, L.M.P., 2009b. Blackspot seabream (*Pagellus bogaraveo*) lipogenic and glycolytic pathways appear to be more related to dietary protein level than dietary starch type. *Aquaculture*, **291**, 101-110.

Directivas Legais

In compliance with what is stated in Decret-Law nº 216/92 of October 13th, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results shown, as well as in their interpretation and the writing of respective manuscripts.

This thesis also includes two scientific papers published in international journals originating from part of the results obtained in the experimental work referenced to as:

Figueiredo-Silva, A.C., Corraze, G., Borges, P., Valente, L.M.P., 2009a. Dietary protein/lipid level and protein source effects on growth, tissue composition and lipid metabolism of blackspot seabream (*Pagellus bogaraveo*). *Aquac. Nutr.* (*doi: 10.1111/j.1365-2095.2009.00649.x*)

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Summary

Find new finfish candidate species is one of the strategies that producers may follow to maximise their sustainability and profitability, ensuring a continuous expansion of the Mediterranean aquaculture industry. The nutritional and economical importance of blackspot seabream (*Pagellus bogaraveo*) in Mediterranean market, associated with the eminent reductions in its capture, requires urgent nutritional studies for the consolidation of this species farming. The reduced growth rates associated to the high propensity of this species to deposit fat (18-21% wet weight), even at low dietary lipid level incorporation (12%), prompt us to test two high protein (50 or 60%)/ low lipid (6 or 10%) dietary level as a first attempt to maximize this species growth and reduce its excessive fat accumulation (Chapter 2). Both, 60P/6L or 50P/10L dietary P/L tested, resulted in high lipid retentions, confirming the sustained propensity of blackspot seabream to deposit fat even at low dietary lipid levels, and suggested the conversion of nutrients other than lipid into body fat. Hence, in Chapter 3, the lipogenic potential of carbohydrate type on blackspot seabream was evaluate using two isolipidic (10%) and isonitrogenous (45%) diets containing either crude starch (CS) or gelatinized starch (GS). Starch type did not cause any significant effect on blackspot seabream body composition or lipogenic pathways, whereas a reduction of 10% dietary protein level (45 to 35%) markedly depressed hepatic activities of lipogenic enzymes. Besides nitrogen level, the work presented and discussed in Chapter 4, demonstrated that blackspot seabream lipid metabolism also depends on dietary nitrogen nature, which explain at some extent the high lipid retentions found when fish meal (FM) is partially replaced by plant protein (PP) sources. The present Thesis also demonstrates the role of the dietary dispensable amino acids (DAA) content on blackspot seabream lipid metabolism, with Ala+Ser playing a preponderant role on species lipogenic pathways regulation. Once the consideration of blackspot seabream by aquaculture industry passes through the feasibility of FM replacement by plant feedstuffs, besides the dietary IAA/DAA balance also the absolute amounts of DAA should be taken into account. Overall, different dietary P/L ratios and protein level have a relevant role on viscera lipid content regulation, while PS and AA nature has mainly affected liver lipid content through lipogenic pathways. In fact, dietary protein (level and nature) play a major role on blackspot seabream lipid metabolism and can contributed, in three months, to increases up to 2% on body lipid contents. The knowledge obtained with this Thesis permit us to suggest that a diet with a dietary P/L of 45P/10L combined with a feed distribution by self feeders appears to be the most adequate for optimizing growth and reducing body lipid content in blackspot seabream.

Resumé

Trouver de nouvelles espèces des poissons candidates pour l'aquaculture est une des stratégies que les producteurs doivent suivre pour assurer la viabilité et la rentabilité de leur production, tout en permettant une expansion de l'aquaculture Méditerranéenne. L'importance nutritionnelle et économique de la dorade rose (*Pagellus bogaraveo*) sur le marché Méditerranéen, associée à la réduction éminente des captures de cette espèce, nécessite de conduire des études nutritionnelles afin de permettre le développement d'une filière aquacole pour cette espèce. Le faible taux de croissance de cette espèce associé à sa forte propension à l'engraissement (18-21% de lipides corporels), même avec une alimentation à faible teneur en lipides (12%), nous ont conduit à évaluer des régimes ayant une teneur en protéines élevée avec un faible taux de lipides, comme une première alternative pour maximiser la croissance et réduire l'accumulation excessive de graisses chez cette espèce (Chapitre 2). Les deux niveaux de protéines/lipides (P/L), 60P/6L ou 50P/10L, ont conduit à des rétentions lipidiques plus élevées, confirmant la forte prédisposition de la dorade rose pour l'accumulation de lipides corporels, même avec de faibles taux de lipides alimentaires, ce qui suggère une synthèse de lipides à partir des autres nutriments (protéines, glucides). Ainsi, dans le Chapitre 3, nous nous sommes intéressés au potentiel lipogenique de glucides chez la dorade rose, en comparant les effets de deux régimes isolipidiques (10%) et isoproéiques (45%) contenant soit de l'amidon brut (CS) soit de l'amidon gélatinisé (GS). Nous avons montré que le type d'amidon n'a pas d'effets significatifs sur la composition corporelle ou les voies lipogénique chez la dorade rose, tandis qu'une réduction de 10% de la teneur en protéines alimentaires (de 45 à 35%) conduit à une nette diminution des activités des enzymes de la lipogenèse hépatique. Outre le taux de protéines alimentaires, les travaux présentés et discutés dans le Chapitre 4, ont démontré que la nature des protéines et des acides aminés alimentaires affectent le métabolisme lipidique de cette espèce, expliquant ainsi la plus forte rétention lipidique observée lorsque la farine de poisson (FM) est partiellement remplacée par des protéines végétales (PP). Ce travail de Thèse nous a aussi permis de montrer le rôle des acides aminés dispensable (DAA) sur la régulation du métabolisme lipidique de la dorade rose, avec Ala+Ser qui jouent un rôle prépondérant sur les voies de la lipogenèse. Le développement de l'aquaculture de la dorade rose doit intégrer les possibilités de remplacement de la FM par des sources protéiques végétales. Dans ce contexte nos travaux ont montré qu'en plus de l´équilibre en IAA/DAA alimentaires, la nature des DAA doit également être prise en compte. L'ensemble de nos travaux ont démontré un rôle prépondérant du rapport P/L et du taux protéique des aliments sur la régulation des dépôts lipidiques au niveau viscéral, alors que la nature des

protéines, en particulier le profil en AA, a principalement affecté le teneur en lipides au niveau hépatique. Chez cette espèce, ce sont les protéines alimentaires (niveau et nature) qui jouent un rôle majeur sur le métabolisme lipidique et peuvent conduire à des augmentations allant jusqu'à 2% de la teneur en lipides corporels, après 3 mois d'alimentation. Les connaissances acquises lors de ce travail de Thèse suggèrent qu'un régime alimentaire avec une teneur P/L de 45P/10L combiné avec une distribution d'aliment à la demande semble le protocole le plus adéquat pour optimiser la croissance et réduire la teneur en lipides corporels chez la dorade rose.

Resumo

Encontrar novas espécies candidatas à aquacultura é uma das estratégias que os produtores podem seguir para maximizar a sua sustentabilidade e rentabilidade, assegurando assim uma contínua expansão da indústria aquícola Mediterrânica. A importância nutricional e económica do goraz (*Pagellus bogaraveo*) no mercado Mediterrânico, associada às reduções eminentes na sua captura, exige a urgente realização de estudos de nutrição para a consolidação da produção desta espécie. Taxas de crescimento reduzidas associadas à alta propensão desta espécie para depositar gordura (18-21% do peso húmido), mesmo quando alimentada com baixos níveis lipídicos (12%), levou-nos a decidir por dietas de alto nível proteico/baixo nível lipídico, como uma primeira tentativa de maximizar o crescimento e reduzir a acumulação excessiva de gordura nesta espécie (Capítulo 2). Ambos os níveis de proteína/lipidos (P/L) testados, 60P/6L ou 50P/10L, resultaram em elevadas retenções lipídicas, confirmando a propensão do goraz para depositar gordura, mesmo quando alimentados com baixos níveis lipídicos, sugerindo a conversão de outros nutrientes que não lípidos em gordura corporal. Assim, no Capítulo 3, procedeu-se à avaliação do potencial lipogénico dos hidratos de carbono em goraz, usando-se para isso dietas isolipidicas (10%) e isoprotéicas (45%) contendo amido cru (CS) ou amido gelatinizado (GS). O tipo de amido não causou qualquer efeito significativo na composição corporal ou nas vias lipogénicas no goraz, enquanto que uma redução no nível proteico da dieta em 10% (de 45 para 35%), reduziu acentuadamente a actividade das enzimas hepáticas da lipogénese. Além do nível proteico, o trabalho apresentado e discutido no Capítulo 4, demonstra que o metabolismo lipídico no goraz depende igualmente da natureza dessa proteína, corroborando as elevadas retenções lipídicas obtidas aquando da substituição parcial da farinha de peixe (FM) por proteína de origem vegetal (PP). A presente Tese demonstra ainda uma regulação do metabolismo lipídico no goraz em função do conteúdo em amino ácidos dispensáveis (DAA) das dietas, exercendo a Ala+Ser um papel preponderante na regulação das vias lipogénicas nesta espécie. A consideração do goraz pela indústria aquícola passa pela viabilidade da substituição da FM por alimentos de origem vegetal. Neste contexto, os resultados obtidos nesta Tese mostram que além do equilíbrio alimentar em IAA/DAA, o seu valor absoluto em DAA deve ser igualmente considerado. Em termos globais, a variação do teor P/L e do nível proteico das dietas exerce um papel relevante na regulação do teor lipídico visceral, enquanto que a natureza da proteína, nomeadamente o seu perfil em AA, afecta particularmente o conteúdo lipídico do fígado pela regulação das vias lipogénicas. De facto, a proteína incorporada nas dietas (nível e natureza) assume particular relevância no metabolismo

lipídico desta espécie, podendo aumentar até 2% os teores lipídicos corporais, em três meses. O conhecimento adquirido na presente Tese, permite sugerir uma dieta com um teor P/L de 45P/10L distribuída a pedido, como o protocolo de alimentação mais adequado à optimização do crescimento e à redução do teor lipídico no goraz.

CHAPTER 1

Aquaculture main constrains and development options

The world aquaculture grew tremendously during the last fifty years from a production of less than a million tonnes in the earlies 1950s to 48.1 million tonnes in 2005, which represents an annual average growth rate of 8.8 percent (FAO 2007). Global fish consumption per capita has increased over the past four decades, rising from 9.0 Kg in 1961 to an estimated 16.5 Kg in 2003 (FAO 2007). Thus, and considering the fisheries failure to meet the growing world demand for aquatic food, aquaculture is predicted to play a major role in meeting an important part of fish world's demands for human consumption. The aquaculture sector is expected to contribute more effectively to global food security, nutritional well-being, poverty reduction and economic development by producing - with minimum impact on the environment and maximum benefit to society - 85 million tonnes of aquatic food by 2030, an increase of 37 million tonnes over the 2005 level (FAO 2007).
From the many factors that determine the supply of aquaculture products and will, to a large extent, also determine the availability and consumption of fish, those summarized below have been outlined in The State of World Fisheries and Aquaculture 2006 (FAO 2007), to play a lead role in the coming decades, namely:

a) Intensification; access to land and water resources;
b) Environmental management;
c) Enhancement of nutritional value of fish for human consumption;
d) Search of alternative feedstuffs for fish meal and fish oil;
e) Diversification of production systems and species.

The extent of the environmental impact of the aquaculture development is mainly dependent on feeding technique and feed composition. Thus, the development of aquaculture industry requires great nutritional efforts towards the search of alternative feedstuffs to fish meal and fish oil and the achievement of reliable species nutritional knowledge in an aquaculture species diversification scenario.

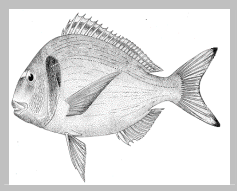
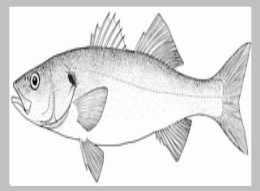
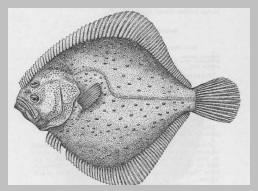
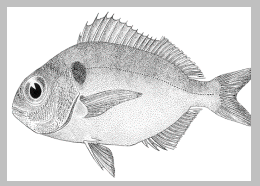
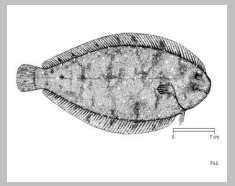
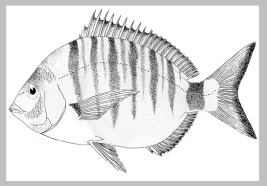
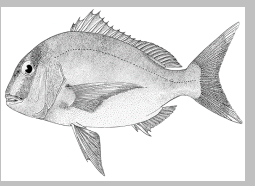
With the predicted global increase in aquaculture production, the demand for aquafeed will continue to grow, as will the demand for fishmeal and fish oil, since feed for intensively farmed fish, still rely heavily on feedstuffs of marine origin (Tacon & Metian 2008). However, according to the International Fishmeal and Fish Oil Organisation, the use of fishmeal in aquafeed is expected to rise by more than 5 percent (from 2.87 to 3.02 million tonnes from 2002 to 2012), while the demand for fish oil will increase by more than 17 percent (from 0.83 to 0.97 million tonnes) from 2002 to 2012 (Tacon & Metian 2008). Thus, alternative protein and oil sources are needed to replace fish meal and fish oil in

aquafeeds, contributing to long-term sustainability of the industry. Considerable progress has been made in finding suitable alternatives to replace marine feedstuffs (fish meal and fish oil) by plant feedstuffs (Gatlin *et al.* 2007; Kaushik & Hemre 2008; Turchini *et al.* 2009). However, the massive global demand on renewable energy such as biodiesel production is seriously affecting the world feeds costs. Demand of producing crops for food and fuel have reached a point of surpassing supplies which resulted in increased feeds costs presenting unique challenges for the livestock and aquaculture industries. This recent economical picture is seriously affecting and challenging the marine feedstuffs replacement by the considered over the last year's potential vegetable alternatives feedstuffs (Hardy 2008).

Over the past 20 years, the successful development of Mediterranean aquaculture has resulted in increased production of European sea bass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), and turbot (*Scophthalmus maximus*) (Table 1). As an effect of increased production, market prices for those cultured fish have declined which has consequently resulted in reduced profits. In this context, finding "new" finfish candidate species is one of the strategies that producers may follow to maximise their sustainability and profitability, ensuring a continuous expansion of the Mediterranean aquaculture industry. Nevertheless, the problems of introducing foreign marine species may represent a threat for aquatic biodiversity. Thus, native rather than exotic species should be first choice for the diversification of the aquaculture sector. In addition, the candidate species should be culture in such a way to match the organoleptic properties of their wild counterparts (Abellán & Basurco 1999).

The advantages on the consideration of Sparids species like red porgy (*Pargus pargus*), white seabream (*Diplodus sargus)* or blackspot seabream *(Pagellus bogaraveo*, Brünnich, 1768) relies on the production techniques similarity to the previously produced species, like gilthead seabream, resulting in a lower cost equipment investment and a more efficient use of existent resources (so called mimetic species) (Divanach 2002). Due to its high commercial value, excellent palatability, scarcity in the fishing grounds and high resistance to diseases in captivity, blackspot seabream has been pointed as a potential candidate in the context of Mediterranean marine fish farming diversity.

Table 1. Main marine fish species in a Mediterranean aquaculture context

Scientific Name	*Sparus aurata*	*Dicentrarchus labrax*	*Scophthalmus maximus*	*Pagellus bogaraveo*	*Solea senegalensis*	*Diplodus sargus*	*Pagrus pagrus*
							
English Name	Gilthead Seabream	European Seabass	Turbot	Blackspot seabream	Senegalese sole	White seabream	Red porgy
French Name	Dorade royale	Bar, Loup	Turbot	Dorade rose	Sole du sénégal	Sar commun	Pagre commun
Portuguese Name	Dourada	Robalo	Rodovalho	Goraz	Linguado	Sargo legítimo	Pargo legítimo
Growth [1]	Average	Average	Fast-growth	Slow-growth*	Average	Slow-growth	Average
Months to reach market [2]	18 - 20	20 - 22	~20	16-36	~12 -24	**	~24
Market size (g) [3]	200-300	200-300	500-1000	300-1000	250-400	300-400	300-400
Market price (Euros) [4]	~3-4	~3-4	~8-13	10-20	7-13	5	12
Fisheries production (t) 2007 [5]	7361	11139	7949	1698	5033	1746	11417
Aquaculture production (t) 2007 [5]	125355	62764	8188	200	36	26	13

* Natural environment

** reach 58 g in 13 months (Abellan & Garcia-Alcazar 1995)

[1] Considering the literature discrepancy (nutrition, temperature, photoperiod and rearing conditions) in what concerns species growth rates, a general classification was adopted (slow, average or fast-growth)

[2] Data obtained from: Dinis *et al.* 1999; Iglesias *et al.* 1987; Kentoyri *et al.* 1995; Genovese *et al.* 1998; Imsland *et al.* 2003; AquaTT 2004; JACUMAR 2004; Sellero *et al.* 2007

[3] Data obtained from: Kentoyri *et al.* 1995; Genovese *et al.* 1998; Imsland *et al.* 2003; Sellero *et al.* 2007

[4] Data obtained from : INE, 2007; FAO, European price report 2008; t-tonnes

[5] Data obtained from : FAO 2009; t-tonnes

Blackspot seabream (*Pagellus bogaraveo*) as a potential aquaculture new species

Biological characteristics

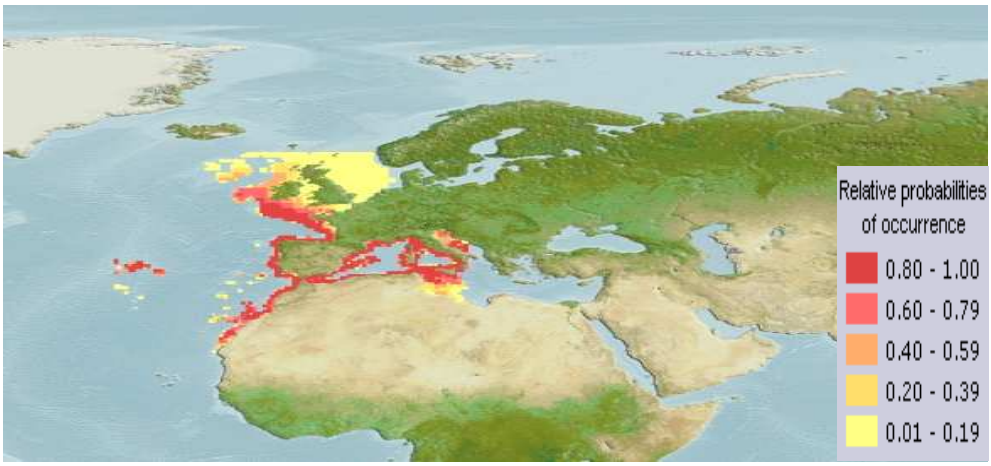


Figure 1. Distribution of blackspot seabream.

The image is hosted at http:www.fishbase.org/summary/SpeciesSummary.php?id=890

Blackspot seabream also known as red seabream is a widespread (65ºN-20ºN, 32ºW-17ºE) demersal fish occurring in the Atlantic from Norway to Cape Blanco, Madeira and the Canaries, being common in the Mediterranean but absent in the Black Sea. The larvae are planktonic and juveniles occur in coastal waters. Adults can be found in depths from the inshore waters, above various bottoms: rocks, sand and mud, down to 400m in the Mediterranean and 700m in the Atlantic (Bauchot & Hureau 1986).

This Sparidae species has an oblong reddish-grey body with a black spot at the origin of the lateral line (adults) just above the pectoral fin, attaining up to 70 cm of maximum size (Abellán & Basurco 1999).

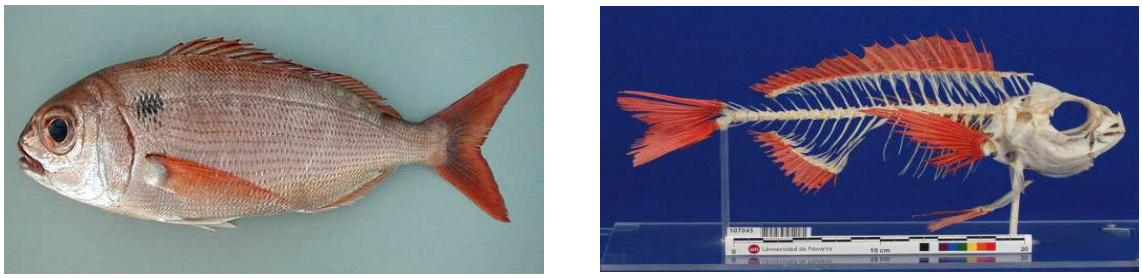


Figure 2. Blackspot seabream morphology

The image on the left is hosted at http:www.fishbase.org/summary/SpeciesSummary.php?id=890

The image on the right is hosted at http://www.unav.es/unzyec/mzna/specs/107045.htm

Blackspot seabream has predominantly carnivorous feeding habits, with the majority of the prey consumed consisting in smaller organisms, mainly fishes and several invertebrates (Morato *et al.* 2001). The diet composition of blackspot seabream depends on the distribution area considered and consequently on prey availability. Moreover, the occurrence of vertical feeding migrations described by Desbrosses (1983) and Uranga (1990) reflects the opportunistic foraging behaviour of the species.

This species exhibits protandric hermaphroditism (Krug 1990), characteristic common among the sparids (Buxton & Garratt 1990). In the Azores, sex transition takes place mostly at fork length sizes of 28-34 cm (Krug 1990) while in Cantabrian waters occurs at smaller sizes (Sánchez 1983). According to Krug (1990) blackspot seabream males mature at an average length of approximately 27.7 cm and females at 34.6 cm, corresponding to 5 and 8 years of age, respectively. Micale *et al.* (2002) suggests that sex inversion in blackspot seabream is related to fish age rather than size, and that maturation occurs earlier in captivity than in the wild. In fact, the earliest spawns are described to occur at 3 and 4 years of age in males and females kept in captivity versus 5 to 8 years in wild (Olivier 1928; Sánchez 1983; Fernandez-Pato *et al.* 1990; Krug 1990). Blackspot seabream spawning season in the natural environment varies in terms of latitude and longitude. In Azorean as well as in Cantabrian waters this species spawn from January/February to April/May with peak activity occurring between February and March (Krug 1990; Sanchéz 1983), whereas in England, spawning starts in September and end in October (Olivier 1928). Females fecundity was estimated to ranging from 73000 to 1500000 mature oocytes. This estimation was based on the number of mature oocytes collected from wild individuals, measuring 29 to 41 cm and with 6 and 11 years of age, respectively (Krug 1990).

Fisheries and economic market value

Blackspot seabream individuals are often found as fresh product in Spain, Italy, Portugal and Morocco markets and occasionally in Tunisia and Greece (Abellán & Basurco 1999). Species fishery is mainly allocated in the VI (Rockall bank), VII (Great Sole area), VIII (Cantabrian Sea) and IX and X (Portugal areas) areas of International Council for the Exploration of the Sea (ICES), where different fleets (France, Portugal, Spain and England, principally) operate (Sánchez 1983). The fisheries are characterized by their peculiar seasonal character with maximum yields from December to February in the VIII area, from June to July in the VII area and during the months of August and September in the VI area (Sánchez 1983; Sellero *et al.* 2007). This seasonality suggests a species

migratory movement depending on seasonal factors, like water temperature, together with the alimentary and reproductive habits (Sánchez 1983). Earlier studies about the migratory habits of blackspot seabream showed an evident movement of the specimens from Cape Peñas shelf (North of Spain) towards western Ireland waters, following up the French coast, while a less portion of the population moves towards Galician coast (West Spain) (Gueguen 1971).

Blackspot seabream is near to commercial extinction due to the over-fishing and/or recruitment failure in some fishing grounds such as the Cantabria Sea. The Mediterranean stock has been heavily exploited leading to this species biomass decline. The decline in the total catches and in the catches per unit of effort has been observed in the Strait of Gibraltar whereas in the ICES divisions VI, VII, VIII the stock is near to collapse (ICES 2002, 2004). Recent data on natural stock size distribution, sex ratio and reproductive seasons (Chilari *et al.* 2006; Erzini *et al.* 2006) indicates the overfishing of the large, mature females, and the subsequent reduction in recruitment, as the main causes for the decline of this species mean size. Considering such, the Andalucía (Spain) government has adopted since 1999 a recovery plan for the stock (Anonymous 2002). However, no other fishery regulations are currently imposed except the minimum landing size, which is set at 12 cm. ICES previous reports (2002,2004) advises that most deep-sea species can only sustain low rates of exploitation and that consequently the fisheries on such species should only be permitted when accompanied by programmes to collect data. Considering the ICES advices, the European Commission proposed in 2006 (EC 2006) a reduction over 50% in deep-sea species fishing effort and quotas, including blackspot seabream. Thus, the reduction on fisheries catches resulted in species market scarcity and then increased market value. The average price for this species, despite being subject to fluctuation depending on catch rates and season, is about 10 € in the Portuguese (INE 2007) and 20 € in the Spanish markets (Sellero *et al*. 2007). The higher average price observed in Spanish markets compared with Portuguese ones is related to high market value during certain seasons, such as Christmas (Sellero *et al*. 2007). As a result, numerous R&D projects coordinated by the private sector in collaboration with research institutes have been financially supported, to foster the farming consolidation of blackspot seabream. To date, Isidro de La Cal (Valdoviño, Coruña, Spain) is the only aquaculture enterprise farming blackspot seabream in the world, with the annual production reaching 200 tonnes in the year of 2007. However, the increase in the aquaculture production of blackspot seabream is being hampered by some environmental policies imposed by the Galician Autonomous Government which negatively affects blackspot seabream economic viability (Soto & Salgado 2007).

Current knowledge on species production cycle and nutrition

The first studies carried out on blackspot seabream mostly refers to preliminary experiments conducted on individuals caught in the natural environment and have mainly dealt with reproduction and disease control, larvae and juveniles culture techniques (Chereguini *et al*. 1990; Peleteiro *et al.* 1994,1997, 2000; Genovese *et al.* 1998; Olmedo *et al.* 1998; Micale *et al.* 2002). The Instituto Español de Oceanografía (IEO) launched in the earliest 90 `s the first research project for the development of blackspot seabream farming practices. The control of a species reproductive cycle is a major condition for its feasibility as an aquaculture candidate. Thus, taking the available data on sexual behaviour, maturity stage or size-weight ratio of wild fish (Sánchez 1983, Krug 1990) as a starting point, the first reproductive and ongrowing studies were carried on individuals caught from the natural environment. The first successful spawning in captivity (20000 fertilized eggs/daily), were obtained from a wild broodstock in 1997 by IEO, under natural temperature flutuations (12-21 ºC) (Peleteiro *et al.* 1997, 2000). Although significant advances have been accomplished since the first trials, the reproduction cycle of blackspot seabream remains to be elucidated. To date, the reproduction is still based on a wild broodstock. The structure of the north-east Atlantic population may indicate a low to moderate (but significant) genetic differentiation between populations at a regional level (Stockley *et al.* 2005). Moreover, Pinera *et al.* (2007) found a high genetic diversity even within populations off Spanish Coasts (Mediterranean and Cantabrian Sea). Considering that blackspot seabream spawns in captivity are still assured by wild breeders, performances are greatly dependent on the parental genetic population structures. Great efforts are hence been devoted to the control of reproduction on this species under captivity in order to start a consistent genetic selection programme. The current knowledge on the blackspot seabream production cycle was summarized by Sellero *et al.* (2007) and is presented in Table 2.

Larval stages

The development of blackspot seabream from hatching to mouth opening takes approximately 115 hours at 14ºC, whereas the digest ive system gets entirely functional 138 hours after hatching (Peleteiro *et al.* 1997). At larval stages, neither the embryonic development (54 h at 24 ºC) nor the consumption of the vitelline sac (138 h at 14 ºC) presents practically any difficulty (Peleteiro *et al.* 1997). Moreover, the larvae feeding was shown to be similar to others Sparidae largely cultivated in aquaculture industry, being mainly constituted by rotifer until the 30th days after hatching (DAH), artemia nauplius from

Table 2. Current knowledge on the blackspot seabream production cycle

		Broodstock	Spawn conditions	Eggs	Larvae	Pre-Ongrowing	Ongrowing
Tanks/sea cages facilities		Tanks 250L	Tanks 10 m3	Tanks 120 L	Open	Tanks 500 L	Cages 2,5x1,5x6 m
	Water circulation	Open	Open	Open	Closed	Open	Sea
Physic-chemical conditions							
	Temperature	NC	NC	14 ºC	19 ºC	NC	NC
	Photoperiod	24 h Dark	NC	Without direct light	24 h Light	NC	NC
	Oxigen					> 6 mg/L	
Density		1fish/m3	1-2 Kg/m3		10 larvae /L	1-3 Kg /m3	0.4 Kg/m3
Survival rate%		30%			20-30%	90%	95%
Eclosion rate%				40-50%			
Nutritional feeding plan							
	Diet type	Wet diet	Wet diet	viteline reserves	Live preys **	Artificial feeds	Artificial feeds
	Diet composition	44P/17L	48P/24L + vit				
	Ration	Ad libitum	2% fish biomass			Ad libitum	0.5-0.7% fish biomass ***
Time-course phase		6 weeks	February-May*	45-50 days length	45-50 days length	100-120 days length	19 months lenght

Adapted from Sellero *et al.* (2007).
* In Galicia, Spain
** Rotifers from the 3rd to the 35 th day after hatching (DAH); Artemia nauplius from the 30 th to the 35 th DAH; Artemia adults from the 35 th to the 50 th DAH; inert diet from the 40th day on.
*** if fed daily or 3% fish biomass if fed 2 to 3 times weekly.
NC- natural conditions

20 to the 50 th DAH, adult artemia from 28 to 50 th DAH, and inert diet afterwards (Olmedo *et at*. 1998). The digestive enzymes activity of blackspot seabream follow the same pattern usually described for other marine finfish larvae, reflecting its ability to digest food from early life stages. Like in other species, blackspot seabream larvae have a strong trypsin and low amylase activity, suggesting low ability to use dietary carbohydrates (Ribeiro *et al.* 2008). Moreover, lipase specific activity showed a rather stability during the early stages of development (Ribeiro *et al.* 2008). In spite of the considerable relevance of this kind of studies to the consolidation of larvae nutritional protocols, further multidisciplinary studies using different diet compositions (level and/or sources of ingredients, etc.) will be essential to gain insight into the blackspot seabream digestive physiology.

Juvenile stages

Pathologies

In terms of blackspot seabream pathology, exophthalmia and inflation of the swim-bladder have been pointed out as the most important ones, and attributed to stress situations. These pathologies frequently result in fish starvation due to their inability to catch the food. In addition, blackspot seabream is hypersensitive to water turbidity either derived from insufficient or inadequate tank cleaning or caused by storms or elements alien to the installation, which might cause massive mortality rates (Peleteiro *et al.* 2000).

Temperature and rearing conditions

According to Divanach (2002) the maximal temperature tolerated by blackspot seabream is around 20-21^{o}C. Studies conducted on the effect of water temperature during pre-ongrowing stage have shown that an increase in water temperature from 16 to 19 ^{o}C improves juveniles growth (Peleteiro *et al*. 2000). Moreover, the growth and survival of this species, during ongrowing stage, was shown to be significantly superior in cages than in tanks (Olmedo *et al.* 2002). According to Genovese *et al.* (1998), blackspot seabream grows from 6 g to 300 g in about 16 months, while JACUMAR (2004) refers that fish reach the marketable size of 460 to 650 g within 28 months. In addition, Sellero *et al.* (2007) points out a 36 months period to reach a higher marketable size of 800-1000 g. Factors such as water temperature and diet composition could explain differences within species for the marketable size required time.

Nutrient requirements

Aquaculture is currently recognized as a viable and profitable enterprise worldwide. Hence, the economic sustainability of the sector drives toward higher yields and faster growth. Considering that feed accounts for more than one-half of the variable operating cost (NRC 1993; FAO 2007) knowledge on nutrition and practical feeding of fish is essential to a successful aquaculture production. Feeds and feedstuffs contain nutrients and energy sources essential for fish growth, reproduction, and health. The dietary requirements for protein and amino acids (AA), lipids, minerals, and vitamins have been established in several teleost fish species (NRC 1993). Although many progresses have been accomplished in terms of blackspot seabream pre-fattening and ongrowing in tanks or sea cages (Peleteiro *et al.* 2000; Olmedo *et al.* 2000; Linares *et al.* 2001; Silva *et al.* 2006; Ozório *et al.* 2009; Valente *et al.* 2009a), specific growth rates (SGR) of this species (0.5-1.2) is still low, when compared with gilthead seabream (1.7) (Santinha *et al.* 1999; Izquierdo *et al.* 2003). Therefore, the successful production of this new species for aquaculture requires the establishment of a nutritionally balanced mixture of ingredients able to support species growth, reproduction and health, and ultimately assure the final product quality.

Dietary Protein

The protein allowances in fish diets are appreciably higher than those in the diets of terrestrial warm-blooded animals. In practice, protein is usually given first priority for being the major and the most expensive dietary component. Protein requirements are primarily species dependent (Table 3). The optimum protein level in the diets for sea bass juveniles was estimated to be around 500 g Kg^{-1} of the diet (Hidalgo & Alliot 1988; Peres & Oliva-Teles 1999). Using semi-purified diets Sabaut & Luquet (1973) estimated the optimum protein requirement for maximum growth of juvenile gilthead seabream to be 400 g Kg^{-1} diet. Recently, this value was re-evaluated to 450 to 460 g Kg^{-1}, based on the same criteria but with practical diets (Santinha *et al.* 1996; Vergara *et al.* 1996). Blackspot seabream protein requirement were established to be in the order of 450 g Kg^{1} (Silva *et al.* 2006) and thus are well within those observed for sea bass or sea bream (Table 3). On the other hand, protein requirements for maximum growth of flatfishes like turbot, dover sole (*Solea solea* L.), Senegalese sole, plaice (*Pleuronectes platessa* L.) and Atlantic halibut (*Hippoglossus hippoglossus*) seems to be higher than for that of sea bass and sea bream ranging between 500 and 650 g Kg^{-1} of the diet (Cowey *et al.* 1972; Berge & Storebakken 1991; Guillaume *et al.* 1991; Aksnes *et al.* 1996; Helland & Grisdale-Helland 1998; Hamre *et al.* 2003; Lee *et al.* 2003; Rema *et al.* 2008). Available data indicates

Table 3. Protein requirements % (dietary dry matter) of some European aquaculture species

Species	Protein source	DP/DE	Protein requirement	Reference
European Seabass	FM	-	**50**	Hidalgo & Alliot 1988
	Miscellaneous	19	**43**	Dias *et al.* 1998
	FM, CPSP	25	**48**	Peres & Oliva-Teles 1999
Gilthead Seabream	Casein, FPC, AA	-	**40**	Sabaut & Luquet 1973
	FM	24	**45**	Santinha *et al.*1996
Turbot	FM	-	**50**	Lee *et al*. 2003
Atlantic halibut	FM	-	**51**	Helland & Grisdalle-Helland 1998
Plaice	Cod muscle	-	**50**	Cowey *et al.*1972
Senegalese sole	Miscellaneous	-	**53**	Rema *et al.* 2008
Blackspot seabream	Miscellaneous	29	**45**	Silva *et al.* 2006
White seabream	FM, CPSP	-	**38-42**	Sá *et al.* 2007
Rainbow trout	Casein and gelatin	-	**40**	Zeitoun *et al.* 1973
Atlantic salmon	FM	25	**55**	Grisdale-Helland & Helland 1997

CPSP – Fish protein concentrate; FM – Fish meal; FPC – Concentrate of fish protein; SBM – Soy bean meal
Miscellaneous – FM and different plant prontein sources

that the optimal protein level of the diets is higher and the protein retention efficiency is lower in marine fish when compared to salmonids, suggesting that conventional energy sources have a limited protein sparing effect in marine fish (Kaushik 1997). Moreover, the optimal dietary protein level is influenced by the optimal dietary protein-to-energy balance, the dietary AA composition and digestibility and the dietary non-protein energy content (Wilson 2002). As the body AA composition does not change significantly among fish

species, major inter-specific differences in the dietary AA requirements are not expected (Table 4), particularly if requirements are estimated by the ideal protein method (Mambrini & Kauhik 1995; Akiyama *et al.* 1997; Kaushik 1998). Therefore, when the AA requirements of a fish species are not known, available data on the AA requirements of other species or the AA profile of their carcasses may be used as a guideline for practical diets formulation. Fishmeal, due to its almost ideal nutritional composition, is the best protein source for fish diets. However, its high price and reduced availability in the international market makes it necessary to be partially or completely replace by alternative protein sources. Hence, considerable research efforts have been expended in evaluating alternative protein sources for use in aquaculture feeds (Gatlin *et al.* 2007; Kaushik & Hemre 2008). Palmegiano *et al.* (2007) has shown that rice protein concentrate (RPC) could replace fish meal up to 20% in diets for blackpot seabream without affecting growth performances or fillet quality in terms of fatty acid composition. However, authors did not present the whole body composition of those fish and hence it is not possible to accurately infer about blackspot seabream RPC nutrient utilization. As protein is generally the most expensive nutrient, the major issue regarding the dietary formulations for fish has been to supply the minimum protein requirement for optimal or maximal growth with an appropriate balance of other nutrients to supply the required energy. This balance is termed as the digestible protein-to-digestible energy ratio (DP/DE).

Dietary DP/DE

Species optimal DP/DE ratio can be reduced if a complementary energy source (lipids or digestible carbohydrates) is supplied to allow protein sparing. With this purpose, aquafeed formulations tend to increase lipid content (high-fat diets) (Hillestad & Johnsen 1994; Grisdale-Helland & Helland 1997; Company *et al*. 1999b; Hemre & Sandnes 1999) and/or carbohydrate (Kaushik & Oliva-Teles 1985; Grisdale-Helland & Helland 1997; Dias *et al.* 1998; Venou *et al.* 2003; Fernández *et al*. 2007) as a non-protein source to enhance growth and spare protein, and to reduce organic matter and N losses to the aquatic systems. Although protein sparing by dietary lipid is widely accepted, the limits to its effectiveness, or the mechanisms by which it might occur, have not been accurately defined. On the other hand, the incorporation of high dietary levels of non-protein energy might alter body composition, particularly through an increase in lipid deposition with adverse effects on yield, product quality, and storage (Hillestad & Johnsen 1994; Jobling *et al*. 1998; Company *et al.* 1999a,b). In addition, the deposition of excessive lipid in the carcass will be clearly a more serious problem in those species that have a noticeable tendency to deposit fat, like the particular case of blackspot seabream.

Table 4. A/E ratios and an estimation of indispensable amino acids (IAA) requirements (as g/16 g N) for four marine fish teleosts

	Sea bass		Sea bream		Turbot		Blackspot seabream
	A/E	**Requirements**	**A/E**	**Requirements**	**A/E**	**Requirements**	**Requirements**
Arg	146.6	4.6	162.4	5.4	144.6	4.8	3.4
Lys	153.6	4.8	149.6	5.0	152.1	**5.0***	**5.0****
Hist	49.8	1.6	49.9	1.7	46.4	1.5	1.3
Ile	84.0	2.6	78.9	2.6	80.6	2.6	2.2
Leu	138.8	4.3	134.3	4.5	140.8	4.6	4.2
Val	91.2	2.9	88.5	3.0	87.5	2.9	2.5
Met+Cys	72.5	2.3	73.1	2.4	82.3	2.7	1.9
Phe+Tyr	83.4	2.6	86.4	2.9	160.3	5.3	4.1
Thr	86.3	2.7	84.8	2.8	86.6	2.9	2.5
Trp	19.4	0.6	18.5	0.6	18.7	0.6	-

Adapted from Kaushik (1998)

A/E ratio = (IAA/total IAA x 1000)

IAA requirement = (requirement for lysine x specific A/E ratio) / A/E for lysine

* The lysine requirement for turbot juveniles was estimated and determined to be 5 g/16 g N by Kaushik (1998) and Peres & Oliva-Teles (2008), respectively.

**Since no data on the lysine requirement are available for blackspot seabream, a lysine requirement of 5 g/16 g N was used. The AA requirements were here estimated taking as a base the muscle AA profile of wild individues (Annex 1).

Due to the inexistence of a specific diet formulation to blackspot seabream, the first trials used diets developed for other marine species, namely gilthead seabream. Therefore, and as suggested by Peleteiro *et al.* (2000), diet composition is a plausible reason for the slow growth and high fat deposition of blackspot seabream. Nutritional studies testing different diet formulations, with highly digestible ingredients are needed to improve blackspot seabream growth and feed efficiency. Moreover, attention should be devoted to the dietary protein, lipids and carbohydrates level and source role on fish lipid metabolism, in order to understand the excessive high build up capacity attributed to blackspot seabream.

Dietary lipids

The ideal inclusion level of dietary lipids is not a fixed value, since it varies with the type of lipid as well as the protein and energy content of the diet. Due to the metabolic interactions among protein, lipid, and carbohydrate, the definition of the exact dietary lipid requirements for fish is not particularly meaningful. However, it has long been accepted that dietary lipid ranging from 10–20% (dry weight basis) is sufficient to allow protein to be effectively utilized for fish growth without depositing excessive lipid in the fish tissues (Cowey & Sargent 1979; Watanabe 1982; Sargent *et al.* 1989; Corraze 2001). According to Linares *et al.* (2001), dietary protein quality and lipid level exerts a clear influence on the growth of blackspot seabream juveniles that seem to have low lipid tolerance (≤12%). Moreover, an excessive lipid deposition has been described to this species under intensive aquaculture system, even at low dietary lipid levels. The high lipid depot, found mainly around viscera and at lower concentration in muscle and liver tissues (Linares *et al.* 2000, 2001; Valente *et al*. 2009b), leads to poor flesh quality and slaughter yield. These poor traits observed in cultured blackspot seabream constitute a serious constraint for its production in semi-intensive and intensive aquaculture systems.

The essentiality of fatty acids (FA) is very difficult to establish, since the criteria of growth rate are less sensitive to essential fatty acids (EFA) supplies than inputs of indispensable amino acid (IAA). Tissue lipid composition varies in relation to FA inputs, essential or not, which does not permit to constitute body lipid composition as a requirement criterion. All vertebrate species have absolute dietary requirements for certain polyunsaturated fatty acids (PUFA). The EFA requirements, investigated so far for marine fish species, indicate that the n-3 EFA requirements can be only met by 20:5n-3 (EPA, eicosapentaenoic acid) together with 22:6n-3 (DHA, docasahexaenoic acid), often collectively called the n-3 highly unsaturated fatty acids (HUFA). This condition results from the adaptation to a combination of the predominant PUFA in the marine food web and the carnivorous

lifestyle of virtually all the marine species investigated so far (Sargent *et al*. 2002). Moreover, the great majority of marine fish, including all species currently farmed or under development for farming, are carnivores, consuming predominantly fish that, a priori, are rich in EPA and DHA derived from phytoplankton via zooplankton. Consequently, marine fish have a poor capacity, when compared to freshwater species, to convert their dietary intake of 18:3n-3 (linolenic acid) to EPA and DHA, which substantiate their essentiality (Sargent *et al.* 2002; Tocher 2003).

Despite the current high costs of fish oil, aquafeed industry continue to use this feedstuff in their formulations because it is traditionally accepted among fish farmers, for its property as appetent and its adequate FA profile, particularly for marine carnivorous species. Nevertheless, the increased costs of fish oil prices and limited supplies together with the increased concern on the long-term sustainability of these finite fishery resources, challenge aquaculture industry to find and implement sustainable alternatives to fish oil, such as vegetable oils (Turchini *et al.* 2009).

Dietary carbohydrates

Carbohydrate play little role as an energy source due to its low abundance in natural diets (Walton & Cowey 1982). According to Wilson (1994), fishes do not have dietary carbohydrate requirement, however, the incorporation of digestible carbohydrate in aquafeeds may spare other dietary nutrients, such as protein and lipids, for energetic proposes. The optimal or recommended dietary level of digestible carbohydrate varies among species, once the extent to which fish in general, and marine species in particular can utilize dietary carbohydrate, also vary. Carbohydrate utilization is also affected by the dietary inclusion level, botanical origin, the complexity of the molecule and the technological treatments applied (Wilson 1994; Hemre *et al.* 2002; Stone 2003; Enes *et al*. 2009). It is generally considered that for carnivorous species, including salmonids and marine fish, the dietary incorporation of digestible carbohydrates should not exceed 20%, whereas for warmwater herbivorous or omnivorous species levels can reach 40% (Wilson 1994; Stone 2003). Overall, carbohydrate processing, like gelatinization or extrusion, generally has a positive effect on starch digestibility and thus on its utilization by fish (Baños *et al.* 1998; Dias *et al.* 1998; Alvarez *et al.* 1999; Peres & Oliva-Teles 2002; Venou *et al.* 2003; Fernández *et al.* 2007). However, the intermediary metabolism of carnivorous fish, like rainbow trout, shows only moderate adaptation to high levels of dietary carbohydrate (Cowey & Walton 1989; Moon 2001). A recent long-term study on blackspot seabream reared in sea cages has shown that different carbohydrate sources (wheat vs. wheat bran) did not affect growth performance, feed utilization or fat deposition during the

grow-out phase (Valente *et al.* 2009a). Nevertheless, further work is needed to clarify if the lack of differences among the treatments could be associate to the extremely low feed consumption and growth rate observed during the experimental period or to a dysfunction on the glucose metabolism regulation as previously hypothesized to explain the low dietary glucose utilisation in fish (Wilson 1994; Enes *et al*. 2009). Thus, the understanding of the nutritional regulation of the glucose metabolism in blackspot seabream requires the integration of studies combining enzyme regulation, nutrient digestibility and growth performance in relation to dietary carbohydrates.

Nutritional regulation of lipid deposition in fish

The longstanding interest in fish lipid nutrition stems from its abundance and uniqueness. Indeed, fish are the most important source of HUFA, known for their important role in animal and human nutrition (Corraze 2001; Sargent *et al.* 2002; Tocher 2003; Ruxton *et al*. 2004; Massaro *et al.* 2008). However, the research on marine lipids gain momentum three decades ago, due to the need of understanding the lipid nutritional requirements of farmed fish, optimizing dietary protein to energy ratio, and boosting growth in farmed fish. Lipids and their constituents FA are, along with proteins, the major dietary nutrients in fish diets, playing major roles as sources of metabolic energy for the maintenance processes, growth, reproduction, serving as a vector during intestinal absorption of liposoluble vitamins and carotenoid pigments, and perhaps most importantly, as structural components of cell membranes (Corraze 2001; Sargent *et al.* 2002; Tocher 2003).

For animal nutrition, the most important lipids classes are the triacylglycerols (TAGs) and phospholipids (PL). TAGs are triesters of glycerol and three FA, and PL are esters of two FA and one phosphoric acid, which is itself linked by a diester bond to another alcohol (usually ethanolamine, choline, serine or inositol). PL are mainly localised in the cell membrane and cellular organelles, representing a relatively constant lipid fraction of the tissues. TAGs are the predominant form of storing FA, comprising the main energy reserve in all animals, and thus, are mainly responsible for the changeability of fish lipid contents. The gross energy yield from the complete oxidation of lipids is about 39.5 kJ g^{-1} in contrast with about 23.6 kJ g^{-1} for proteins and 17.2. kJ g^{-1} for carbohydrates (Blaxter 1989).

Numerous biotic and abiotic factors are known to influence lipid content in fish, such as genetic, nutritional, endocrine and environmental, with diet playing a preponderant role. Therefore, body lipid content can easily be modulated by controlling the feeding regime (Shearer *et al.* 1997; Jobling *et al.* 1998; Johansson *et al.* 2000). Lipid storage in fish tissues depends, firstly, on the availability of circulating TAGs originated from both exogenous (diet) and endogenous sources (fatty acid synthesis *de novo* or lipogenesis). Indeed, energy intake and balance more than dietary fat level *per si* appears to influence whole body fat storage in fish (Shearer 1994), with surplus non-fat energy being converted into fat though lipogenic pathways (Hellerstein *et al.* 1996). Therefore, to get a general overview on nutritional regulation of lipid deposition, the role of lipid, carbohydrate and protein content on fish diets is considered in the current Thesis and shortly reviewed. Detailed aspects concerning dietary lipid digestion, absorption, transport to peripheric tissues and mitochondrial or peroxisomal β-oxidation in fish fall outside the scope of this work and have been reviewed elsewhere (Kunau *et al.* 1995; Corraze 2001; Sargent *et al.* 2002; Tocher 2003).

Fatty acid synthesis *de novo*

Although carbohydrate, fat, or protein may all be present in excess in a diet, TAGs are the only substantial storage form for surplus energy in animals. The capacity for glycogen storage is limited, and no protein whose sole function is to serve as an AA reservoir has been identified in animals. Thus, no mechanism exists for the direct storage of a long-term surplus of either carbohydrates or protein in the diet. These simple considerations lead to the inference that the body must be capable of transforming surplus non-fat energy into fat, through fatty acid synthesis *de novo* or lipogenesis (Hellerstein *et al.* 1996).

The lipogenic pathway in fish is assumed to be basically similar to that operating in mammals (Iritani *et al.* 1984; Henderson & Sargent 1985). However, in fish, lipogenesis mainly occurs in liver while in mammals occurs in adipose tissue (Henderson & Sargent 1985). In fact, the activity of lipogenic enzyme is substantially higher in hepatic than in adipose tissues, implying that liver is undoubtedly the main site for lipogenesis in fish whilst adipose tissue is adapted for the uptake and storage of exogenous and endogenous FA (Lin *et al.* 1977a,b; Henderson & Sargent 1985). Lipogenesis requires acetyl-CoA units as the primary substrate and reducing power provided by NADPH.

The considerable reducing power required for lipogenesis could be produced by the following reactions:

a) Through the oxidative phase of pentose phosphate pathway, glucose-6-phosphate undergoes dehydrogenation and decarboxylation generating NADPH and ribulose-5-phosphate. This reaction is catalysed by glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) both enzymes requiring $NADP^+$ as a hydrogen acceptor;

G6PD

Glucose-6-phosphate + $NADP^+$ → 6-phosphoglucone-1.5 lactone+ NADPH + H^+

b) Throughout the pyruvate-malate cycle, malate undergoes oxidative decarboxylation to pyruvate and CO2, generating NADPH from $NADP^+$ in a reaction catalyzed by malic enzyme (ME, EC 1.1.1.40);

ME

L-Malate + $NADP^+$ → Pyruvate + CO_2 + NADPH

c) Through the conversion of citrate to isocitrate and then to α-ketoglutarate by the cytosolic $NADP^+$-dependent isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42) may provide additional NADPH source for lipogenesis.

NADP-IDH

Isocitrate + $NADP^+$ → α-ketoglutarate + NADPH + CO_2

It is well established that the activity of these NADPH-forming enzymes varies significantly depending to the species, but also with the hormonal and nutritional state of the animal (García-Jiménez *et al.* 1993; Barroso *et al.* 1994,1997,1998;1999,2001; Kletzien *et al.* 1994; Sánchez-Muros *et al.* 1996). In humans and monogastric animals (except birds) the penthose phosphate pathway is the main provider of reducing equivalents followed by the pyruvate-malate cycle are (Iritani *et al.* 1984). In ruminants, the pyruvate-malate cycle seems to be inoperative and thus the NADPH is mainly provided by pentose phosphate pathway and NADP-IDH. Regardless of the dietary nutrition conditions, NADPH reducing equivalents are mainly provided by G6PD in rainbow trout (Walzem *et al.* 1991; Barroso *et al.* 1994; Hung & Storebakken 1994), European sea bass (Dias *et al.* 1998), Senegalese sole (Dias *et al.* 2004) and gilthead seabream (Menoyo *et al.* 2004). On the other hand,

NADPH is mainly provided by ME and NADP-IDH in Atlantic salmon (Menoyo *et al.* 2003) and white sturgeon (Hung *et al.* 1989; Fynn-Aikins *et al.*1992).

The two carbon acetyl-coA unit is the primary substrate for lipogenesis and is ultimately produced in mitochondria (Fig. 3) through the following routes:

a) The oxidative decarboxylation of pyruvate (carbohydrates source);
b) The oxidative degradation of some AA (protein source);
c) The β-oxidation.

In the lipogenic pathway, the two-carbon acetyl-CoA unities are firstly carboxylated by the acetyl-CoA carboxylase (ACoAC, E.C. 6.4.1.2) to malonyl-CoA. Then, malonyl-CoA is converted to palmitate (16:0) and stearate (18:0) FA by the fatty acid synthetase complex (FAS, EC 2.3.1.38), via a series of condensation and reduction reactions involving the utilization of NADPH (Volpe & Vagelos 1976; Wakil *et al.* 1983; Sargent *et al.* 1989; Nelson & Cox 2004).

FAS

$$\textbf{Acetyl-CoA + 7 Malonyl-CoA + 14 NADPH + 14H}^{+}\rightarrow \textbf{Palmitic acid + 7 CO}_2\textbf{ + 8 CoA + 14 NADP}^{+}\textbf{ + 6 H}_2\textbf{O}$$

As the acetyl-CoA is generated in the mitochondria whereas lipogenesis takes place in the cytosol, the acetyl-CoA unities are transported from the interior of the mitochondria to the cytosol as citrate. Under conditions that favour FA synthesis, mitochondrial citrate synthetase (CS, EC 2.3.3.1) catalyses the formation of citrate from acetyl-CoA and oxaloacetate (Fig. 3). Citrate could then be exported from the mitochondria and cleaved by the ATP-citrate lyase (ACL, EC 4.1.3.8) to produce oxaloacetate and acetyl-CoA, the latter becoming the cytosolic substrate for FA synthesis. The oxaloacetate can then be reduced to malate by cytosolic NAD-malate dehydrogenase (MDH, EC 1.1.1.37). Malate, in turns, undergoes oxidative decarboxylation to pyruvate and CO2, generating simultaneously NADPH, in a reaction catalyzed by ME, as described above. Pyruvate can reenter the mitochondria, where it recombines with CO2 to form oxaloacetate and thus completing the cycle (Fig. 3).

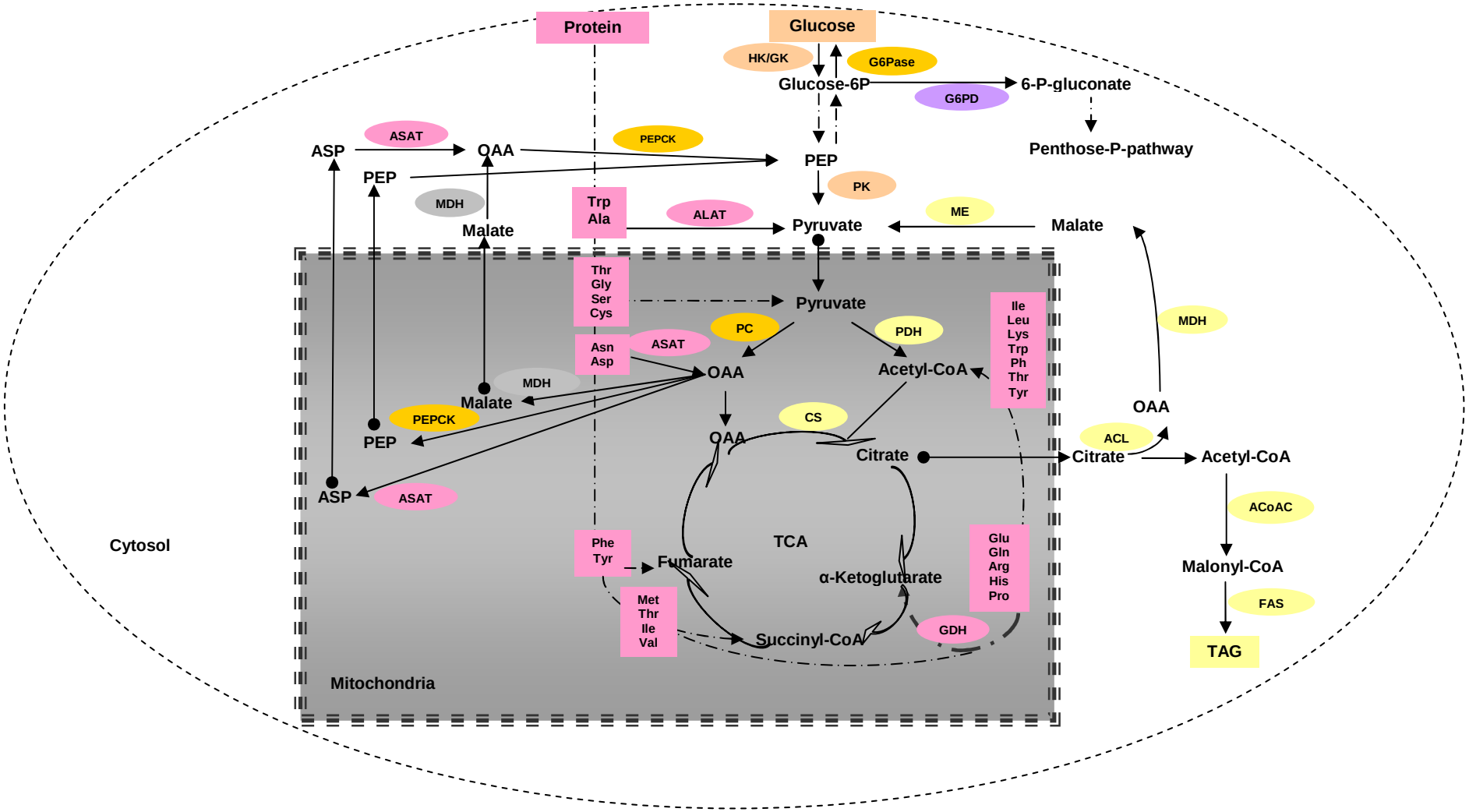


Figure 3. Simplified view of the pathways involved in conversion of carbohydrates and protein to fatty acids in liver.
Legend: HK, hexokinase; **GK**, glucokinase; **G6Pase**, glucose-6-phosphatase; **G6PD**, glucose-6-phosphate dehydrogenase; **PK**, pyruvate kinase; **ME**, malic enzyme; **PEPCK,** phosphoenolpyruvate carboxykinase; **MDH**, malate dehydrogenase; **ACL**, ATP-citrate lyase; **ACoAC**, acetyl-CoA carboxylase; **FAS**, fatty acid synthetase; **ALAT**, alanine aminotranferase; **ASAT**, aspartate aminotranferase; **GDH**, glutamate dehydrogenase; **PC**, pyruvate carboxylase; **PDH**, pyruvate deshydrogenase; **CS,** citrate synthetase**; TAG,** triacylglycerols**; OAA**, oxaloacetate**; PEP,** phosphoenolpyruvate**; TCA,** tricarboxylic acid.

A short review of the pathways leading to the acetyl-CoA generation from non-lipid precursors (carbohydrates and proteins) is presented in the following sections, and a schematic overview is presented in Figure 3. The production of acetyl-CoA from β-oxidation is driven to supply the organism with additional energy under unfavourable energy state. Thus, the acetyl-CoA generated from β-oxidation is primarily directed into energy production rather than fatty acid synthesis *de novo.* Indeed, the lack of acetyl-coA and malonyl-CoA has an inhibitory effect on FAS and thus down-regulates lipogenesis (Nelson & Cox 2004).

Fatty acid synthesis from dietary carbohydrates

Glucose plays a key role in mammalian energetics but in fish its importance as a metabolic fuel seems limited (Hemre *et al.* 2002; Stone 2003; Enes *et al.* 2009). Although the enzymes for the major glucose metabolic pathways, such as glycolysis, pentose phosphate shunt, gluconeogenesis, and glycogen synthesis, have been detected in fish, the regulation of carbohydrate metabolism differs among species and, in some aspects, is distinct from that of mammals (Cowey & Walton 1989). Rates of glucose turnover and glucose oxidation in fish are one or two orders of magnitude below those of mammals of comparable body size and only in some unique species, such as eels or tunas, mammalian-type rates are found (Hemre *et al.* 2002). Yet, dietary carbohydrates can exert important indirect influence on fish intermediary metabolism.

Liver plays a key role in regulating body metabolism in response to the nutritional status and thus in response to dietary carbohydrate, affecting a cascade of events in other tissues and organs. The excess of blood glucose, resulting from ingestion, is uptaken by the liver to be either stored as glycogen or oxidised through glycolysis and pentose phosphate pathway, and ultimately used for FA synthesis (Greenway 2004; Nelson & Cox 2004). The carbohydrate entering the pentose phosphate pathway generates reducing power in the form of NADPH (Fig. 3) required for lipogenesis (Walton & Cowey 1982). Glycolysis and gluconeogenesis (Fig. 4) are opposite metabolic pathways involved in the degradation and synthesis of carbohydrates, respectively, and play a major role in maintaining glucose homeostasis. Furthermore, the more efficient regulation of plasma glucose found in omnivorous fish was suggested to occur when carbohydrates are mainly channelled into glycolysis and lipogenesis pathways, decelerating the gluconeogenesis route (Shimeno *et al.* 1993). The conversion of glucose to pyruvate (glycolysis) begins with the phosphorylation of glucose by hexokinase (HK, EC 2.7.1.1) and glucokinase (GK, EC 2.7.1.2) to produce glucose-6-phosphate, a molecule that can also be used in other metabolic pathways, such as glycogenesis and the pentose-phosphate pathways, and

thus playing a key role in the intermediary metabolism. The regulation of glycolytic pathway is dependent on the activities of key enzymes such as GK, 6-phosphofructo-1-kinase (PFK-1, EC 2.7.1.11) and pyruvate kinase (PK, EC 2.7.1.40). Phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32), fructose-1,6-bisphosphatase (FBPase-1, EC 3.1.3.11) and glucose-6-phosphatase (G6Pase; EC 3.1.3.9) catalyze the reverse reactions of glycolytic pathways, and thus are key steps in gluconeogenesis.

Depending on the nutritional and hormonal regulation, pyruvate is converted into acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex (PDH, EC 1.2.4.1) and can either be catabolized (oxidation via the TCA cycle) or used for FA synthesis (isoprenoid, cholesterol or steroid synthesis). Since no net formation of glucose from acetyl-CoA occurs (Walton & Cowey 1982), the conversion of pyruvate into acetyl-CoA is a major control point of the intermediary metabolism, as it represents an irreversible loss of carbohydrate. The contribution of carbohydrates as substrates for the hepatic lipogenic enzymes is well documented in higher vertebrates (Iritani 1992; Hellerstein *et al.* 1996; Hillgartner & Charron 1998). In addition, the Guru Walla model (Pasquet *et al.* 1992) notably evidenced that under unusual dietary conditions (high carbohydrate intake), important rates of lipogenesis can occur, resulting in a substantial increase of body fat content in human. As observed in mammals, the enzymes involved in carbon flux from carbohydrates into FA were shown to be up-regulated by dietary carbohydrates in fish, such as rainbow trout (Hung & Storebakken 1994; Brauge *et al.* 1995), coho salmon (*Oncorhynchus kisutch*) (Lin *et al.* 1977a,b), channel catfish (Likimani & Wilson 1982), Nile tilapia (*Oreochromis niloticus*) (Shimeno *et al.* 1993), European sea bass (Dias *et al.* 1998) and gilthead seabream (Fernández *et al.* 2007).

The dietary level of carbohydrate and their complexity affects carbohydrate utilization. In several fish species the dietary incorporation of gelatinized starch resulted in higher liver lipogenic activity (Shiau & Chen 1993; Shiau & Lin 1993; Shiau & Liang 1995; Hung *et al.* 1989; Arnesen *et al.* 1995; Robinson & Li 1995; Deng *et al.* 2000). Numerous studies have shown that a potential or, perhaps, detrimental effect of feeding high digestible carbohydrate diets is the excessive lipid deposition in the flesh. In fact, a high dietary digestible carbohydrate content resulted in increased fat deposition in rainbow trout (Kaushik & Oliva-Teles 1985), Atlantic salmon (Grisdale-Helland & Helland 1997), European sea bass (Dias *et al.* 1998) and gilthead sea bream (Venou *et al.* 2003; Fernández *et al.* 2007).

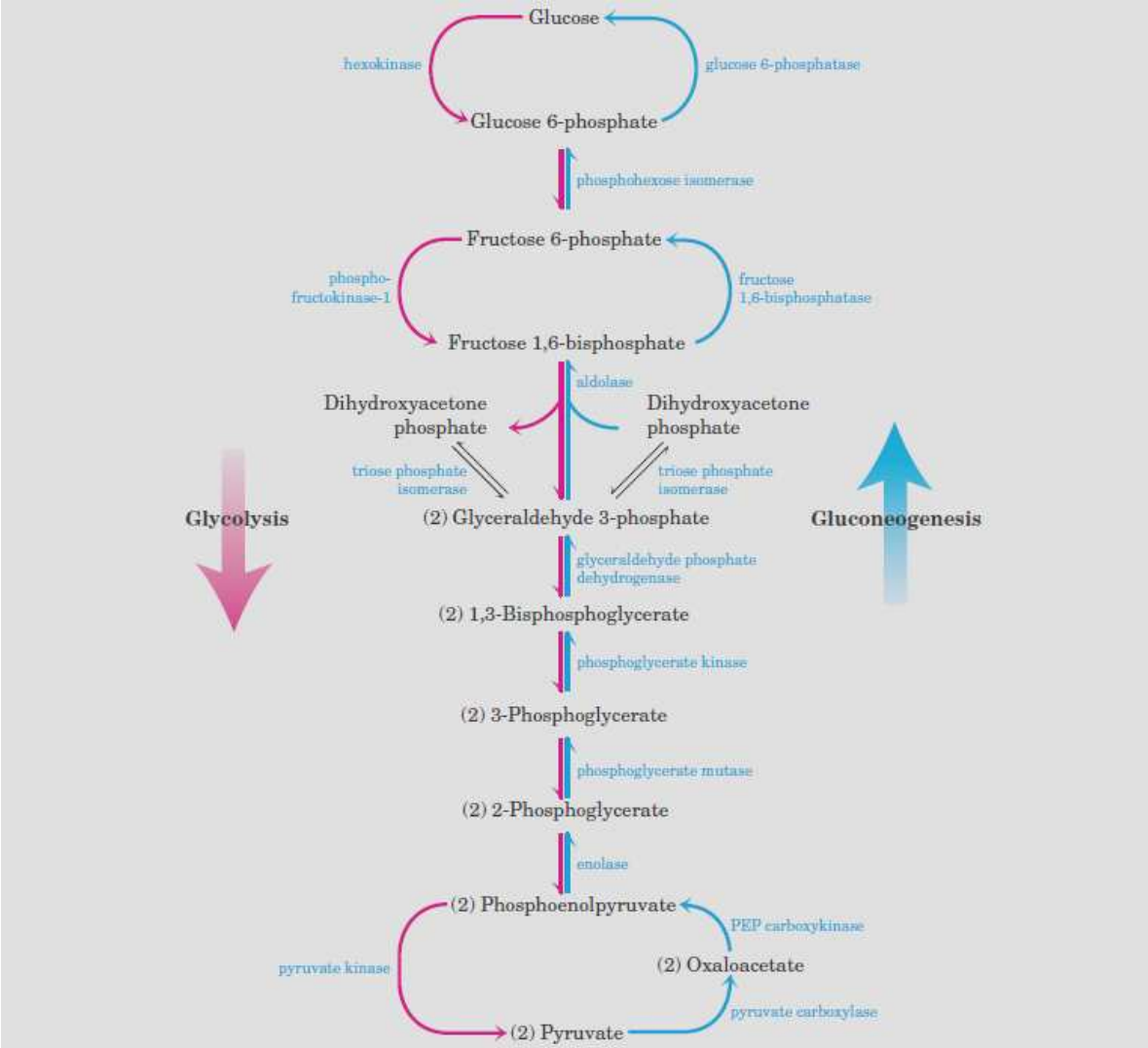


Figure 4. Schematic view of glycolysis and gluconeogenesis pathways.

Adapted from Nelson & Cox 2004.

On the other hand, other studies reported no effect of carbohydrate on whole body composition in rainbow trout (Alvarez *et al.* 1999), European sea bass (Enes *et al.* 2006a) or in gilthead seabream (Peres & Oliva-Teles 2002; Enes *et al.* 2008a). In fact, Hemre & Kars (1997) following radiolabelled glucose course showed the limited ability of cod to convert glucose into lipids. On the other hand, the dietary inclusion of digestible carbohydrates has little effect on ME and FAS activities in Senegalese sole, but positively enhanced G6PD activities (Dias *et al.* 2004). It seems therefore, that carbohydrate stimulates lipogenesis in fish, mainly by the increase of reducing power (NADPH) and less through the release of carbon skeletons to form FA (Hemre *et al.* 2002).

Fatty acid synthesis from dietary protein

Protein is the main component of the natural diets of carnivorous fish which parcially explain their low ability to use carbohydrates (Hemre *et al.* 2002; Stone 2003; Enes *et al.* 2009). If too much protein is supplied in the diet, only part of it will be used to make new proteins, and the remainder will be converted into fat and energy (Wilson 2002). Therefore, even if glucose derived from the carbohydrate metabolism is considered the primary substrate for fatty acid synthesis in omnivorous mammals and in humans (Hilgartner *et al.* 1995; Hellerstein *et al*. 1996), carbon skeletons derived from the AA catabolism through conversion into pyruvate or TCA cycle intermediates (Fig.3) are apparently the major precursors of acetyl-CoA in fish.

Following their release from ingested proteins and their absorption from the gastrointestinal tract or even directly entring into the blood stream via intravenous line, AA experience one of three major metabolic reactions (Young & Fukagawa, 1988):

a) Used as a substrate for the net synthesis of new proteins and maintenance of tissue and organ proteins;
b) Used as precursors for many metabolically non-protein nitrogen-compounds, such as creatine, epinephrine, serotonin, and the polyamines, as well as providing nitrogen and carbon skeletons for the dispensable amino acids (DAA) synthesis;
c) Converted to other AA and/or enter catabolic pathways where its carbon skeletons are incorporated into carbohydrates and lipids molecules.

Cowey & Walton (1989) summarized the enzymes and catabolic pathways for each AA in fish, and this information still holds today. AA catabolism occurs mainly in the liver where the amino group is firstly removed (transdeamination) into intermediate compounds that can be further metabolized in the TCA cycle to yield energy or used as substrates for synthesis of other compounds like glucose or lipids.

Glutamate dehydrogenase (GDH, EC 1.4.1.2) catalyses the amination of *α*-ketoglutarate and the deamination of glutamic acid (Glu), and thus plays a key role in the AA catabolic pathways. Aminotransferases or transaminases catalyse the transfer of an amino group from one AA into a ketoacid to form another AA, like the particular case of alanine (ALAT, EC 2.6.1.2) and aspartate aminotranferase (ASAT; EC 2.6.1.1).

GDH

Glutamate + $NADP^+$ ↔ α-ketoglutarate + Ammonia + $NADPH^+$

ASAT

Aspartate + α-ketoglutarate ↔ Glutamate+ oxaloacetate

ALAT

Alanine + α-ketoglutarate ↔ Glutamate + pyruvate

AA catabolic pathways converge to form only six main products (Fig.5), being all of them possible TCA cycle intermediates and thus subject to complete oxidation into CO2 and H_20. On the other hand, the carbon skeletons could also divert to gluconeogenesis, ketogenesis or lipogenesis depending at some extend of their nature:

a) Phenylalanine, tyrosine, isoleucine, leucine, tryptophan, threonine, and lysine carbon skeletons are ultimately broken down to produce acetoacetyl-CoA and/or acetyl-CoA, for ketone bodies and/or FA synthesis, respectively;
b) Arginine, glutamine, histidine, proline, glutamate are converted to α-ketoglutarate;
c) Isoleucine, methonine, threonine and valine are converted to succinyl-CoA;
d) Phenylalanine and tyrosine are converted to fumarate;
e) Asparagine and aspartate are converted to oxaloacetate;
f) Alanine, cysteine, glycine, serine, threonine and tryptophan are converted to pyruvate.

The AA converted into α-ketoglutarate, succinyl-CoA, fumarate, and/or oxaloacetate can lead to glucose and glycogen production by glucogenic and glycogenic pathways, respectively, and thus are designated as the glucogenic AA. The AA converted into acetoacetyl-CoA and/or acetyl-CoA can yield ketone bodies and/or lipids, and thus are designated as ketogenic AA. However, the division between ketogenic and glucogenic AA is not sharp; five AA - tryptophan, phenylalanine, tyrosine, threonine, and isoleucine – could be both ketogenic and glucogenic.

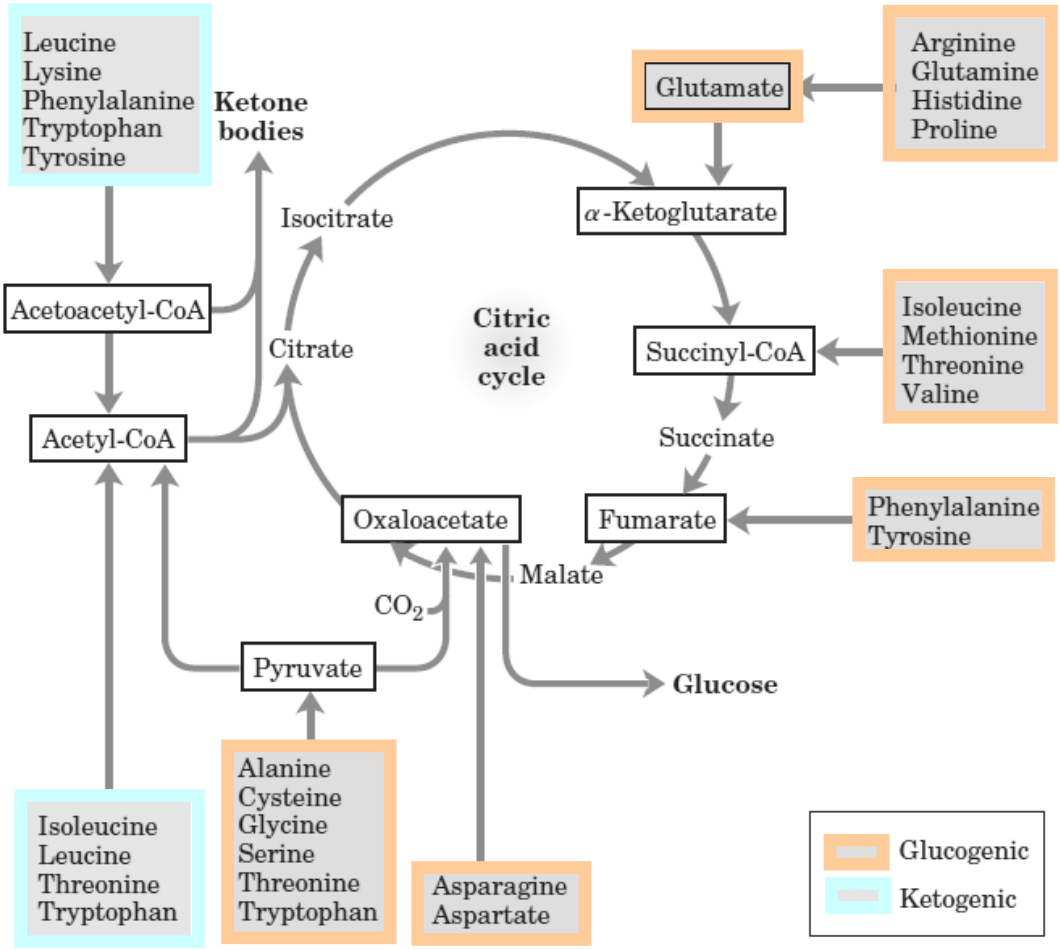


Figure 5. General overview of AA conversion into TCA intermediates or substrates diverted to gluconeogenesis, ketogenesis or lipogenesis

Adapted from Nelson & Cox (2004)

Even if the effect of dietary protein on hepatic lipogenesis has been less studied than either fat or carbohydrate, several studies recognized protein level and protein nature as potent regulator of lipid biosynthesis in both high vertebrates and fish. Herzberg & Rogerson (1981) reported increased lipogenic enzymatic activities in young rat fed with increasing protein levels. Conversely, an inverse relationship between dietary protein level and in vitro hepatic lipogenesis rate was found in chickens (Rosebrough *et al.* 1996, 2002). Such discrepancies might result from the fact that the major lipogenic tissues differ between rats and chickens and also from the different dietary protein levels tested in those studies. Using a ^{13}C-labelled protein tracer, Campbell *et al.* (1998) reported that the increase in protein intake markedly enhanced in vivo FA synthesis rate in rainbow trout, with considerable amount of dietary protein carbon skeletons being incorporated into lipids. Indeed, rainbow trout fed extremely low dietary protein levels diminish the lipid biosynthesis pathway (Walzem *et al.* 1991). Moreover, increasing dietary protein levels have been shown to up-regulate European seabass ACoAC activities, suggesting that the

carbon residues from AA catabolism constitutes probably a major source of acetyl-CoA in this species (Dias *et al.* 1998, 2003).

Due to fish meal high price and reduced availability in the international markets, numerous studies devoted to the evaluation of alternative protein sources for use in aquaculture feeds (Gatlin *et al.* 2007; Kaushik & Hemre 2008; Tacon & Metian 2008). Despite the interest in the evaluation of protein-rich plant ingredients as alternatives to fish meal, few studies have dealt with the effects of dietary protein sources at a metabolic level. The modification of body lipid content as well as lipogenesis by protein nature as been demonstrated in fish (Goméz-Requeni *et al.* 2003; Kaushik *et al.* 2004; Dias *et al.* 2005) like evidenced earlier in higher vertebrates (Iritani *et al.* 1986, 1996; Kayashita *et al.* 1996; Padmakumarannair *et al.* 1998). In rats, soybean or buckwheat protein decreased activity of G6PD, ME and FAS when compared to casein and fish proteins (Herzberg & Rogerson 1984; Iritani *et al.* 1986, 1996; Kayashita *et al.* 1996). Similarly, hepatic lipogenic activity was down-regulated in European seabass (Dias *et al.* 2005) fed soybean meal and in gilthead seabream (Gómez-Requeni *et al.* 2003) fed soybean meal supplemented with Glu. Even though, it becomes difficult to clearly distinguish whether these effects on lipogenesis are simply due to dietary AA or due to some compounds present in soybean meal such as trypsin inhibitors, saponins, phytoestrogens, phytosterol, isoflavones suggested to affect cholesterolemia and hepatic lipogenic enzymes (Anderson & Wolf 1995; Potter 1995; Kaushik *et al.* 1995; Gómez-Requini *et al.* 2003; Dias *et al.* 2005).

The mechanisms underlying the modulation of fatty acid synthesis *de novo* by protein nature are ill-defined, but dietary AA composition has been cited as one of the major factors (Herzberg 1991). In carp, *in vitro* FA synthesis rate from ^{14}C-alanine and ^{14}C-glutamate was markedly higher than that from ^{14}C-glucose (Nagai & Ikeda 1972; Shikata & Shimeno 1997). In addition, Henderson & Sargent (1981) reported that the incorporation of ^{14}C-glucose into TAG was much lower than in ^{14}C-alanine incorporation in liver slices of rainbow trout. A review of salmonid fishes metabolism by Walton & Cowey (1982) showed that a wide variety of the biochemical processes in salmonids require glutamine (Gln) or glutamate (Glu) as source of amino-nitrogen, or require the carbon skeleton of these compounds (α-ketoglutarate) to act as a amino-nitrogen acceptor. Although Gln can donate amino groups, its involvement in other processes of intermediary metabolism is minimal and, generally, is not as rate limiting as those of Glu (Nelson & Cox 2004). Hughes (1985) has firstly evidenced the potential effects of DAA in fish growth and nutrient utilization. In this study, lake trout (*Salvelinus Namaycush*) showed better growth but also higher fat content when fed Glu rather than glycine, as the major source of DAA.

Indeed, it has been suggested that several DAA may become conditionally essential when the rate of endogenous synthesis is constrained by the availability of appropriates quantities of metabolic nitrogen (Reeds 2000; Li *et al.* 2009). Therefore, considering the role of dietary protein nature and its respective AA profile on fish lipogenic pathways, besides the dietary IAA/DAA ratio also the plant protein AA profile must be taken into attention when fish meal is being replaced by plant protein sources.

Biosynthesis of highly unsaturated fatty acids

The FA supplied by the food and those endogenously synthesised by the animal, can be bioconverted, to a certain extent, into FA with longer or more unsaturated chains. However, FA composition of the dietary lipids has an undoubtedly greater influence on tissue FA composition than FA coming from lipogenesis (Watanabe 1982; Henderson & Tocher 1987; Sargent *et al.* 1989). In fish, the main newly synthesised FA are palmitate (16:0), stearate (18:0) and myristate (14:0), in different proportions depending on the species. These saturated FA synthesised by fatty acid synthesis *de novo* could be either deposited or converted into monounsaturated fatty acids (MUFA), such as 18:1 n-9, by Δ9 desaturases. The bioconversion of PUFA includes elongation (addition of two carbons) and desaturation (addition of a double bound), assured by several enzymes like Δ6 and Δ5 desaturase (Fig. 7). The extent to which fish can convert C18 PUFA into C20/22 HUFA varies between species, being high in freshwater and low in marine fish species (Sargent *et al.* 2002). Similarly to mammals, freshwater fish present high bioconversion capacity of C18 FA into HUFAS (Corraze 2001) with C18 FA (linoleic and linolenic acid) becoming the truly EFA. The inability of marine fish to elongate and desaturate 18:3n-3 to EPA (20:5n-3) and DHA (22:6n-3) has been earlier described by Sargent *et al.* (1989). Moreover, the ineffective step of this pathway has been recently identified as the Δ5-desaturase, in gilthead sea bream or the C18–C20 elongase in turbot (Ghioni *et al.* 1999). The result is that the n-3 HUFAs, DHA and EPA, must all be supplied in the diet for normal growth and development of marine fish (Sargent *et al.* 1999). Synthesis of EPA is achieved by Δ6 desaturation of 18:3-n3 to produce 18:4n-3 that is elongated to 20:4n-3 followed by Δ5 desaturation (Cook 1996), with synthesis of DHA from EPA requiring two further elongation steps, a second Δ6 desaturation and a peroxisomal chain shortening step (Sprecher 2000).

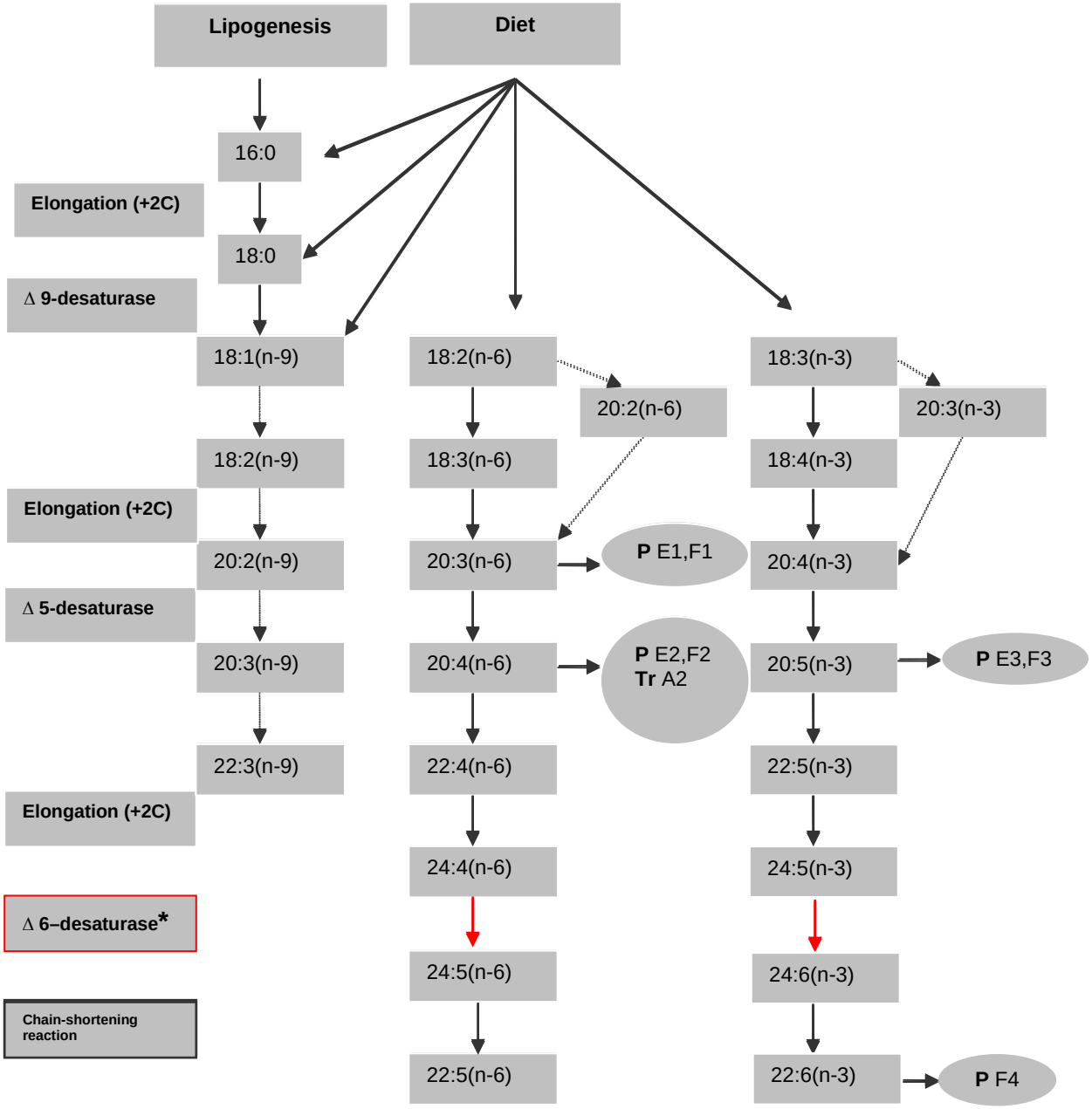


Figure 7. Diagram of main routes for bioconversion of FA and prostaglandins (P) and tromboxanes (Tr). The Δ6* enzyme acting on C 24 FA may or not be the same enzyme (Δ6) that acts on C18 FA.
Adapted from Corraze (2001) and Tocher (2003)

In addition, marine fish are also unable to synthesise the n-6 HUFA. Hence, the requirement of arachidonic acid (20:4n-6; ARA), which is an essential component of membrane phospholipids and a precursor for bioactive eicosanoids must also be considered in marine fish diets.

Lipid deposition

Stored lipids are generally TAG resynthesised in the tissues from free circulating FAs liberated by lipase action or coming from the endogenous synthesis *de novo*. Consequently, both dietary lipid level and the rate of newly synthesized FA contribute, even at different proportions, to fish whole body lipid contents. Feeding fish with a high dietary lipid levels undoubtedly contributes to augment flesh lipid contents (Shimeno *et al.* 1993; Shearer 1994; Grisdale-Helland & Helland 1997; Alvarez *et al.* 1998; Company *et al.*1999a,b and revisions by Sargent *et al.* 2002 and Tocher 2003). Moreover, high dietary lipid levels may enhance the lipid deposition in the edible parts of the fish (i.e. trimmed fillet) as well as in the "waste" compartments such as the viscera and the abdominal belly wall (Hillestad & Johnsen 1994; Weatherup *et al.* 1997; Jobling *et al.* 1998; Company *et al.* 1999a,b), with significant implications on fish final quality.

Lipogenesis is tightly regulated by the dietary lipid level. In contrast to the stimulatory effect of dietary carbohydrate and protein, the addition of fat in particular PUFA reduces the rate of FA synthesis and the activities of the lipogenic enzymes in mammals (Stabile *et al.* 1998; Clarke 2001) and in fish (Alvarez *et al.* 2000). High fat diets were shown to depress the activity of several lipogenic enzymes in salmonids (Lin *et al.* 1977b; Arnesen *et al.* 1993; Alvarez *et al.* 1998; Gélineau *et al.* 2001) and in marine fish like seabass (Bautista *et al.* 1988; Dias *et al.* 1998). On the other hand, flatfish species like turbot and Senegalese sole showed surprisingly little response to increasing dietary lipid levels (Regost *et al.* 2001; Dias *et al.* 2004). Marine fish, that naturally consume diets rich in lipid, are not likely to biosynthesize fatty acids *de novo* to a significant extent, while freshwater species present higher lipogenic capacity since lipid-rich prey are much less common in fresh water than in the sea (Tocher 2003).

The location of lipid depots varies widely among species, serving as a criterion for differentiating between several categories of fish. Nevertheless, adipose tissue is the major lipid storage tissue of most teleost species (Sheridan 1988, 1994; Corraze 2001; Tocher 2003). Hence, the viscerosomatic index (VSI) (Jobling *et al.* 1998; Rasmussen 2001) and the viscera lipid content (Hillestad *et al.* 1998; Rasmussen *et al.* 2000) are both positively correlated with dietary lipid level. In salmonids, like rainbow trout, fat deposition mainly occurs in visceral adipose tissue, and to a lesser extent in muscle, within adipocytes scattered between myofibers (Weatherup *et al.* 1997; Corraze & Kaushik 1999). In marine species such as the European seabass, lipids are mainly accumulated in liver and viscera (Corraze & Kaushik 1999) whereas in flatfish like turbot (Andersen &

Alsted 1993; Regost *et al*. 2001), Atlantic Halibut (Berge & Storebakken 1991; Martins *et al.* 2007) and Senegalese sole (Borges *et al.* 2009) subcutaneous fat also have a considerable contribution to fish whole body lipid content.

In farmed blackspot seabream, fat deposition mainly occurs in visceral adipose tissue (70-73%), and to a lesser extent in muscle (15%) and liver (14-16%) (Valente *et al.* 2009b). The distribution of lipid depots among fish tissues is a major issue for fish processors. High visceral lipid depots represents an economical concern as viscera is discarded as a by-product during fish processing, whereas big muscle fat depots might have strong implications in the fillet product quality. Hence, understanding the regulation of fish lipid deposition is a very important issue for both producers and processors. Although reared fish are usually fatter than their wild congeners, this difference becomes less evident when feed formulation is developed according to species requirement. Therefore, question arises on how the manipulation of feed composition and feeding regime may influences body lipid content in blackspot seabream.

Endogenous lipids, synthesised via lipogenesis, and dietary lipids, not used for supply of energy, leads to lipids depots in tissues. Formation of tissue lipid depots involves transport of both absorbed and de novo synthesized lipids in peripheral tissues as lipoproteins, and release of FA from triacylglycerol-rich core of circulating lipoproteins by lipoprotein lipase (LPL) for uptake by tissues (Fig. 8). Fish plasma contains a similar range of lipoproteins to mammalian plasma, namely, chylomicrons, very-low-density lipoprotein (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL), the latter formed, in mammals at least, by the combined actions of LPL and lecithin cholesterol acyl transferase (LCAT) on LDL (Sheridan 1988). LPL is recognized to have a key role on lipid deposition regulation. LPL is an important enzyme that catalyzes the hydrolysis of TGA from circulating chylomicrons and VLDL into free fatty acids (FFA) and 2-monoacylglycerols, and thus constitutes a rate-limiting step in the lipid transport into peripheral tissues (Sato *et al.* 1999). The resulting FFA could then be re-esterified and stored, as occurs in adipose tissue, or used as an energy source by peripheral tissues, mainly in muscle and heart (Auwerx *et al.* 1992).

In fish, LPL has been identified at the molecular level in various species, including zebrafish (*Danio rerio*) (Arnault *et al.* 1996), rainbow trout (Arnault *et al.* 1996; Lindberg & Olivecrona 2002; Richard *et al.* 2006a), red sea bream (Oku *et al.* 2002; Liang *et al.* 2002a,b), and recently in gilthead seabream (Saera-Vila *et al.* 2005). In contrast to mammals, LPL activity and/or expression has also been detected in the liver of adult fish

(Black *et al.* 1983; Liang *et al.* 2002a,b; Richard *et al.* 2006a,b; Saera-Vila *et al.* 2005). Moreover, both LPL activity and expression have been showed to be markedly higher in adipose tissue than in muscle or liver (Lindberg & Olivecrona 1995, 2002; Arantzamendi *et al*. 2003; Saera-vila *et al.* 2005; Richard *et al.* 2006a), which usually correlates well with the major lipid storage organs in fish.

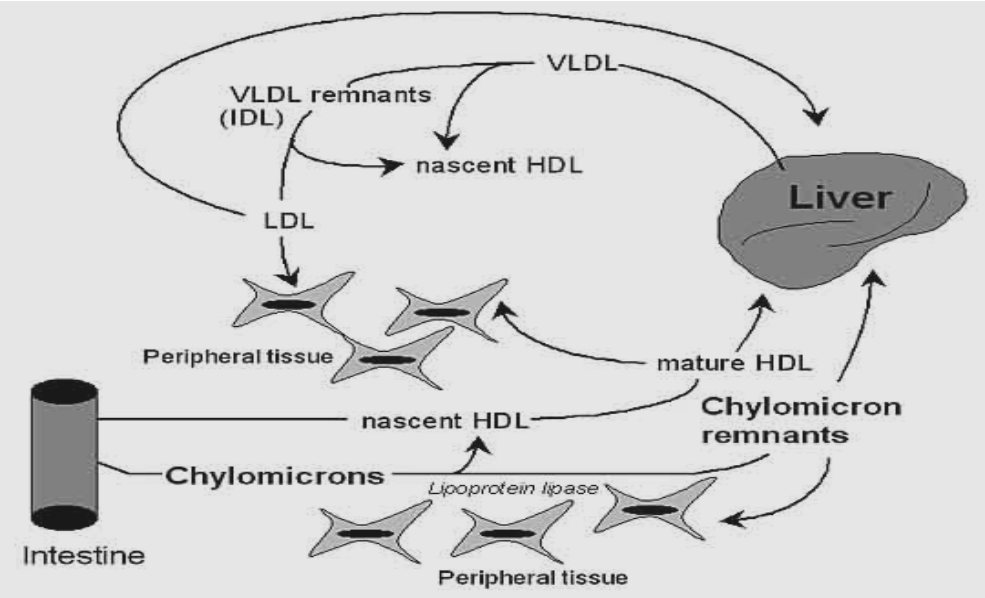


Figure 8. Schematic view of lipid transport.

The exogenous pathway comprises the synthesis of chylomicrons from dietary lipids absorbed by the intestine. HDL, high-density-lipoproteins; IDL, intermediate-density-lipoproteins; LDL, low-density-lipoproteins; VLDL, very-low-density-lipoproteins

In both, mammals and fish, LPL activity is down regulated in adipose tissue during fasting tissue while it does not change or increases in skeletal muscle or heart (Black & Skinner 1986; Doolittle *et al.* 1990; Lladó *et al.* 1999; Ruge *et al.* 2005; Albalat *et al.* 2006). Furthermore, a regulatory effect of LPL by both dietary FA and protein source has been described in fish (Liang *et al*. 2002 a,b; Richard *et al*. 2006a; Saera-Vila *et al*. 2005). However, there is very little information about the possible hormonal regulation underlying this nutritional effect on LPL activity or expression in fish.

It is well documented that body composition (water, protein, lipid, ash) of fish and utilization of nutrients are not constant, but change throughout the life cycle and are affected by endogenous (e.g., physiological state, strain, size) and exogenous (e.g., season, diet composition, feeding method) factors (Lovel 1980; Carter *et al*. 2001). Although insulin is an anabolic hormone in fish, information regarding insulin control of lipid deposition and mobilization is relatively scarce (Navarro *et al.* 2005). It was recently

found that insulin exerts a stimulatory effect on LPL activity in the adipose tissue of rainbow trout (Albalat *et al.* 2006) and gilthead seabream (Albalat *et al.* 2007). Besides insulin, GH and insulin-like growth factors (IGFs) are known to play a major role in fish development and metabolism (Pérez-Sánchez & Le Bail 1999; Company *et al.* 2001; Reinecke *et al.* 2005). Peptide hormones such as ghrelin, leptin, corticotrophin-releasing factor (CRF), bombesin, cholecystokinin (CCK), galanin, and neuropeptide Y are also implicated in the control of energy homeostasis (De Pedro & Bjornsson 2001; Leaver *et al.* 2008). Like in mammals, numerous hormones and metabolites involved in fish nutrient metabolism, showed daily fluctuations suggesting that fishes are not in the same physiological state throughout the day (Boujard & Leatherland 1992a; Spieler 1992; Boujard 2001). Indeed, the plasmatic levels of hormones like insulin and growth hormone (GH) are known to positively correlate with feed intake and ultimately to fish growth performances (Plisetskaya *et al*. 1991; Gélineau *et al*. 1996; Rungruangsak-Torrissen *et al.* 1999). The physiological significance of these endocrine variations relating to feeding time might be correlated to differences in growth rate of fish and particularly to differences in nutrient partitioning (Bolliet *et al.* 2001a; Carter *et al.* 2001). Taking all this into consideration, to understand at which point a same diet can be differently metabolized by a species depending on the feeding method would also be of prime interest for growth and nutrient deposition optimization of a new species.

Recognised the importance of macronutrients on fish lipid deposition, the regulation of this important process is a major issue for aquaculture purposes. Hence, the development of a nutritional formula that optimizes the conversion of dietary protein, lipid and carbohydrate into fish body protein and not fat, creating an attractive product for both producers and consumers, is a constant challenge to nutritionists.

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Objectives of the Thesis

The nutritional and economical importance of blackspot seabream (*Pagellus bogaraveo*) in Mediterranean market, associated with the eminent reductions in its capture, requires urgent consistent nutritional studies for the consolidation of blacskpot seabream farming. Knowing that the optimal level of dietary crude protein should be about 45% (Silva *et al*. 2006) and considering the low lipid tolerance (12%) of this species (Linares *et al.* 2001), the work carried out along this thesis aimed to understand the regulatory mechanism beyond energetic metabolism that leads to excessive lipid deposition in this species. Hence, the following specific objectives are proposed:

a) Evaluate the effects of different dietary protein/lipid levels on the growth performance, body composition and nutrient utilization of blackspot seabream contributing to a general insight into lipid metabolism of this new species.
b) Gain knowledge on carbohydrate utilization by blackspot seabream. Considering the high fat building capacity of the species, even when low lipid diets are used, the regulatory effect of carbohydrates on glucose metabolism and its relation to lipogenic pathways will be investigated.
c) Evaluate the dietary inclusion of protein-rich plant ingredients as alternatives to fish meal. Taking into account the reviewed modulation of lipid metabolism by protein nature, the replacement of fish meal by plant protein will be first evaluated in terms of growth performance, feed utilization, and lipid metabolism. In a subsequent work, the responsiveness of blackspot seabream lipid metabolism to an inadequate dietary amino acid (AA) ratio, varying the dietary dispensable AA content (DAA), will also be considered.
d) The restriction of food availability to predefined day times could profoundly affect fish behaviour and physiology. Thus, in a complementary work, two different feeding methods (hand feeding vs self-feeding) will be evaluated on blackspot seabream growth, nutrient utilization, lipogenic and glycolytic metabolism. In addition, the depiction of the blackspot seabream diel feeding pattern will be of prime interest to fish farmers in order to establish species optimal feeding protocols.

Based on the available data, this work was designed and proposed to give an insight into the nutritional regulation of the mechanisms beyond blackspot seabream energetic metabolism, as a first attempt to understand the basis for its excessive fat accumulation.

CHAPTER 2

Dietary protein/lipid level and protein source effects on growth, tissue composition and lipid metabolism of blackspot seabream (*Pagellus bogaraveo*).

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Abstract

A study was carried out to determine the effects of fishmeal (FM) replacement by plant protein (PP) on growth, body composition and lipid metabolism of blackspot seabream fed different protein/lipid levels. Four experimental diets were formulated to contain two protein (P) and lipid (L) levels (60P/6L or 50P/10L), varying in their protein source (100% FM or 50% FM: 50% PP). Dietary inclusion of PP did not affect growth of fish fed 60P/6L, although fish fed 50P/10L exhibited lower FBW and DGI. Fish fed 60P/6L presented the highest protein and the lowest lipid content. FM replacement by PP has decreased muscle n-3 whereas the n-6 fatty acids increased. G6PD and FAS were depressed in fish fed 50P/10L. FAS was significantly increased with 60P/6L PP which was positively correlated with lipid retention data. Those results suggest the conversion of other nutrient than lipid (protein and/or carbohydrates) into corporal fat. Hepatic LPL activity was lowest in fish fed PP diets. Plasma glucose peaked 1-2 h postfeeding, in all groups and was generally higher with 60P/6L FM. This work shown that besides dietary P/L level, protein source (PS) has a strong effect on species lipogenesis and lipid retention. Hence, the 50P/10L FM diet was the most cost effective for blackspot seabream juveniles.

Introduction

Because of its high commercial value, excellent palatability and scarcity in the fishing grounds, the blackspot seabream (*Pagellus bogaraveo*) has been recently produced in the Atlantic coast. Considerable progress has been made in terms of pre-fattening and ongrowing in tanks and cages (Peleteiro *et al.* 1994, 2000; Olmedo *et al.* 2000), but growth rates of cultured blackspot seabream are very low, when compared with other farmed Sparidae such as gilthead seabream (Santinha *et al.* 1996, 1999; Izquierdo *et al.* 2003; Gómez-Requeni *et al.* 2003, 2004; Sitjá-Bobadilla *et al.* 2005) and generally associated with a high lipid deposition (Linares *et al.* 2000, 2001; Silva *et al.* 2006; Ozório *et al.* 2009). However, and as suggested by Peleteiro *et al* (2000) the identified problems during the ongrowing phase could be corrected by specific feed formulations and by optimizing the species production systems.

Silva *et al.* (2006) has recently estimated that the optimal level of dietary crude protein for blackspot seabream juveniles would be about 450 g kg^{-1} dry matter (DM). Considering the high dietary protein requirements of carnivorous species, fish meal (FM) replacement by alternative plant protein (PP) sources is fundamental for the sustainable development of aquaculture industry. Moreover, FM market availability fluctuations seriously increased the prices of this important protein source which is the single most important and expensive dietary nutrient. The large majority of studies confirm the idea that high dietary PP levels (>40% of total protein) depress growth and feed efficiency (Ballestrazzi *et al.* 1994; Robaina *et al.* 1995; Mambrini *et al.* 1999; Burel *et al.* 2000; Refstie *et al.* 2000). Nevertheless, almost total FM replacement by PP sources was shown to be feasible, when amino acids (AA)-supplemented diets were used (Kaushik *et al.* 1995, 2004; Gómez-Requeni *et al.* 2004; Sitjá-Bobadilla *et al.* 2005; Dias *et al.* 2005).

Blackspot seabream excessive fat accumulation in carcass, muscle, liver and particularly around viscera (Linares *et al.* 2000, 2001; Silva *et al.* 2006; Ozório *et al.* 2009) remains an important problem to be solved. Several studies developed in higher vertebrates reported that dietary protein level and source affect lipid deposition, fatty acid bioconversion and alter serum and liver lipids (Lindholm & Eklund 1991; Terasawa *et al.* 1994; Potter 1995; Aoyama *et al.* 2000). In fish, dietary PS and AA unbalances are also known to affect fish metabolic pathways (Gómez-Requeni *et al.* 2003, 2004; Dias *et al.* 2005; Sitjá-Bobadilla *et al.* 2005). Dias *et al.* (2005) have showed that FM dietary replacement by soybean meal affects seabass liver lipogenesis decreasing glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME) and fatty acid synthetase (FAS). Moreover, the ingestion of

dietary PP sources has a hypocholesterolemic effect on gilthead seabream (Gómez-Requeni *et al.* 2004; Albalat *et al.* 2005; Sitjá-Bobadilla *et al.* 2005), rainbow trout (Kaushik *et al.* 1995) and European sea bass (Robaina *et al.* 1995, 1999; Kaushik *et al.* 2004; Dias *et al.* 2005).

The aim of this study was to evaluate the effects of partial FM replacement by PP on the growth performance, body composition and nutrient utilization of blackspot seabream (*Pagellus bogaraveo*) fed different protein/lipid levels, contributing to a general insight into lipid metabolism of this new species. Protein requirement for blackspot seabream maintenance was estimated as 4.3 g Kg^{-1} day^{-1} (Silva *et al.* 2006), which is quite superior to values previously reported for rainbow trout (2.6 g Kg^{-1} day^{-1}) (Kaushik & Gomes 1988), European sea bass (2.0-2.8 g Kg^{-1} day^{-1}) (Ballestrazzi *et al.* 1994) and gilthead seabream (0.86g Kg^{-1} day^{-1}) (Lupatsch *et al.* 1998). Thus, and as suggested by Silva *et al.* (2006) crude protein requirements for maximum weight gain would be above 400 g kg^{-1}DM. Considering this, dietary protein/lipid level of 50P/10L or 60P/6L was adopted in this study to ensure maximal blackspot seabream growth and to help understanding lipid metabolism. To this end, and for the first time in blackspot seabream, liver lipogenic enzymes activity, glycaemia and plasma lipids were analysed. Furthermore, considering the key role of lipoprotein lipase (LPL) in lipoprotein metabolism, its activity was also determined in muscle, liver and adipose tissue. This work must be considered an attempt to understand some of the mechanisms associated with blackspot seabream growth and nutrient utilization.

Material and methods

Experimental diets

Four experimental diets were formulated to contain two different crude protein (P)/lipid (L) levels (60P/6L or 50P/10L) either with fish meal (FM) as the main protein source (PS) or with the replacement of 50% FM by wheat gluten (PP). All ingredients were supplied by Sorgal S.A. (Ovar, Portugal) and were finely grounded, mixed and dry pelleted through a 2.4-mm die at 50 ºC (CPM, C-300 model). Ingredients, proximate composition and gross energy of the experimental diets are presented in Table 1 and the dietary fatty acid profiles in Table 2. Additionally, the theoretical composition of some fish indispensable amino acids (IAA, % of protein) is given in Table 1, based on Sorgal S.A. ingredient composition information.

Table 1. Ingredients and proximate composition of the experimental diets with different dietary protein/lipid levels (P/L) and protein sources (PS: fish meal, FM and plant protein, PP).

	Dietary treatments			
	60P/6L		50P/10L	
	FM	PP	FM	PP
Ingredients (%)				
Fish meal [a]	83.2	42.9	67.2	35.7
Wheat gluten [b]	-	34.2	-	29.8
Wheat [c]	15.9	20.2	267	269
Fish oil	-	1.8	5.2	6.7
Mineral mix [d]	0.5	0.5	0.5	0.5
Vitamin mix [e]	0.2	0.2	0.2	0.2
Choline chloride [f]	0.1	0.1	0.1	0.1
Lutavin E50 [g]	0.05	0.05	0.05	0.05
Lutavin C35 [h]	0.04	0.04	0.04	0.04
Proximate composition				
Dry matter (DM) (%)	91.9	91.9	91.6	91.6
Protein (% DM)	62.2	64.0	53.4	56.9
Lipids (% DM)	6.3	6.1	10.7	10.4
Ash (% DM)	17.0	13.3	14.4	9.9
Starch (% DM)	9.9	14.8	15.4	18.7
Energy (MJ kg^{-1} DM)	19.4	20.8	20.1	21.6
DP/DE mg kg^{-1}	33.3	31.9	27.5	27.4
Theoretical IAA composition (% protein) [i]				
Lysine	7.86	4.94	7.72	4.98
Methionine + cysteine	3.91	3.88	3.90	3.89
Tryptophan	1.13	1.05	1.13	1.05
Threonine	4.38	3.57	4.33	3.58

[a] Fish meal composition: DM: 91.3 %; Protein: 71.5 % DM; Lipids: 7.3 % DM
[b] Wheat gluten composition: DM: 90.1 %; Protein: 83.8 % DM; Lipids: 1.6 % DM.
[c] Micronized wheat composition: DM: 87.6 %; Protein: 14.4 % DM; Lipids: 2.4 % DM.
[d] Minerals (g or mg kg^{-1} diet): Mn (manganese sulphate), 20 mg; I (potassium iodide), 0.6 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.4 mg; Mg (magnesium sulphate), 500 mg; Zn (Bioplex, (Alltech)), 30 mg; Se (Sel-Plex 2000, (Alltech)), 0.3 mg; Fe (iron sulphate), 40 mg; Ca (calcium carbonate), 2.15 g; dibasic calcium phosphate, 5 g; KCl, 1 g; NaCl, 0.4 g.
[e] Vitamins (IU or mg kg^{-1} diet): Vitamin A, 8000 IU; vitamin D3, 2000 IU; vitamin E, 100 mg; vitamin K, 10 mg; vitamin B12, 0.02 mg; vitamin B1, 15 mg; vitamin B2, 25 mg; vitamin B6, 15 mg; folic acid, 10 mg; biotin, 1 mg; vitamin C, 100 mg; betaine, 500 mg; inositol, 300 mg; nicotinic acid, 100 mg; pantothenic acid, 50 mg;
[f] Choline chloride, 1000 mg kg^{-1} diet
[g] Lutavin E50: vitamin E, 300 mg kg^{-1} diet;
[h] Lutavin C35: vitamin C, 500 mg kg^{-1} diet;
[i] Theoretical composition for some indispensable amino acids (IAA, % of protein), based on Sorgal S.A. ingredient composition information.

Table 2. Fatty acid composition of the experimental diets (g/100 g total fatty acid) with different dietary protein/lipid levels (P/L) and protein sources (PS: fish meal, FM and plant protein, PP).

	Dietary treatments			
	60P/6L		50P/10L	
	FM	PP	FM	PP
10:0	1.5	1.9	0.6	1.5
14:0	8.5	7.2	8.3	7.4
15:0	0.7	0.6	0.7	0.7
16:0	25.2	23.9	22.6	22.8
17:0	0.6	0.4	0.5	0.5
18:0	4.0	2.9	3.5	3.1
20:0	0.2	0.2	0.2	0.2
Saturates	40.7	37.2	36.6	36.3
16:1	8.1	6.4	8.1	7.1
17:1	0.2	0.2	0.3	0.3
18:1	11.2	12.2	13.6	14.3
20:1	1.0	1.6	2.0	2.5
22:1	0.5	1.2	1.6	1.9
24:1	0.1	0.1	0.1	0.1
MUFA	21.2	21.8	25.8	26.4
14 PUFA	0.6	0.6	0.3	0.4
16:2 n-4	0.6	0.4	0.9	0.5
16:3 n-4	1.6	0.9	1.0	0.7
16:4 n-1	1.8	1.2	1.5	1.1
18:2 n-6	4.5	15.3	4.8	10.6
18:3 n-6	0.2	0.2	0.2	0.2
20:2 n-6	0.1	0.1	0.2	0.2
20:3 n-6	0.1	0.1	0.1	0.1
20:4 n-6	1.0	0.6	0.8	0.6
22:2 n-6	0.1	0.0	0.1	0.1
Σ n-6	5.9	16.3	6.1	11.7
18:3 n-3	0.7	1.4	1.0	1.4
18:4 n-3	1.8	1.7	2.4	2.3
20:3 n-3	0.0	0.0	0.1	0.1
20:4 n-3	0.4	0.4	0.8	0.5
20:5 n-3	9.8	7.0	9.3	7.5
21:5 n-3	0.3	0.2	0.3	0.3
22:5 n-3	1.1	0.8	1.0	0.8
22:6 n-3	8.5	6.3	8.0	6.2
Σ n-3	22.6	17.8	22.9	19.0
PUFA	33.1	37.3	32.7	33.5
Sat/PUFA	1.2	1.0	1.1	1.1
n3/n6	3.8	1.1	3.8	1.6

Growth trial

Experiments were directed by trained scientists (following FELASA category C recommendations) and were conducted according to the European Economic Community animal experimentation guidelines Directive of 24 November 1986 (86/609/EEC). Blackspot seabream juveniles (*Pagellus bogaraveo*) were obtained from a fish farm

(Grupo Isidro de la Cal, Valdoviño, Coruña, Spain), and acclimated to the experimental conditions for 3 weeks before the beginning of the trial. The growth trial was conducted at the experimental facilities of CIIMAR, Porto. Homogenous groups of 37 juveniles with an average initial body weight of 37.5 g (Table 4) were randomly distributed among 12 square fibre glass tanks (500 L), in a recirculating water system. Triplicate groups of fish for each treatment were fed by hand to apparent satiety, two times a day (09.30 and 18.00 h) for 103 days. Each tank was supplied with filtered, heated (19 ± 1 ºC) saltwater (33 g L^{-1}), at a flow rate of 2 L min^{-1}. The pH, ammonia, nitrites, nitrates and phosphates in the water were monitored during the entire trial and maintained at levels compatible with marine species. Fish were exposed to natural photoperiod. Every four weeks, fish were bulk weighed under moderate anaesthesia (ethylene glycol monophenyl ether at 50 ppm) and data on feed distributed were recorded. Prior to sampling, fish were fasted for 24 h and killed by a sharp blow on the head. At the beginning of the experiment a pooled sample of nine fish from the initial stock were taken and stored at -20 ºC for subsequent whole body composition analyses. The dorsal muscle and the liver from other nine fish were also collected, immediately frozen in liquid nitrogen and stored at -80 ºC for the posteriors muscle lipid content and liver lipogenic enzymes analyses. At the end of the growth trial, three fish per tank were sampled and stored at -20 ºC for subsequent whole body composition. Muscle, liver and adipose tissues were collected, frozen in liquid nitrogen and stored at -80 ºC for subsequent individual analyses of muscle total lipid and fatty acids (nine fish per treatment) and liver lipogenic enzymes activity (nine fish per treatment). Liver, muscle and adipose tissue of 9 fish per treatment were also collected for determining LPL activity. Blood collection was carried out, after a slight anaesthesia, in <3 min, at each sampling point, to avoid plasmatic metabolites response induced by handling. Samples were taken from the caudal vein, with syringes and collecting tubes containing 15-20 µl of sodium fluoride and potassium oxalate (4% each), from three fish per tank, at nine different times after the last meal (30 min, 1; 2; 4; 6, 9; 12; 16; 24 h). Fish sampled at the first five times (30 min, 1; 2; 4; 6 h) have been identified to assure that the same fish would not be sampled twice. Plasma was obtained after centrifugation (6000 *g* for 10 min at 4 ºC) and stored at -80 ºC until further glucose, cholesterol (CHOL), triacylglycerol (TAG) and non-esterified fatty acids (NEFA) analysis.

Digestibility trial

The apparent digestibility coefficients (ADC) of the dietary components of the four diets were assessed after incorporation of 0.1% of yttrium oxide, as inert marker, to a grounded portion of each diet. The mixtures were then dry pelleted through a 2.4-mm die at 50 ℃ (CPM, C-300 model). Four homogenous groups of ten fish (mean body weight of 90 g)

were randomly stocked into four digestibility tanks (50 L), specially constructed according to the Guelph system protocol (Cho *et al.* 1982) and adapted to the new conditions for 15 days. The experimental fish were subjected to a natural photoperiod, water temperature was maintained at 19 ± 1 ℃ and salinity at 33 g L^{-1}. The four diets were randomly assigned by tank, and tested in two following 15 days periods that were hence considered the experimental unit (*n* 2, replicates). The first two days of each 15 day period were used for acclimation to the feed and no faeces were collected. This time period was deemed sufficient for the fish to achieve complete evacuation of previous meals. Fish were fed once daily until apparent satiety and faeces were collected every morning over a four week period. After collection, faeces were centrifuged pooled and frozen at –20 ^{o}C. The ADC's were calculated according to Maynard *et al.* (1969).

Feed, body composition and faeces analyses

Whole fish from each tank were ground, pooled and fresh moisture content was determined. Fish and faeces were subsequently freeze-dried before further analysis. Feed, whole body samples and faeces were analyzed for DM (105 ºC for 24 h), ash by combustion in a muffle furnace (550 ℃ for 12 h), c rude protein (Micro-Kjeldahl; N x 6.25) after acid digestion, lipid content by petroleum ether extraction (at Soxhlet 40-60 ºC), gross energy in an adiabatic bomb calorimeter (IKA, Werke C2000) and starch according to Thivend *et al.* (1972). Yttrium oxide concentrations were determined in both diets and faeces samples by atomic absorption spectrophotometry (SpectrAA 220FS, Varian), after a chemical digestion with HNO_3 (Reis *et al.* 2008).

Plasma metabolites assays

Plasma glucose, CHOL, TAG and NEFAs were determined using enzymatic commercial kits: nº 61269; nº 61218; nº 61236 from Bio-Mérieux, Marcy-L'Etoile, France and NEFA-C from Wako, Neuss, Germany, respectively.

Total lipids and fatty acids analyses

Total lipids were extracted and measured gravimetrically according to Folch *et al*. (1957) using dichloromethane instead of chloroform. Fatty acid methyl esters were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride methanol according to Santha & Ackman (1990) and were analyzed in a Varian 3800 gas chromatograph (Varian, Les Ulis, France). The chromatograph was equipped with a DB Wax fused silica capillary column (30 m X 0.25 mm internal diameter, film thickness: 0.25 µm, J & W Scientific, Folsom, CA, USA). Helium was used as carrier gas (1 mL min^{-1}) and the

thermal gradient was 100 to 180 ºC at 8ºC min^{-1}, 180-220 ºC at 4 ºC min^{-1} and a constant temperature of 220 ºC during 20 min. Injection was made in a split mode (ratio 1:40) with 1 µl injected. Injector and flame ionization detector temperatures were 260 and 250 ºC, respectively. Fatty acid methyl esters were identified by comparison with known standards mixtures (Sigma189-19, St Louis, MO, USA) and quantified using the STAR computer package (Varian).

Lipogenic enzymes and lipoprotein lipase assay

Liver samples were homogenised in three volumes of ice-cold buffer (0.02 mol L^{-1} Tris–HCl, 0.25 mol L^{-1} sucrose, 2 mmol L^{-1} EDTA, 0.1 mol L^{-1} NaF, 0.5 mmol phenylmethyl sulphonyl fluoride, 0.01 mol L^{-1} β-mercaptoethanol, pH 7.4) and centrifuged at 30 000 *g,* at 4 ºC for 20 min. Selected lipogenic enzyme activities were assayed on supernatant: glucose-6-phosphate dehydrogenase G6PD (EC 1.1.1.49) according to Bautista *et al.* (1988) and fatty acid synthetase (FAS, EC 2.3.1.38) according to the methodology of Chang *et al.* (1967) and modified by Chakrabarty & Leveille (1969). LPL (EC 3.1.1.34) was determined in muscle, liver and adipose tissue following the procedure described by Bengtsson-Olivecrona & Olivecrona (1992). Enzyme activity units IU, defined as µmoles of substrate converted to product, per min, at assay temperature, were expressed per mg of hepatic soluble protein (specific activity) or per gram of tissue. Soluble protein content of tissues was determined on supernatant by the method of Bradford (1976). The unknown protein content of the samples was determined using a standard curve with well known protein (bovine serum albumin, BSA, Sigma, St. Louis, USA) concentrations (0 to 100 mg protein mL^{-1}).

Statistical analysis

Statistical analyses followed methods outlined by Zar (1996). All data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Bartlett tests, and then submitted to a two-way ANOVA with protein/lipid level and protein source as main effects, using the STATISTICS 6.0 package (StatSoft, Inc., Tulsa, OK, USA). When these tests showed significance ($P<0.05$), individual means were compared using Tukey test. Tank average values for feed intake, growth, body composition, nutrient accretion and faeces analysis were used as experimental units for statistical analyses.

Results

Data on ADC of the main dietary nutrients and energy are reported in Table 3. High protein (>95%) and starch (>95%) digestibilities were observed in all dietary treatments. The ADC of dietary protein and energy were not significantly influenced by the dietary treatments. However, starch ADC's were significantly affected by different dietary P/L levels and PS. Starch digestibilities decreased with increasing dietary starch levels and were lowest in fish fed 50P/10L PP diet which corresponds to the diet having the highest starch level.

Table 3. Apparent digestibility coefficients (ADC, %) of the different experimental diets with different dietary protein/lipid levels (P/L) and protein sources (PS: fish meal, FM and plant protein, PP).

	Dietary treatments				Two-way ANOVA		
	60P/6L		50P/10L		P-value		
ADC%	FM	PP	FM	PP	P/L	PS	x
Dry matter	77.2 ± 5.1	75.2 ± 6.0	85.3 ± 0.3	78.1 ± 4.3	0.170	0.240	0.501
Protein	95.9 ± 0.1	96.9 ± 0.5	97.0 ± 0.1	96.2 ± 0.8	0.623	0.802	0.049*
Energy	92.3 ± 0.9	93.7 ± 1.0	94.0 ± 0.7	92.5 ± 1.1	0.802	0.302	0.319
Starch	99.6 ± 0.1	98.2 ± 0.6	98.3 ± 0.5	95.6 ± 0.6	0.005	0.004	0.116

Values are means ± standard deviation (n = 2).
Absence of superscript letters indicates no significant interaction between the two factors (P/L vs PS) (P>0.05).
* Without significant differences after *pos-hoc* analysis

At the end of the growth trial all fish more than doubled their initial body weight, displaying significant differences among dietary treatments (Table 4). The P/L level had no effect on growth, but significant differences were found in the digestible DM, energy and starch intakes. In general, fish fed 50P/10L diet presented both significantly higher feed conversion rates (FCR), voluntary feed intake and digestible nutrient intake compared with those fed 60P/6L diet. The dietary inclusion of plant protein significantly reduced final body weight (FBW) and daily growth index (DGI), when low protein diets were used (50P/10L). In addition, the partial substitution of FM by PP decreased digestible DM and lipid intake, while increased starch digestible intake.

The PS did not influence whole body composition. Fish fed the high protein/low lipid level (60P/6L) diets presented the highest protein (FM: 17.5; PP: 17.1 %) and the lowest lipid content (FM: 11.7; PP: 13.9 %) (Table 5). Different P/L levels or PS did not affect either liver or muscle lipid content when expressed as % DM (Table 5). However, when expressed as % of wet weight, liver lipid content was highest in fish fed PP diets. Hepatosomatic index was not

affected by dietary treatments while different dietary P/L ratios have slightly (*P*=0.046) affected viscerosomatic index, with the highest value found in fish fed 50P/10L FM diet. Nitrogen gain of fish fed the 60P/6L diet was significantly higher (229-239 mg Kg^{-1} ABW day^{-1}) than that of fish fed the 50P/10L diet (206-215 mg Kg^{-1} ABW day^{-1}), while an opposite trend was observed in lipid gain. Protein and lipid retentions were significantly affected by both P/L level and PS. Fish fed 60P/6L diets displayed the highest protein and lipid retention. Furthermore, in fish fed 60P/6L diets, FM replacement by PP significantly increased lipid retention (127 to 188%).

Table 4. Growth performance and feed utilization efficiency of blackspot seabream fed different dietary protein/lipid levels (P/L) and protein sources (PS: fish meal, FM and plant protein, PP) over 103 days at 19 ± 1 ºC

	Dietary treatments				Two-way ANOVA		
	60P/6L		50P/10L		P-value		
	FM	PP	FM	PP	P/L	PS	x
Growth							
Initial body weight (g)	37.7 ± 0.1	37.6 ± 0.1	37.6 ± 0.1	37.6 ± 0.1	0.385	0.995	0.540
Final body weight (g)	90.2 ± 0.5[ab]	89.4 ± 2.2[ab]	94.2 ± 2.2[a]	85.2± 4.9[b]	0.947	0.019	0.038
FCR [a]	1.3 ± 0.04	1.3 ± 0.04	1.5 ± 0.1	1.6 ± 0.1	0.000	0.817	0.119
VFI [b]	1.1 ± 0.03	1.0 ± 0.02	1.2 ± 0.1	1.2 ± 0.1	0.000	0.072	0.836
PER [c]	1.2 ± 0.04	1.3 ± 0.04	1.3 ± 0.1	1.1 ± 0.1	0.278	0.112	0.043*
DGI [d]	1.1 ± 0.01[ab]	1.1 ± 0.04[ab]	1.2 ± 0.03[a]	1.0 ± 0.1[b]	0.940	0.020	0.037
Digestible Intake (g or kJ Kg^{-1} ABW [e] day^{-1})							
Dry matter	8.1 ± 0.3	7.5 ± 0.2	10.6 ± 0.7	9.1 ± 0.4	0.000	0.003	0.141
Protein	6.3 ± 0.2	6.2 ± 0.1	6.4 ± 0.4	6.4 ± 0.3	0.241	0.772	0.793
Energy	188.0 ± 6.0	193.4 ± 3.9	233.4 ± 14.7	233.9 ± 10.7	0.000	0.615	0.673
Starch	1.0 ± 0.03	1.5 ± 0.03	1.9 ± 0.1	2.2 ± 0.1	0.000	0.000	0.064

Values are means ± standard deviation (*n* = 3).
[a,b] Mean values within a row with unlike superscript letters showed a significant interaction between the two tested factors (P/L vs PS) (*P*<0.05).
[a] FCR, Feed conversion ratio = dry feed intake/weight gain.
[b] VFI, Voluntary feed intake = 100 x crude feed intake/average body weight/day
[c] PER, Protein efficiency ratio = weight gain/crude protein intake.
[d] DGI, Daily growth index = 100 x ((Final body weight)$^{1/3}$ - (Initial body weight)$^{1/3}$)/days
[e] ABW, Average body weight = (Final body weight + Initial body weight)/2
*Without significant differences after *pos-hoc* analysis

Muscle fatty acid composition is shown in Table 6. Compared to the initial profile, we observed in all groups an increase in saturated FA and a decrease in n-3 PUFA. Muscle FA composition reflected in general terms that of the diets.

Table 5. Whole body composition (% or MJ kg^{-1} of wet weight), nutrient gain and retention, and tissue total lipid content (% wet weight, WW, or % dry weight, DW) of blackspot seabream fed different dietary protein/lipid levels (P/L) and protein sources (PS: Fish meal, FM and Plant protein PP).

	Dietary treatments				Two-way ANOVA		
	60P/6L		50P/10L		P-value		
	FM	PP	FM	PP	P/L	PS	x
Final body composition [a]							
Moisture (%)	65.9 ± 1.3	64.2 ± 0.9	64.6 ± 0.9	64.1 ± 1.0	0.308	0.116	0.369
Protein (%)	17.5 ± 0.5	17.1 ± 0.5	16.0 ± 0.4	16.5 ± 0.2	0.003	0.744	0.096
Lipid (%)	11.7 ± 1.6	13.9 ± 0.1	14.7 ± 0.8	15.0 ± 1.0	0.010	0.071	0.147
Energy (MJ kg^{-1})	8.5 ± 0.5	9.4 ± 0.3	9.3 ± 0.3	9.5 ± 0.5	0.098	0.060	0.230
Liver total lipids							
%WW	12.2 ± 2.1	15.7 ± 4.9	14.1 ± 2.9	22.2 ± 3.8	0.095	0.031	0.330
%DW	34.8 ± 4.3	40.0 ± 13.1	38.3 ± 5.7	51.5 ± 4.8	0.176	0.108	0.452
Muscle total lipids							
%WW	3.4 ± 0.5	3.8 ± 0.8	3.8 ± 1.4	3.9 ± 0.8	0.335	0.387	0.740
%DW	13.6 ± 1.8	14.9 ± 2.5	15.0 ± 4.8	15.6 ± 2.7	0.389	0.415	0.717
HSI (%) [b]	1.2 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.2	0.105	0.335	0.960
VSI (%) [c]	5.0 ± 1.0	5.3 ± 0.9	5.7 ± 1.0	5.3 ± 1.1	0.046	0.950	0.096
Gain [(mg or g) Kg^{-1} ABW[d] day^{-1}]							
Nitrogen (mg)	238.6 ± 10.0	228.7 ± 9.9	215.2 ± 3.4	205.7 ± 16.9	0.007	0.170	0.980
Lipids (g)	0.9 ± 0.2	1.1 ± 0.01	1.3 ± 0.1	1.2 ± 0.2	0.010	0.226	0.053
Retention (%) of intake							
Protein	22.9 ± 0.9	22.5 ± 1.4	20.4 ± 1.4	19.3 ± 1.4	0.005	0.035	0.645
Lipid	127.3 ± 29.5 [b]	188.0 ± 6.4 [a]	98.0 ± 5.9 [b]	100.8 ± 15.0 [b]	0.000	0.012	0.019
Energy	32.0 ± 2.8	36.9 ± 2.3	31.9 ± 0.7	29.5 ± 3.9	0.039	0.449	0.047*

Values are means ± standard deviation (*n*=3 for whole body composition, nutrient gain and retention; *n*=9 for liver and muscle total lipids, HSI and VSI)
[a,b] Mean values within a row with unlike superscript letters showed a significant interaction between the two factors (P/L vs PS) (*P*<0.05).
[a] Initial body composition was: moisture 66.0 %; protein 15.8 % DM; lipid 13.3 % DM and energy 90 MJ kg^{-1}.
[b] HSI, Hepatosomatic index = 100 X liver weight/body weight
[c] VSI, Viscerosomatic index = 100 X weight of viscera/body weight
[d] ABW, Average body weight = (final body weight + initial body weight)/2
* Without significant differences after *pos-hoc* analysis

Total saturated fatty acids ranged between 37 to 39% of total fatty acids and were not affected by the different dietary P/L levels or PS. Stearic acid (18:0) percentage was systematically higher in muscle than in the diet. Moreover, a significant effect of the dietary P/L level was observed, with fish fed 60P/6L diet displaying the highest 18:0 values.

Table 6. Muscle fatty acid composition of blackspot seabream fed different dietary protein/lipid levels (P/L) and protein sources (PS: fish meal, FM and plant protein, PP) (g/100 g total fatty acid)

		Dietary treatments				Two-way ANOVA		
		60P/6L		50P/10L		P-value		
	Initial	FM	PP	FM	PP	P/L	PS	x
10:0	0.5	1.2 ± 0.2	0.9 ± 0.2	1.1 ± 0.5	1.1 ± 0.4	0.658	0.287	0.162
14:0	4.5	5.2 ± 0.5	5.0 ± 0.4	5.1 ± 0.8	5.5 ± 0.7	0.327	0.626	0.127
16:0	19.3	24.7 ± 0.8	23.7 ± 1.1	23.5±1.7	23.7 ± 0.7	0.117	0.364	0.125
17:0	0.4	0.4± 0.02 ^{b}	0.3 ± 0.02^{c}	0.4±0.02^{a}	0.4 ± 0.02^{a}	0.000	0.000	0.002
18:0	5.8	6.6 ± 0.3	6.5 ± 0.3	6.2 ± 0.3	6.2 ± 0.4	0.002	0.777	0.653
20:0	0.2	0.2 ± 0.01	0.2 ± 0.02	0.2 ± 0.02	0.2 ± 0.01	0.017	0.121	0.441
Saturates	31.2	39.2 ± 1.0	37.4 ± 1.3	37.4 ± 2.4	38.0 ± 0.9	0.242	0.307	0.028*
16:1	5.5	5.9 ± 0.4	5.6 ± 0.3	5.9±0.5	6.1 ± 0.5	0.037	0.657	0.088
17:1	0.2	0.3 ± 0.04	0.3 ± 0.1	0.4±0.1	0.4 ± 0.4	0.000	0.954	0.749
18:1	19.8	22.2 ± 1.8	24.7 ± 1.6	20.8 ± 1.8	20.9 ± 2.3	0.000	0.045	0.071
20:1	1.9	1.5 ± 0.1 ^{c}	1.6 ± 1.3^{bc}	1.9 ± 0.2^{a}	1.8 ± 0.1^{ab}	0.000	0.865	0.031
22:1	1.5	0.5 ± 0.1 ^{c}	0.7 ± 0.2^{bc}	1.1 ± 0.4^{a}	0.8 ± 0.1^{ab}	0.000	0.469	0.023
MUFA	29.0	30.7 ± 2.1	33.1 ± 1.8	30.4 ± 2.1	30.3 ± 2.4	0.038	0.116	0.075
12PUFA	0.1	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.844	0.378	0.057
14PUFA	0.2	0.3 ± 0.04^{ab}	0.2 ± 0.03^{b}	0.3 ± 0.1^{ab}	0.3 ± 0.1^{a}	0.004	0.664	0.016
16:2 n-4	0.3	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.03	0.3 ± 0.02	0.008	0.023	0.459
16:3 n-4	0.4	0.5 ± 0.04^{a}	0.3 ± 0.06^{c}	0.4 ± 0.1^{b}	0.4 ± 0.1^{bc}	0.437	0.000	0.003
16:4 n-1	0.6	0.5 ± 0.1^{a}	0.4 ± 0.04^{b}	0.5 ± 0.1^{ab}	0.5 ± 0.1^{a}	0.359	0.012	0.005
18:2 n-6	8.8	6.0 ± 0.6	8.7 ± 0.4	5.8 ± 0.2	8.6 ± 0.6	0.189	0.000	0.797
18:3 n-6	0.2	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.03	0.2± 0.01	0.590	0.000	0.857
20:2 n-6	0.3	0.2 ± 0.02	0.3 ± 0.03	0.2 ± 0.02	0.4 ± 0.1	0.462	0.000	0.367
20:3 n-6	0.1	0.2 ± 0.0^{b}	0.3 ± 0.1^{a}	0.2 ± 0.02^{b}	0.2 ± 0.02^{b}	0.000	0.000	0.001
20:4 n-6	0.7	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.040	0.000	0.329
Σn-6	10.1	7.3 ± 0.6	10.0 ± 0.5	7.0 ± 0.2	9.8 ± 0.6	0.220	0.000	0.895
18:3 n-3	1.1	0.7 ± 0.1	0.9 ± 0.04	0.8 ± 0.03	1.0 ± 0.1	0.000	0.000	0.398
18:4 n-3	1.0	0.8 ± 0.1	0.7 ± 0.04	1.0 ± 0.1	1.0± 0.1	0.000	0.575	0.125
20:4 n-3	0.7	0.5 ± 0.04	0.5 ± 0.04	0.7 ± 0.04	0.6 ± 0.1	0.000	0.062	0.491
20:5 n-3	7.0	5.4 ± 0.5	4.4 ± 0.4	5.9 ± 0.6	5.1 ± 0.4	0.000	0.000	0.446
21:5 n-3	0.3	0.2 ± 0.02	0.2± 0.02	0.2 ± 0.04	0.2 ± 0.01	0.001	0.000	0.630
22:5 n-3	2.4	1.7 ± 0.2	1.4 ± 0.2	1.9 ± 0.4	1.5 ± 0.2	0.124	0.000	0.555
22:6 n-3	10.2	7.1 ± 1.1	5.5 ± 1.0	8.4 ± 1.9	6.2 ± 0.8	0.023	0.000	0.544
Σn-3	22.9	16.5 ± 1.7	13.5 ± 1.6	18.9 ± 2.9	15.9 ± 1.3	0.001	0.000	0.804
PUFA	34.6	25.6 ± 2.1	24.9 ± 2.1	27.7 ± 2.8	27.2 ± 1.8	0.007	0.428	0.864
Sat/PUFA	0.9	1.5 ± 0.1	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.1	0.011	0.922	0.579
n3/n6	2.3	2.3 ± 0.2	1.3 ± 0.1	2.7 ± 0.4	1.6±0.1	0.000	0.000	0.315

Values are means ± standard deviation (n = 9).
^{a,b,c} Mean values within a row with unlike superscript letters showed a significant interaction between the two factors (P/L vs PS) (P<0.05). * Without significant differences after pos-hoc analysis

The monounsaturated fatty acids (MUFA) in flesh lipids were mainly represented by 18:1 (oleic acid). Muscle MUFA levels were higher than those supplied by the diets with fish fed the 60P/6L with PP diets attaining the highest proportion. Flesh percentages of linoleic acid

(18:2n-6) were higher in fish fed diets with 50% substitution of FM by wheat gluten due to the high levels of this fatty acid in those diets.

Table 7. Effects of different dietary protein/lipid level (P/L) and protein sources (PS: fish meal, FM and plant protein, PP) in blackspot seabream enzymes activities

	Dietary treatments				Two-way ANOVA		
	60P/6L		50P/10L		P-value		
	FM	PP	FM	PP	P/L	PS	x
G6PD [a]							
Liver							
IU g^{-1} tissue	13.1 ± 4.3	14.6 ± 4.5	12.4 ± 2.1	10.7 ± 2.6	0.063	0.949	0.179
mIU mg^{-1} protein	103.8 ± 32.6^{ab}	136.7 ± 31.8^{a}	108.1 ± 28.6^{ab}	94.6 ± 18.0^{b}	0.054	0.312	0.020
FAS [b]							
Liver							
IU g^{-1} tissue	2.6 ± 1.1	3.2 ± 0.2	2.1 ± 1.2	1.8 ± 0.9	0.004	0.699	0.179
mIU mg^{-1} protein	20.5 ± 7.8^{b}	30.0 ± 3.2^{a}	22.4 ± 8.1^{ab}	16.5 ± 6.8^{b}	0.019	0.454	0.003
LPL							
Liver							
mIU g^{-1} tissue	23.7 ± 8.0	20.9 ± 8.6	28.6 ± 12.6	17.3 ± 3.5	0.838	0.033	0.183
mIU mg^{-1} protein	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.01	0.236	0.032	0.246
Muscle							
mIU g^{-1} tissue	26.6 ± 12.7	19.8 ± 8.2	16.0 ± 4.9	19.4 ± 10.4	0.095	0.594	0.115
mIU mg^{-1} protein	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.142	0.770	0.075
Adipose tissue							
mIU g^{-1} tissue	22.8 ± 8.3	20.5 ± 9.5	24.4 ± 11.8	18.6 ± 8.0	0.965	0.220	0.601
mIU mg^{-1} protein	1.4 ± 0.7	1.3±0.9	1.6 ± 0.9	1.4 ± 0.6	0.642	0.592	0.841

Values are means ± standard deviation (n = 9).
[a,b] Mean values within a row with unlike superscript letters showed a significant interaction between the two factors (P/L vs PS) (P<0.05).
[a] Initial G6PD activity was: 4.2 IU g^{-1} tissue or 50.2 mIU mg^{-1} protein
[b] Initial FAS activity was: 0.2 IU g^{-1} tissue or 2.8 mIU mg^{-1} protein

The proportion of muscle n-3 PUFA was affected by different P/L levels and PS. Fish fed 50P/10L diets presented the highest values mainly due to an increased accumulation of eicosapentaenoic (20:5n-3; EPA) and docosahexaenoic (22:6n-3; DHA) fatty acids compared to fish fed diets 60P/6L. Total n-3 PUFA ranged from 16.5% to 18.9% in fish fed FM diets and from 13.5 to 15.9% in those fed PP diets, for 60P/6L and 50P/10L levels, respectively. FAS and G6PD activities assayed in blackspot seabream liver are reported in Table 7. In general terms, there was a significant interaction between P/L levels and protein source for both enzymes activity.

Table 8. Plasma glucose (mg dL^{-1}), total Chol, TAG and NEFA (g L^{-1}) determined up to 24h after the last meal

		60P/6L		50P/10L		Two-way ANOVA P-value		
Time		**FM**	**PP**	**FM**	**PP**	**P/L**	**PS**	**x**
0.5	**Glucose**	76.8 ± 14.2‡	74.7 ± 19.8	66.3 ± 16.1	72.1 ± 25.5‡	0.325	0.778	0.552
	Chol	2.3 ± 0.4†‡	1.7 ± 0.5	2.2 ± 0.5†‡	1.9 ± 0.5†‡	0.845	0.013	0.296
	TAG	3.3 ± 1.5†‡	2.4 ± 0.9	2.5 ± 1.1	3.4 ± 1.5	0.850	0.997	0.048
	NEFA	0.13 ± 0.05§¶	0.11 ± 0.03‡§	0.16 ± 0.06$^{†‡§‖}$	0.14 ± 0.04$^{‡§‖}$	0.056	0.191	0.794
1	**Glucose**	161.2 ± 62.4†	108.0 ± 22.2	115.7 ± 41.2	103.1 ± 20.3†‡	0.078	0.024	0.152
	Chol	2.7 ± 0.4†	1.8 ± 0.4	2.6 ± 0.7†	2.0 ± 0.4†‡	0.943	0.000	0.336
	TAG	3.3 ± 0.7†‡	2.8 ± 1.5	3.4 ± 1.7	4.0 ± 1.4	0.186	0.881	0.242
	NEFA	0.13 ± 0.04$^{a§‖¶}$	0.07 ± 0.02 ^{b§}	0.13 ± 0.04$^{a†§‖}$	0.13 ± 0.03$^{a§‖}$	0.010	0.008	0.026
2	**Glucose**	158.5 ± 42.9^{a†}	107.3 ± 43.0^{b}	100.9 ± 31.5^{b}	117.5 ± 31.2$^{ab †}$	0.068	0.178	0.011
	Chol	2.9 ± 0.9†	1.7 ± 0.2	2.5 ± 0.4†	2.0 ± 0.6†‡	0.687	0.000	0.092
	TAG	3.7 ± 1.8†	2.2 ± 1.1	3.5±1.2	3.6 ± 1.1	0.269	0.145	0.095
	NEFA	0.09 ± 0.03¶	0.07 ± 0.02§	0.12±0.06§	0.11 ± 0.03$^{‖}$	0.024	0.269	0.723
4	**Glucose**	135.0 ± 42.4†‡	91.4 ± 13.0	102.5 ± 50.1	91.6 ± 28.8†‡	0.291	0.083	0.284
	Chol	2.0 ± 0.6†‡	1.7 ± 0.4	2.2 ± 0.6†‡	1.8 ± 0.5†‡	0.468	0.106	0.788
	TAG	3.6 ± 1.5†‡	2.8 ± 1.4	2.8 ± 0.8	3.3 ± 2.1	0.845	0.790	0.324
	NEFA	0.12 ± 0.03§¶	0.10 ± 0.03§	0.15 ± 0.04$^{†‡§‖}$	0.12 ± 0.1$^{§‖}$	0.150	0.180	0.870
6	**Glucose**	117.9 ± 48.9†‡	102.9 ± 49.2	11.6 ± 55.9	91.0 ± 28.8†‡	0.640	0.363	0.888
	Chol	2.3 ± 0.3†‡	2.0 ± 0.4	1.9 ± 0.6†‡	2.0 ± 0.4†‡	0.381	0.764	0.184
	TAG	4.2 ± 1.7^{ab†‡}	2.5 ± 0.8^{b}	2.4 ± 1.5^{b}	5.6 ± 2.4^{a}	0.372	0.264	0.002
	NEFA	0.15 ± 0.05$^{‡§‖¶}$	0.09 ± 0.02§	0.14 ± 0.06$^{§‖}$	0.12 ± 0.01$^{§‖}$	0.554	0.015	0.101
9	**Glucose**	84.3 ± 35.5†‡	91.9 ± 44.2	96.9 ± 43.6	73.3 ± 31.0‡	0.849	0.613	0.325
	Chol	2.3 ± 0.5†‡	1.5 ± 0.3	2.3 ± 0.5†‡	2.0 ± 0.4†‡	0.143	0.006	0.287
	TAG	2.1 ± 0.6†‡	2.9 ± 1.0	3.5 ± 1.0	3.3 ± 2.3	0.175	0.583	0.461
	NEFA	0.24 ± 0.08†‡	0.13 ± 0.02‡§	0.21 ± 0.08$^{†‡‖}$	0.18±0.1$^{†‡§‖}$	0.663	0.012	0.108
12	**Glucose**	69.6 ± 20.1‡	89.7 ± 51.9	89.7 ± 32.9	71.3±27.2†‡	0.970	0.943	0.238
	Chol	2.6 ± 0.5†‡	1.6 ± 0.6	2.7 ± 0.5†	2.4±0.5†	0.045	0.009	0.132
	TAG	2.2 ± 0.5†‡	2.5 ± 1.4	3.8 ± 1.1	3.6±2.5	0.044	0.972	0.746
	NEFA	0.26 ± 0.05†	0.22 ± 0.06†	0.23 ± 0.06†‡	0.22±0.1†	0.513	0.345	0.526
16	**Glucose**	72.0 ± 27.0‡	63.2 ± 14.3	80.1 ± 39.4	66.1 ± 19.8‡	0.530	0.236	0.781
	Chol	2.6 ± 0.4^{a†}	1.6 ± 0.3^{b}	1.6 ± 0.5^{b‡}	2.4 ± 0.5^{a†}	0.441	0.545	0.000
	TAG	2.2 ± 1.3†‡	2.3 ± 0.8	2.4 ± 1.0	3.5 ± 1.0	0.046	0.083	0.138
	NEFA	0.21 ± 0.06$^{†‖}$	0.23 ± 0.07†	0.19±0.05$^{†‡§‖}$	0.19 ± 0.01†‡§	0.133	0.547	0.731
24	**Gluc**	83.5 ± 40.2‡	75.3 ± 23.5	89.7±23.9	75.4 ± 26.3‡	0.752	0.269	0.760
	Chol	1.6 ± 0.6‡	1.5 ± 0.4	1.7 ± 0.4‡	1.3 ± 0.4‡	0.589	0.199	0.502
	TAG	1.6 ± 0.6‡	2.1 ± 0.9	1.8 ± 0.4	2.7 ± 1.2	0.177	0.033	0.434
	NEFA	0.19 ± 0.05$^{†‡§‖}$	0.17 ± 0.05†‡	0.24 ± 0.06†	0.21 ± 0.05†‡	0.015	0.162	0.673
P-value One-Way ANOVA - Time	**Glucose**	0.000	0.036	0.125	0.002			
	Chol	0.000	0.484	0.001	0.001			
	TAG	0.022	0.828	0.030	0.077			
	NEFA	0.000	0.000	0.000	0.000			

Values are means ± standard deviation (n = 9).
[a,b] Mean values within a row with unlike superscript letters showed an interaction between the two factors (P/L vs PS) (P<0.05).
[† ‡ § ‖ ¶] Mean values within a row unlike superscript symbols indicates significant differences between time within treatments (P<0.05)

FAS activity was significantly higher in fish fed PP diet than in fish fed FM diet, but only at 60P/6L, and was positively correlated with lipid retention data (Pearson correlation = 0.75). Moreover, when PP was used, both enzymes were depressed in fish fed 50P/10L by the increase in dietary lipid level (60P/6L versus 50P/10L). Blackspot seabream LPL activities were within the same range of values in the various tissues when expressed as mIU g^{-1} tissue (Table 7). In muscle and adipose tissue no dietary effects were found in LPL activity. However, in adipose tissue LPL specific activities (mIU g^{-1} protein) were considerably higher (five- to sevenfold increase) than in muscle or liver. In liver LPL activity (expressed as mIU g^{-1} tissue or as specific activity) was significantly affected by PS, being lowest in fish fed PP diets.

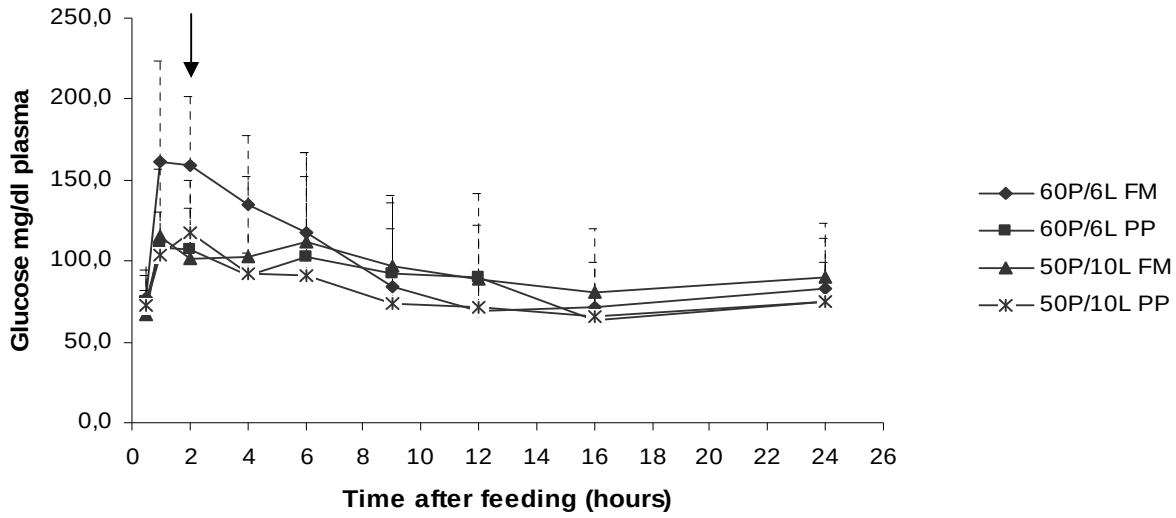

Figure 1. Plasma glucose levels (mg dL^{-1}) measured at different times up to 24 h after the last meal (*n* 9). Glucose peak is signalled by an arrow.

Data on plasma glucose, CHOL, TAG and NEFA levels analyzed at several times after the last meal are presented in Table 8. The glucose peak occurred 1-2 h postprandially (Fig. 1) in all dietary treatments. Nevertheless, plasma glucose levels were generally higher in fish fed the 60P/6L FM diet that showed significant higher values 2 h after the last meal (158.5 mg dL^{-1} plasma) compared to other treatments. In all cases, basal glucose levels were restored between 9 and 16 h after last meal. PS significantly affected plasma total CHOL levels at several sampling times: 0.5; 1; 2; 9 and 12 h. Plasma TAG was only significantly affected by PS 24 h after the last meal, with the highest value found in fish fed the PP diets. Plasma cholesterolemia and NEFA levels were generally higher in fish fed

FM-based diets and lower in those fed PP diets. In addition, the different P/L levels have only slightly affected CHOL levels or TAG (P≥0.044).

Discussion

The ADC observed for all nutrients, were generally high and in accordance with ADC for carnivore species (NRC, 1993). ADC values were not affected by dietary treatments, except for starch which decreased (from 99.6 to 95.6%) with increasing starch dietary levels (from 100 to 190 g kg^{-1} DM). Similar results were previously reported in others species (Bergot & Breque 1983; Enes *et al.* 2006a) and for blackspot seabream (Silva *et al.* 2006). Starch digestibilities observed in this study were in general accordance with those described for seabream (Venou *et al.* 2003; Enes *et al.* 2008a) or sea bass (Peres & Oliva-Teles 2002; Enes *et al.* 2006a) when fed treated starches. It must be taken into consideration that the main dietary starch source utilized in this experiment was micronized treated wheat, known to generally increase starch digestibility (NRC 1993), and thus high starch digestibilities were predictable.

Previous studies with blackspot seabream have registered low growth rates (Peleteiro *et al.* 1994; Olmedo *et al.* 2000) when compared with other Sparidae such as gilthead seabream (Santinha *et al.* 1996; 1999; Izquierdo *et al.* 2003; Gómez-Requeni *et al.* 2004; Sitjá-Bobadilla *et al.* 2005). In previous studies, improvements of both FCR (4.2-1.6) and DGI (0.7-1.4) were reached with increasing dietary protein levels up to 400 g kg^{-1} DM (40%), but no further increases were obtained with higher protein levels (Silva *et al.* 2006). Data on DGI (1.0-1.2), obtained in the present work were higher that the ones reported by Olmedo *et al.* (2000) but slightly lower than those found by Silva *et al.* (2006). Differences in initial body weight, rearing temperature and genetic origin of the fish can explain those differences. In addition, the present results have evidenced the possibility of replacing 10% protein (60-50%) by an increase of 4% in dietary lipid (6-10%) without affecting growth performance. Nevertheless, compared with 60P/6L, FCR increased in fish fed 50P/10L probably due to a significantly higher fed intake but lower nutrient retention.

The 50% replacement of fish meal (FM) by wheat gluten in diets for blackspot seabream seems to be possible without any adverse effect on growth, only when high protein levels are used. Indeed, DGI and FBW were significantly reduced in fish fed the low dietary protein levels (50%) containing vegetable sources. The feasibility of FM replacement by PP sources has been shown to be highly variable among species. Several studies have shown that FM can be replaced at least up to 60-75% without significantly effects on

growth of gilthead seabream (Gómez-Requeni *et al.* 2004; Sitjá-Bobadilla *et al.* 2005) or sea bass (Kaushik *et al.* 2004; Dias *et al.* 2005). Furthermore, almost total replacement of FM by a mixture of PP sources has also been shown to be possible in rainbow trout (Kaushik *et al.* 1995) or more recently in European sea bass (Kaushik *et al.* 2004). In the large majority of these studies, vegetable diets were supplemented with IAA such as lysine and methionine which allowed PP to almost replace FM without significant effects on growth. Data on quantitative IAA requirements of blackspot seabream is not available, but it has been suggested that differences between species would be minor (Mambrini & Kaushik 1995). In addition, the dietary theoretical percentages are within the recommendations for most cultivated species (Kaushik 1998). Considering such, impaired growth registered in fish fed 50P/10L PP was probably due to a lower protein retention and lower nitrogen gain (although without statistical meaning) rather than IAA deficiencies. Low palatability and complex synergistic interactions among antinutritional factors, attributed to plant protein sources (NRC 1993), could also be responsible for the reduced growth verified with 50/10 PP diet. Nevertheless, the determination of blackspot seabream IAA requirements would eventually allow higher PP inclusion, even at low protein levels, through balancing by addition of crystalline AA.

In this experiment, different P/L ratios (60P/6L or 50P/10L) have significantly affected both protein and lipid whole body content, but no PS effect was found. Blackspot seabream juveniles fed 60P/6L diets presented the highest protein (17.1 to 17.5 % WW) and the lowest lipid contents (11.7 to 13.9 % WW) comparing to those fed 50P/10L. In sea bass, the use of soy protein as the main dietary protein source has significantly reduced fat content (Dias *et al.* 2005), whereas a significant fat increase was observed when increasing levels of FM were replaced by a mixture of PP sources (Kaushik *et al.* 2004), indicating a clear PS effect on body fat contents.

Protein and lipid retention were significantly influenced by both P/L level and PS. Even at low dietary lipid levels (6-10% DM) lipid retentions were generally extremely high (98-188%), attaining maximal values in fish fed 60P/6L diets. Those results suggest the conversion of other nutrient than lipid (protein and/or carbohydrates) into corporal fat. Nevertheless, considering the excessive fat accumulation (>18%) previously described for this species (Linares *et al.* 2000, 2001; Silva *et al.* 2006; Ozório *et al.* 2009) positive improvements were reached with this experiment, and body fat content is well within the values found for other species such as gilthead seabream (Santinha *et al.* 1996, 1999) or European seabass (Dias *et al.* 1998, 2005; Kaushik *et al.* 2004). Liver and muscle lipid content of blackspot seabream were also lower than those previously reported for this

species (Ozório *et al.* 2009), displaying values similar to those described for gilthead seabream (Santinha *et al.* 1999; Izquierdo *et al.* 2003) or sea bass (Izquierdo *et al.* 2003). Dietary treatments have affected neither liver nor muscle lipid content when expressed by DM. However, when expressed by wet weight, the liver lipid content showed a slight increase in fish fed PP diets. Such result does not seem to be related to lipogenesis or LPL activity, but fish individual variability on lipid and/or DM content might explain this result.

Muscle fatty acid composition generally reflects the dietary profile, as showed in previous studies (Bell *et al.* 2002; Izquierdo *et al.* 2003; Mourente & Bell 2006), though specific fatty acids were selectively retained. At the end of experimental period, total saturated (37-39%) and monounsaturated (30-33%) fatty acids were the main flesh fatty acid classes irrespective of the dietary treatment. The observed accumulation of stearic acid (18:0) may result from an elevated species lipogenesis capacity as suggested by the high FAS activities displayed by this fish species. An accumulation of the monounsaturated (MUFA) fraction content was also observed in muscle, essentially due to the accumulation of 18:1 and 20:1 fatty acids. Although PS did not affect total saturated, monounsaturated or PUFA lipid fractions, the n-3 and n-6 fractions varied significantly and displayed opposite trends; muscle n-6 content increased, essentially due to an accumulation of linoleic acid (18:2n-6; LA), while a decrease in eicosapentanoic (20:5n-3; EPA) and docosahexaenoic (22:6n-3; DHA) fatty acids significantly contributed to lower muscle n-3 percentages in fish fed PP diets. Nevertheless, muscle percentages of DHA were similar to those presented in PP diets indicating a selective retention of this fatty acid. On the contrary, EPA proportions were lower in muscle than in diets suggesting that this fatty acid is more efficiently catabolised than DHA (Bell *et al.* 2002; Stubhaug *et al.* 2005). The higher assimilation of DHA over EPA implies that DHA might have a higher biological value than EPA during the ongrowing phase. Selective retention of DHA might be a very important characteristic of this species, as the intrinsic structure of this FA is inherently resistant to temperature and pressure changes (Sargent *et al.* 2002), being particularly advantageous for marine demersal fish species.

Activities found for hepatic lipogenic enzymes in blackspot seabream such as G6PD and FAS were respectively lower and higher than those found in gilthead seabream (Gómez-Requeni *et al.* 2003) or seabass (Dias *et al.* 1998, 2005; Richard *et al.* 2006b), but well within the range of values observed for red seabream (Iritani *et al.* 1984). Low protein/high lipid diets (50P/10L) significantly decreased G6PD and FAS hepatic activities. In most teleosts, it has been observed an inhibitory effect of dietary lipid level on lipogenesis

(Likimani & Wilson 1982; Arnesen *et al.* 1993; Alvarez *et al.* 1998; Dias *et al.* 1998). In higher vertebrates, besides protein level, the dietary protein quality is also known to affect lipogenic enzymes activities (Iritani *et al.* 1986, 1996; Kayashita *et al.* 1996; Padmakumarannair *et al.* 1998). Although the present results showed no significant effects of PS on lipogenic enzymes, fish fed high PP diets showed the highest FAS specific activities due to a significant interaction between the two dietary factors (P/L level and PS). These results are not in completely agreement with those found by Dias *et al.* (2005), who demonstrated that dietary PP sources affect fat deposition and lipogenic potential in European sea bass, decreasing G6PD and malic enzyme (ME) activities. The higher dietary FM replacement by different PP sources level (up to 80%) used by Dias *et al.* (2005) together with differences in PP utilization by fish species are probably in the basis of such differences found on hepatic lipogenesis. Concerning FAS, results were shown to be dependent on the PP source, decreasing in sea bass fed soy rich diets but increasing with corn-gluten meal based diets. Dias *et al.* (2005) suggested that these results could be related to the dietary AA imbalance, particularly a deficiency of the exclusively ketogenic AA such as lysine, which leads to increase catabolism of other AA, leaving thus more carbon chains and glucogenic substrates available for lipogenesis. Assuming that dietary IAA percentages are within the recommendations for most cultivated species (Kaushik 1998), the increased FAS activity verified with 60P/6L PP diet has probably resulted from an inadequate AA ratio to each other. In addition, the increase in hepatic FAS activity corroborates well with the extremely high lipid retention observed in this group of fish (Pearson correlation = 0.75).

Lipoprotein lipase (LPL) activity or expression has been showed to be markedly higher in adipose tissue than in muscle or liver tissues in rainbow trout and seabream (Lindberg & Olivecrona 1995, 2002; Arantzamendi *et al.* 2003; Saera-Vila *et al.* 2005; Richard *et al.* 2006a). In the present work LPL activities (expressed as mIU g^{-1} tissue) were similar among the three tissues analysed and no relationship was found between LPL activity and the main lipid depot sites. However, regarding specific activities, a five- to sevenfold higher activity is found in adipose tissue than in muscle or liver tissues which may be due to the differences between these tissues protein content. These similarities between tissues LPL were also found in European seabass (Richard *et al.* 2006b) suggesting a specie specific LPL control.

Several studies concerning LPL activity or expression, showed a tissue-specific regulation by nutritional conditions in rainbow trout (Lindberg & Olivecrona 2002; Arantzamendi *et al.* 2003; Albalat *et al.* 2006; Richard *et al.* 2006b), red sea bream (Liang *et al.* 2002a,b),

gilthead sea bream (Arantzamendi *et al.* 2003; Saera-Vila *et al.* 2005) and European sea bass (Richard *et al.* 2006a). The regulatory effect of dietary fatty acids on LPL gene expression has been reported in previous works (Raclot 1997; Liang *et al.* 2002a,b; Richard *et al.* 2006a). In red seabream LPL mRNA levels in liver increased with dietary long-chain n-3 PUFA while high oleic acid had the inverse effect in adipose tissue (Liang *et al.* 2002b). Similarly, the observed effect of PS on blackspot seabream liver LPL activity could be related to changes in dietary FA composition. Dietary FM replacement by wheat gluten significantly decreased n-3 PUFA contents in diets, which possibly explain the associated reduction in liver LPL activity. This decrease in hepatic LPL activity implies a reduction of liver fatty acid uptake which might be down-regulated by the increased fatty acids levels provided by FAS. Moreover, this inverse dietary effect on LPL (down-regulated) and FAS (up-regulated) activity could explain the final similarity in hepatic lipid contents of fish fed 60P/6L PP diet. In contrast, in gilthead seabream full FM replacements by PP was shown to decrease LPL expression in adipose tissue and enhance it in hepatic tissue, whereas skeletal tissue remained unaffected (Saera-Vila *et al.* 2005).

The length of hyperglycaemic period is species and condition dependent, depending on species-specific mechanism of glucose homeostasis (Moon 2001). The rates of glucose absorption and time to maximum plasma glucose concentration occurrence are then important to understand plasma glucose regulation and metabolic nutrient relationships. Blackspot seabream juveniles fed the different dietary treatments presented a plasma glucose peak between 1 and 2 h after the last meal. These results are in agreement with those found for common carp and red seabream which showed plasma glucose peaks at 1 and 2 h, respectively, after an oral administration of glucose (Furuichi & Yone 1981). Similar plasma peak times (1-3 h) were observed in gilthead seabream, whereas in several other species the peak was reached 3-6 h after glucose injection (Furuichi & Yone 1981; Hemre *et al.* 1995; Lin & Shiau 1995; Garcia-Riera & Hemre 1996; Peres *et al.* 1999; Robaina *et al.* 1999; Gisbert *et al.* 2003). In general, the more carnivorous the species, the longer time needed to clear a glucose load (Moon 2001). In our study, the high standard deviations, possibly due to individual genetic variability observed for plasma metabolites, and for glucose in particular, bring some difficulty to results interpretation. The blackspot seabream ability to restore the basal glucose levels was observed between 9-16 h after the last meal for all dietary treatments. Robaina *et al.* (1999) observed higher glucose levels in sea bass fed 30% wheat gluten meal diets compared with those fed FM diets. These differences were explained by the lesser amounts of digestible starch content in these diets. In the present work dietary starch levels ranged between 100 and 190 g kg^{-}

^{1}, increasing with vegetal protein incorporation. Curiously, the high plasmatic glucose level was observed in fish fed the low starch dietary level (100 g kg^{-1}). In mammals, the hexokinase IV reaction constitutes an important rate-limiting step in the removal of glucose from circulation by the liver (Hornichter & Brown 1969). Upregulation of this enzyme by high carbohydrate diets has been known for a long time in mammals (Borrebaek *et al.* 1970) and fish (Borrebaek *et al.* 1993). Considering this, a carbohydrate upregulation of hexokinase IV activity may be hypothesized to explain the rapidly clearance of glucose from plasma in fish fed high carbohydrate levels. The probably high AA content in 60P/6L FM diets and consequently high gluconeogenic AA content could explain the highest plasma glucose levels in fish fed this diet. Further work on glucose metabolism will be useful in order to explain the role of dietary nutrients as protein and carbohydrates on glucose metabolism regulation.

High PP diet (60P/6L PP) has lowered from 26 to 41% fish CHOL (at 0.5 and 2 h postprandial time, respectively) and NEFA plasma levels. Several studies have previously showed a decrease in lipid deposition and mesenteric fat together with hypocholesterolemia in gilthead seabream (Gómez-Requeni *et al.* 2004; Albalat *et al.* 2005; Sitjá-Bobadilla *et al.* 2005) as well as in other teleostean species (Kaushik *et al.* 1995, 2004; Regost *et al.* 1999; Robaina *et al.* 1999; Dias *et al.* 2005) when FM is replaced by PP. In mammals, an inverse correlation between plasma TAG level and LPL activity, increasing lipid tissue uptake, have been shown (Zampelas *et al.* 1994; Kayashita *et al.* 1996). Similarly, in our study an inverse correlation between plasma TAG and liver LPL activity was also observed, particularly in fish fed FM diets.

The present work has shown that besides dietary P/L level, PS has a strong effect on blackspot seabream lipogenesis, with excessive PP leading to high hepatic FAS activity and consequently extremely high lipid retention (Pearson correlation = 0.75). In addition, 50% FM replacement seems to be feasible on blackspot seabream, but only when a high dietary protein level is used (60%). Therefore, wheat gluten does not appear to be a good PS to replace FM in diets for blackspot seabream. Moreover, 50P/10L FM based diet was the most cost effective. Future studies should be conducted to investigate the species AA optimal ratio to each other and their different rates of absorption and catabolism, preventing AA utilization for other purposes than protein synthesis, such as lipogenesis or gluconeogenesis.

CHAPTER 3

Blackspot seabream (*Pagellus bogaraveo*) lipogenic and glycolytic pathways appear to be more related to dietary protein level than dietary starch type.

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Abstract

The lipogenic potential of carbohydrate type on blackspot seabream was determined using two isolipidic (10%) and isonitrogenous (45%) diets containing either crude (CS) or gelatinized corn starch (GS). A third diet containing a lower protein level (35%) and gelatinized starch (GS) was also tested to determine if any lipogenic effect detected was due to carbohydrate and not from excess dietary protein. The three diets were formulated to have similar energy (21 kJ g^{-1} DM) content. Triplicate groups of fish (24 g initial body weight) were fed each dietary treatment by hand to apparent satiety for 90 days. Both starch type and dietary protein level significantly affected growth. Fish fed the CS diet increased voluntary feed intake (VFI) and digestible protein intake resulting in both higher final body weight (FBW) and daily growth index (DGI). Although the lower protein level (35%) increased VFI, this increase was not enough to reach the same digestible protein intake observed in fish fed the high protein level (45%), resulting in lower FBW and DGI.
Despite the differences observed in growth, nutrient gain and hence whole body composition were not affected by starch type (CS vs GS) or dietary protein level (35 vs 45%). Similarly, glycaemia was not affected by starch type or dietary protein level in any of the treatments, with the highest values being observed at 4 h post feeding. On the other hand, both starch type and dietary protein level significantly affected insulin levels 2 h after feeding. Plasma lipid levels showed no significant differences at 2 and 24 h post feeding, regardless of dietary treatment. A reduction of 10% dietary protein level (45 to 35%) markedly depressed (> 40%) hepatic activities of lipogenic enzymes (glucose-6-phosphate dehydrogenase, G6PD; malic enzyme, ME; fatty acid synthetase, FAS), and significantly increased PK activities at each postprandial time (2, 4 and 24 h). Starch type did not cause any significant effect on hexokinase (HK), glucokinase (GK), pyruvate kinase (PK) (glycolysis) or glucose-6-phosphatase (G6Pase) (gluconeogenesis) postprandial hepatic activities. Blackspot seabream lipogenic and glycolytic pathway regulation appear to be more related to dietary protein level than to dietary starch type.

Introduction

Blackspot seabream has high commercial value, excellent palatability and the scarcity in the fishing grounds has resulted in industrial production of around 250 tonnes in 2007 in the north of Spain. Several studies have been recently devoted to this species nutrition and Figueiredo-Silva *et al.* (2009a) considered a diet containing 50% crude protein and 10% crude lipid to be the most appropriated for blackspot seabream juveniles. However, even at such low dietary lipid level, lipid retention is remarkably high (> 100%) (Figueiredo-Silva *et al.* 2009a). Those results suggest the conversion of nutrients other than lipid (protein and/or carbohydrates) into body fat. Hence, understanding the nutritional regulation of blackspot seabream metabolic pathways would contribute to improving feed formulation and thus their production by the aquaculture industry.

In the last decade, finding alternative energy sources has become an internationally recognized priority for sustaining the rapidly expanding aquaculture industry (Tacon 2003). Low-cost dietary energy provided by carbohydrate rich cereal grains, like wheat and corn, has been extensively utilized in aquaculture feed manufacturing in order to minimize the amount of protein used. Nevertheless, in carnivorous fish species, the capacity to digest complex carbohydrates is limited and mainly depends on the carbohydrate source and type, on its dietary inclusion level and on the technological treatment applied (gelatinization or extrusion) (Wilson 1994; Hemre *et al.* 2002; Stone 2003). Diets with high digestible starch content (20 to 30%) have efficiently improved fish growth in gilthead seabream (Venou *et al.* 2003; Fernández *et al.* 2007) and rainbow trout (Baños *et al.* 1998; Alvarez *et al.* 1999). However, other studies have demonstrated that neither the level nor type of starch affected growth of Senegalese sole (Dias *et al.* 2004), European sea bass (Enes *et al.* 2006a) or gilthead seabream (Enes *et al.* 2008a). Moreover, high dietary levels of non-protein energy such as digestible carbohydrate have been shown to stimulate hepatic lipogenic enzymes (Likimani & Wilson 1982; Dias *et al.* 1998; Alvarez *et al.* 1999; Metón *et al.* 1999; Fernández *et al.* 2007) and contribute to higher fat deposition (Kaushik & Oliva-Teles 1985; Grisdale-Helland & Helland 1997; Dias *et al.* 1998; Venou *et al.* 2003; Fernández *et al.* 2007).

The main objective of the present work was to determine the lipogenic potential of carbohydrate in blackspot seabream using two different starch types, crude (CS) or gelatinized starch (GS), at the same protein level (45%). Several studies have shown that dietary protein *per si* is a potent regulator of lipid biosynthesis in higher vertebrates (Herzberg & Rogerson 1981; Rosebrough *et al.* 1996) including fish (Shikata & Shimeno

1997; Dias *et al.* 1998, 2003; Alvarez *et al.* 1999). For that reason, a third diet containing gelatinized starch (GS) with a lower protein level (35%) was also tested to determine if any lipogenic effect detected was due to carbohydrate and not from excess protein. Dietary effects on glucose (glycolysis and gluconeogenesis) and lipid (lipogenesis) metabolic pathways as well as on important regulatory hormones (insulin and cortisol) were also evaluated for the first time in this fish species.

Material and methods

Diets

Two isolipidic (10%) and isonitrogenous (45%) diets were formulated to contain crude (CS) or gelatinized corn starch (GS). A third diet containing a lower protein level (35%) and gelatinized starch (GS) was also tested. The three diets were formulated to contain similar energy content (21 kJ g^{-1} DM). All ingredients were supplied by Sorgal S.A. (Ovar, Portugal) and were finely grounded, mixed and dry pelleted through a 2.4 mm die at 50 ºC (CPM, C-300 model). Ingredient and proximate composition of the diets are presented in Table 1 and the dietary fatty acid profiles in Table 2.

Growth trial

Experiments were directed by trained scientists (following FELASA category C recommendations) and were conducted according to the European Economic Community animal experimentation guidelines Directive of 24 November 1986 (86/609/EEC). Juvenile blackspot seabream (*Pagellus bogaraveo*) were obtained from a fish farm (Grupo Isidro de la Cal, Valdoviño, Coruña, Spain) and transported to the our facilities at CIIMAR, Porto, Portugal. After a quarantine and acclimatization period (4 weeks), groups of 31 juveniles with an average body weight of 24 g (Table 4) were randomly distributed among 9 square fibre glass tanks (500 L), in a recirculating water system. Triplicate groups of fish for each treatment were fed by hand to apparent satiety, twice a day (09.00 and 18.00 h) for 90 days. Each tank was supplied with filtered, heated (19 ± 1 ºC) saltwater (33 g L^{-}) with dissolved oxygen content above 8 mg L^{-1} at a flow-rate of 2 L min^{-1}. The pH, ammonia, nitrites and nitrates in the water were monitored during the entire trial and maintained at levels compatible with marine species. Fish were exposed to natural photoperiod. Every four weeks, fish were bulk weighted under moderate anaesthesia (ethylene glycol monophenyl ether at 50 mg/L) and data on weight gain and feed fed recorded.

Table 1. Ingredient and proximate composition of experimental diets

	Diet		
	CS	**GS**	**GS**
	45		**35**
***Ingredients* (%)**			
Fish meal 67/10/15 Prime [a]	48.00	48.00	31.50
Wheat gluten	2.50	2.50	2.50
CPSP G	2.50	2.50	2.50
Squid meal	2.50	2.50	2.50
Raw corn starch [b]	25.00	-	-
Gelatinized corn starch [c]	-	25.00	25.00
Wheat bran [d]	14.50	14.50	30.00
Fish oil	4.00	4.00	5.00
Vitamins and mineral mix [e]	0.25	0.25	0.25
Choline chloride [f]	0.10	0.10	0.10
Lutavin E50 [g]	0.05	0.05	0.05
Lutavin C35 [h]	0.05	0.05	0.05
Betafin S1 [i]	0.05	0.05	0.05
Europelin [j]	0.50	0.50	0.50
Proximate composition			
Dry matter (DM) (%)	89.9	91.1	90.7
Crude protein (% DM)	45.1	45.2	35.1
Crude lipid (% DM)	9.1	8.41	9.2
Starch (%DM)	24.8	23.6	26.5
Ash (% DM)	10.8	10.5	9.1
Gross energy (kJ g^{-1} DM)	21.0	21.2	21.2
Digestible protein (DP, %DM)	36.8	35.8	24.7
Digestible energy (DE, kJ g^{-1} DM)	15.8	16.4	15.5
DP/DE mg kJ^{-1}	23.3	21.9	16.0

[a] Fish meal: Dry matter: 90.2 %;Protein: 74.5 % DM; Lipids: 8.5 % DM.
[b] Raw starch, Copan, Leiria, Portugal: Dry matter: 86.7 %; Protein: 0.4 (% DM); Lipids: 1.0 %DM; Starch: 93.9 %DM.
[c] Gelatinized starch, Cerestar, Barcelona Spain.: Dry matter: 91.9 %; Protein: 0.4 % DM; Lipids: 0.5 %DM; Starch: 88.3 %DM.
[d] Wheat bran: Dry matter: 87.4 %; Protein: 16.9 % DM; Lipids: 2.5 %DM; Starch: 19.8 %DM); Fibre: 13.3 % DM.
[e] Vitamins (mg or IU/kg diet): Vitamin A, 8000 IU; vitamin D3, 1700 IU; vitamin K3, 10 mg; vitamin B12, 0.02 mg; thiamin, 8 mg; riboflavin, 20 mg; vitamin B6, 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; nicotinic acid, 70 mg; pantothenic acid, 30 mg.
Minerals (g or mg/kg diet): Mn (manganese oxide), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc oxide) 30 mg; Se (sodium selenite) 0.3 mg; Fe (iron sulphate), 60 mg; Ca (calcium carbonate), 2.15 g; dibasic calcium phosphate, 5 g; KCl, 1 g; NaCl, 0.4 g.
[f] Choline chloride, 1000 mg kg^{-1} diet.
[g] Lutavin E50: vitamin E, 300 mg kg^{-1} diet.
[h] Lutavin C35: vitamin C, 500 mg kg^{-1} diet.
[i] Betafin S1: betain, 500mg/kg diet.
[j] Europelin: Binder.

Prior to sampling, fish were fasted for 24 h and killed by a sharp blow on the head for enzyme determinations. At the beginning and at the end of the feeding trial a pooled sample of 9 fish per treatment were taken and stored at -20 ºC for subsequent whole body composition analyses. Muscle and liver tissues were collected for analysis of total lipid and fatty acids (9 fish per treatment), lipogenic enzymes (9 fish per treatment), glycolytic and gluconeogenic hepatic enzymes (6 fish per treatment). For these last analyses, livers were equally divided into two parts, one for glycolytic and another for gluconeogenic enzyme assays. All samples were frozen in liquid nitrogen and stored at -80 ºC for subsequent analyses. Blood collection was carried out in 12 fish per treatment at 3 postprandial different times (2; 4 and 24 h), and in less than 3 min at each sampling time to avoid any plasma metabolite response induced by handling stress. Samples were taken from the caudal vein, with syringes and collection tubes containing 15 to 20 µL of sodium fluoride and potassium oxalate (4% each). Plasma was obtained after centrifugation (6000 x *g* for 10 min at 4 ºC) and stored at -80 ºC for glucose, cholesterol (CHOL), triacylglycerol (TAG), insulin and cortisol analysis.

Digestibility trial

The apparent digestibility coefficients (ADC) of the dietary components of the three diets were assessed after incorporation of 0.1% of yttrium oxide, as inert marker, to a grounded portion of each diet. The mixtures were then dry pelleted through a 2.4 mm die at 50 ℃ (CPM, C-300 model). Groups of 10 fish (mean body weight of 75 g) were stocked into 6 digestibility tanks of 50 L tanks specially constructed according to the Guelph system (Cho *et al.* 1982) and adapted to the new conditions for 15 days. The fish were subjected to a natural photoperiod, water temperature was maintained at 19 ± 1 ℃ and salinity at 33 ‰. The 3 diets were randomly assigned by tank (n=2). The first two days of the digestibility period were used for acclimation to the feed and no faeces were collected. This time period was deemed sufficient for the fish to achieve complete evacuation of previous meals. Fish were fed once daily until apparent satiety and faeces were collected each morning over a four-week period. After collection, faecal samples were centrifuged, pooled and frozen at –20 ^{o}C. The ADCs were calculated according to Maynard *et al.* (1969).

Feed, body composition and faeces analyses

Whole fish from each tank were ground, pooled and fresh moisture content determined. Fish and faeces were subsequently freeze-dried before further analysis. Feed, whole body samples and faeces were analyzed for dry matter (105 ºC for 24 h), ash by combustion in a muffle furnace (550 ℃ for 12 h), crude protein (micro-Kjeldahl; *N* x 6.25) after acid

digestion, lipid content by petroleum ether extraction (at Soxhlet 40-60 ºC), and gross energy in an adiabatic bomb calorimeter (IKA, Werke C2000).

Table 2. Fatty acid composition of the experimental diets (g/100 g total fatty acid)

	Diet		
	CS	**GS**	**GS**
	45		**35**
10:0	1.5	1.2	0.5
14:0	5.4	5.2	4.8
16:0	18.0	17.3	17.1
17:0	0.5	0.4	0.4
18:0	3.5	3.4	3.2
20:0	0.3	0.3	0.3
ΣSaturates	29.7	28.4	26.8
16:1	6.0	5.8	5.5
17:1	0.2	0.3	0.3
18:1	13.0	12.6	13.6
20:1	3.4	3.4	3.9
22:1	3.9	4.0	4.6
ΣMUFA	26.5	26.1	28.0
14 PUFA	0.2	0.2	0.2
16:2 *n*-4	0.6	0.6	0.5
16:3 *n*-4	0.7	0.6	0.5
16:4 *n*-1	1.2	1.1	0.9
18:2 *n*-6	5.5	5.4	8.9
18:3 *n*-6	0.2	0.2	0.2
20:2 *n*-6	0.2	0.2	0.3
20:4 *n*-6	0.7	0.7	0.7
Σn-6	6.7	6.6	10.2
18:3 *n*-3	1.1	1.1	1.4
18:4 *n*-3	2.0	2.0	2.0
20:3 *n*-3	0.1	0.1	0.2
20:4 *n*-3	0.7	0.7	0.7
20:5 *n*-3	10.9	10.9	9.7
21:5 *n*-3	0.4	0.4	0.4
22:5 *n*-3	1.5	1.6	1.3
22:6 n-3	13.7	13.8	12.2
Σn-3	30.4	30.4	27.8
PUFA	39.7	39.6	40.2
Sat/PUFA	0.8	0.7	0.7
n-3/n-6	4.5	4.6	2.7

Feed and faeces starch content was determined according to Thivend *et al.* (1972). Yttrium oxide concentrations were determined in both diets and faeces samples by atomic absorption spectrophotometry (SpectrAA 220FS, Varian), after digestion with HNO_3 (Reis *et al.* 2008).

Total lipid and fatty acids analyses

Total lipid was extracted and measured gravimetrically according to Folch *et al.* (1957) using dichloromethane instead of chloroform. Fatty acid methyl esters were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride methanol according to Santha & Ackman (1990) and analyzed in a Varian 3800 gas chromatograph (Varian, Les Ulis, France). The chromatograph was equipped with a DB Wax fused silica capillary column (30 m X 0.25 mm internal diameter, film thickness: 0.25 µm, J & W Scientific, Folsom, CA, USA). Helium was used as carrier gas (1 mL/min) and the thermal gradient was 100 to 180 ºC at 8 ºC/min, 180-220 ºC at 4ºC/min and a constant temperature of 220 ºC during 20 min. Injection was made in a split mode (ratio 1:40) with 1 µL injected. Injector and flame ionization detector temperatures were 260 and 250 ºC, respectively. Fatty acids were identified by comparison with known standard mixtures (Sigma 189-19, St Louis, MO, USA) and quantified using a Star computer package (Varian).

Enzyme activity

Lipogenic enzymes

Liver samples for lipogenic enzyme assays were homogenised in three volumes of ice-cold buffer (0.02 mol/L Tris–HCl, 0.25 mol/L sucrose, 2 mmol/L EDTA, 0.1 mol/L NaF, 0.5 mmol phenylmethyl sulphonyl fluoride, 0.01 mol/L β-mercaptoethanol, pH 7.4) and centrifuged at 30 000 x *g*, at 4 ºC for 20 min. Selected lipogenic enzyme activities were assayed in the supernatant: glucose-6-phosphate dehydrogenase G6PD (EC 1.1.1.49) assayed at 30 ºC according to Bautista *et al.* (1988), malic enzyme (ME, EC 1.1.1.40) assayed at 30 ºC according to Ochoa (1955) and fatty acid synthetase (FAS, EC 2.3.1.38) assayed at 37 ºC according to the methodology of Chang *et al.* (1967) as modified by Chakrabarty & Leveille (1969).

Glycolytic and gluconeogenic enzymes

Liver samples for glycolytic enzymes assays were homogenized in five volumes of ice-cold buffer (80 mM Tris; 5 mM EDTA; 2 mM DTT; 1 mM benzamidine; 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, pH 7.6) and centrifuged at 900 x *g* at 4 ºC for 10 min. The resulting supernatant was separated in two fractions, one for hexokinase (HK;

EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) assays and one to measure L-type pyruvate kinase (PK; EC 2.7.1.40). The HK (low Km HKs) and GK (high Km HK or HK IV) activities were measured at 37 ℃, using 0.5 mM and 100 mM of glucose, respectively, as described previously (Tranulis *et al.* 1996; Panserat *et al.* 2000a). The assay for GK activity in frozen samples necessitated a correction by measuring glucose dehydrogenase (EC 1.1.1.47) activity as described by Tranulis *et al.* (1996). PK activities were assayed in the supernatant resulting from a second centrifugation at 10 000 x *g* at 4 ºC for 20 min, according to Foster & Moon (1986). To measure glucose-6-phosphatase (G6Pase; EC 3.1.3.9) activity, microsomes were obtained from blackspot seabream livers samples as previously described by Panserat *et al.* (2000b). Microsomes were suspended in buffer (100 mM NaH_2PO_4; 25 mM Na_2HPO_4; 2mM EDTA; 1mM DTT, pH 7), without further treatment. The procedure followed was that of Alegre *et al.* (1988), monitoring the increase in absorbance at 340 nm (NADH appearance) using purified glucose dehydrogenase in excess as the coupling enzyme.

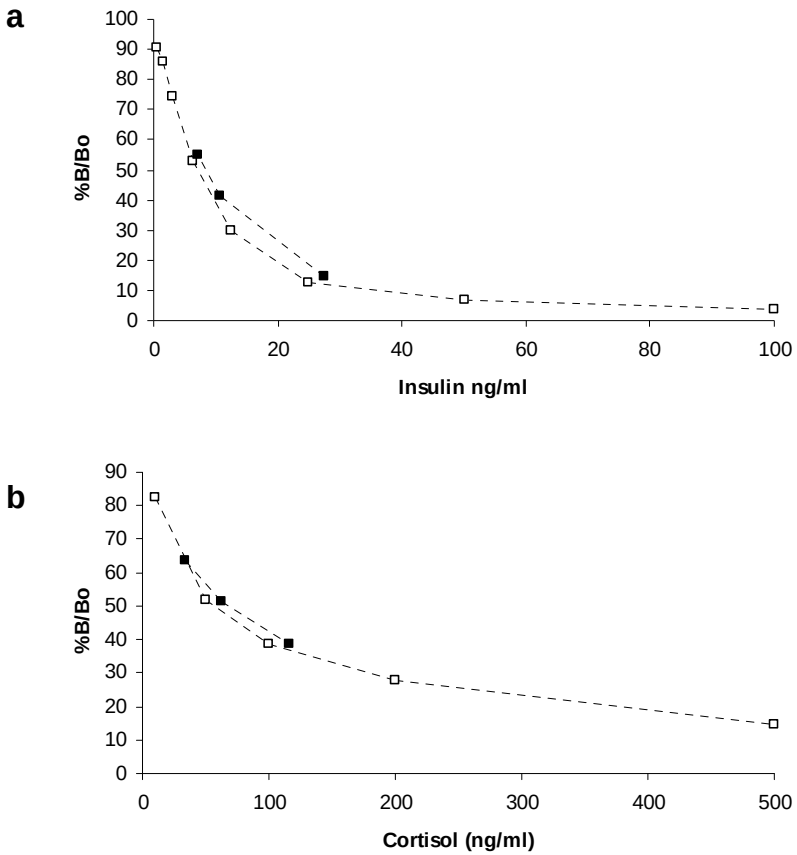

Figure 1. Validation of the radioimmunoassay method for blackspot seabream plasma samples. The parallelism between the standard curve (white diamonds) and plasma serial dilutions (black diamonds) demonstrates the validity of the a) insulin and b) cortisol method for this species.

Specific enzyme activities

Enzyme activity units IU, defined as µmoles of substrate converted to product, per min, at assay temperature, were expressed per mg of hepatic soluble protein (specific activity) or per g of tissue. Soluble protein content of tissues was determined on the supernatant fractions by the method of Bradford (1976) using bovine serum albumin (BSA) as standard (Sigma, St Louis, MO, USA).

Plasma metabolite assays

Plasma glucose, cholesterol (CHOL) and triacylglycerol (TAG) were determined using commercial kits: Glucose RTU (nº 61269); Cholesterol RTU (nº 61218) and Triglycérides Enzymatique PAP 150 (nº 61236) from Bio-Mérieux, Marcy-L'Etoile, France. Plasma insulin levels were measured by radioimmunoassay using bonito insulin as the standard and rabbit anti-bonito as antiserum, according to the method described by Gutiérrez *et al.* (1984) and validated for blackspot seabream (Fig. 1). Cortisol was determined with an enzymatic radioimmunoassay kit (Coat-A-Count Cortisol, Los Angeles, CA, USA) after being validated for blackspot seabream plasma samples (Fig. 1).

Statistical analysis

The data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Bartlett tests. The use of one-way ANOVA with diet as a main effect would test a distorted hypothesis which might not meaningfully reflect both the starch type and protein level effects. Hence, data were submitted to an ANOVA incomplete design (Type VI Sums of Squares) of Statistics 7.0 package (StatSoft, Inc., Tulsa, OK, USA) to test the original hypothesis given in the observed cells (effect of starch type, ST and protein level, PL). These statistical analyses followed methods outlined by Hocking (1996). Moreover, two individual one-way ANOVA analyses were performed between 45 CS and 45 GS and between 45 GS and 35 GS data (results not shown) and the statistical results obtained confirmed with an incomplete design ANOVA. Significant differences were considered when $P<0.05$.

Results

Apparent digestibility coefficients (ADCs) of the main dietary nutrients and energy were significantly affected by protein level, whereas starch type (CS vs GS) only significantly affected starch and lipid ADCs (Table 3).

Table 3. Apparent digestibility coefficients (ADC %) of nutrients and energy

	Diet			Main effects ANOVA *P*-value*	
	CS	GS	GS		
	45		35	ST	PL
ADC%					
Protein	81.6±1.8	79.2±0.2	70.4±1.1	0.150	0.005
Lipids	85.0±0.8	92.9±2.2	81.5±2.9	0.041	0.015
Energy	74.4±1.1	77.2±1.8	73.0±0.9	0.125	0.046
Starch	97.1±0.2	99.1±0.1	97.7±0.0	0.001	0.002

Mean values and standard deviations (SD) (n=2)
**P*-value for starch type (ST) and protein level (PL) main effects

Blackspot seabream juveniles fed gelatinized (GS) diets had significantly higher starch ADC than those fed crude starch (CS). In addition, fish fed the lowest protein level (35%) diet had the lowest protein, lipid and energy ADCs. Starch type (CS vs GS) and dietary protein level (45 vs 35%) significantly affected blackspot seabream growth performance (Table 4). Fish fed CS diet increased voluntary feed intake (VFI) and consequently nutrient and energy intake, resulting in higher final body weight (FBW) and daily growth index (DGI), when compared to the GS fed fish. On the other hand, fish fed the GS diet had significantly improved protein efficiency ratio (PER). The reduction of 10% dietary protein level (45 to 35%) resulted in higher VFI and consequently daily lipid, starch and energy intake. However, this increase was not enough to reach the daily protein intake observed with the higher protein diets (45%), resulting in reduced FBW and DGI.

Despite the differences observed among dietary treatments on growth parameters and nutrient intake, whole body composition as well as nutrient gain (Table 5) were not affected by dietary starch type (CS vs GS) or protein level (45 vs 35%). Nevertheless, a significant decrease in nitrogen loss (1.1 vs 0.9 g/ABW/day) was attained when dietary CS was replaced by GS. Fish fed the high-protein diet (45%) had increased lipid retention and energy retention tended to follow the same trend (P=0.06) when compared with those fed the low-protein diet (35%).

Table 4. Blackspot seabream final growth performance at 19 ± 1 ºC

	Diet			Main effects ANOVA *P*-value*	
	CS	GS	GS		
	45		35	ST	PL
Growth					
Initial body weight (g)	23.8±0.1	23.8±0.2	23.8±0.1	0.990	0.719
Final body weight (g)	78.3±2.1	73.3±1.9	68.8±0.6	0.010	0.016
FCR [a]	1.7±0.1	1.5±0.03	1.8±0.1	0.023	0.0011
DGI [b]	1.6±0.04	1.5±0.03	1.4±0.01	0.007	0.008
PER [c]	1.4±0.1	1.5±0.03	1.6±0.1	0.020	0.103
VFI [d]	2.0±0.1	1.7±0.01	1.9±0.1	0.003	0.004
Digestible intake (g or kJ/kg ABW [e]/d)					
Protein	7.2±0.4	6.0±0.0	4.8±0.2	0.001	0.001
Lipids	1.5±0.1	1.3±0.01	1.5±0.1	0.004	0.018
Energy	304.3±15.0	275.3±1.7	300.6±11.8	0.018	0.031
Starch	4.7±0.2	3.9±0.0	5.0±0.2	0.002	0.0003

Mean values and standard deviations (±SD) are presented for each parameter (n=3)
*P-value for starch type (ST) and protein level (PL) main effects
[a] FCR, Feed conversion ratio = dry feed intake/weight gain.
[b] DGI, Daily growth index = 100 x ((Final body weight)$^{1/3}$ - (Initial body weight)$^{1/3}$)/days
[c] PER, Protein efficiency ratio = weight gain/crude protein intake.
[d] VFI, Voluntary feed intake = 100 x crude feed intake/average body weight/day
[e] ABW, Average body weight = (final body weight + initial body weight)/2

There were no significant differences in viscerosomatic index (VSI) among the experimental groups, whereas fish fed the low protein diet (35 GS) had the highest hepatosomatic index (HSI) (Table 5).

Muscle and liver fatty acid (FA) compositions of blackspot seabream are shown in Table 6 and 7, respectively, and in general terms reflect the diet profiles (Table 2). PUFA was the main class present in muscle (34 to 38%), followed by saturates (31 to 32%) and MUFA (27 to 30%). A different pattern was observed in liver with MUFA (36 to 43%) representing the major FA class, followed by saturated (30 to 35%) and PUFA (18 to 29%). Moreover, *n*-3 PUFA content was considerably higher in muscle (ranging from 26 to 29%) than liver (ranging from 13-19%). Starch type had no effect on FA composition of muscle and induced only minor changes in liver FAs. On the contrary, dietary protein level strongly affects FA composition in both tissues.

Table 5. Whole body composition (% or kJ g^{-1} of wet weight, (ww)), nutrient gain and energy retention in blackspot seabream fed the different dietary treatments

	Diet			Main effects ANOVA *P*-value*	
	CS	GS	GS		
	45		35	ST	PL
Final body composition [a]					
Moisture %	63.8±1.23	64.1±1.8	65.1±0.5	0.786	0.351
Protein %	17.8±0.7	17.8±0.7	17.3±0.4	0.935	0.343
Lipid %	13.9±1.1	13.4±1.3	12.7±1.0	0.555	0.512
Energy kJ/g	9.4±0.7	9.1±0.7	8.8±0.3	0.498	0.576
HSI % [b]	1.2±0.27	1.1±0.1	1.5±0.2	0.293	0.0001
VSI % [c]	7.3±1.8	7.5±2.0	7.1±1.0	0.740	0.593
Nitrogen gain (mg/ kg ABW [d]/d)	350.8±15.2	335.6±22.0	306.8±9.7	0.298	0.075
Nitrogen loss (g/ kg ABW [d]/d)[e]	1.1±0.1	0.9±0.03	0.8±0.04	0.008	0.086
Lipids gain (g/ kg ABW [d]/d)	1.6±0.2	1.4±0.2	1.3±0.16	0.325	0.295
Energy gain (kJ/ kg ABW [d]/d)	112.1±11.3	101.5±12.4	91.8±4.3	0.243	0.282
Retention (%) intake					
Protein	25.0±2.2	27.6±2.0	28.1±1.4	0.133	0.077
Lipid	90.1±14.9	101.3±16.8	70.0±10.5	0.376	0.036
Energy	27.5±4.2	28.5±3.7	22.3±0.9	0.739	0.060

Mean values and standard deviations (±SD) are presented for each parameter (*n*=3)
*P-value for starch type (ST) and protein level (PL) main effects
[a] Initial body composition was: moisture: 64.2%; protein :16.3%ww; lipid :14.9%ww and energy :9.4 kJ/g.
[b] HSI, Hepatosomatic index = 100 X liver weight/body weight
[c] VSI, Viscerosomatic index = 100 X weight of viscera/body weight
[d] ABW, Average body weight = (final body weight + initial body weight)/2
[e] Nitrogen loss = N intake- N gain

Liver total saturated FAs were significantly higher in fish fed the high-protein diets than in those fed the low-protein diet, mainly due to an increase in 14:0 and 16:0 FAs. In muscle, saturated FA contents were similar (31 to 32%) among all dietary treatments. Fish fed the low protein diet (35%) had the lowest MUFA percentages in both muscle and liver, due to the low level of 18:1 (oleic acid), and higher proportions of linoleic (18:2*n*-6) and linolenic (18:3*n*-3) acids, resulting from the higher content of these FAs in this diet. Muscle *n*-3 PUFA fraction was unaffected by dietary treatment while in liver a significantly increase was observed in fish fed 35 GS diet, mainly due to the higher percentages of 22:5*n*-3 and 22:6*n*-3 FAs. The higher muscle and liver *n*-6 FA contents found in fish fed the 35 GS diet consequently had a lower *n*-3/*n*-6 FA ratio.

Table 6. Muscle total lipid content (% wet weight, WW, or % dry weight, DW) and fatty acid composition (g/100 g total fatty acid) of blackspot seabream fed the different dietary treatments.

	Diet			Main effects ANOVA *P*-value*	
	CS	GS	GS		
	45		35	ST	PL
Fatty acid (FA)					
10:0	1.7±0.5	1.8±0.4	1.8±0.8	0.596	0.840
14:0	3.2±0.4	3.1±0.3	3.2±0.4	0.490	0.761
16:0	19.7±0.6	18.8±0.5	18.4±1.8	0.132	0.442
17:0	0.4±0.04	0.4±0.02	0.5±0.1	0.443	0.001
18:0	6.4±0.6	6.4±0.4	6.1±0.3	0.692	0.179
20:0	0.3±0.04	0.2±0.02	0.3±0.04	0.707	0.218
ΣSaturates	31.9±1.1	31.1±0.7	31.0±2.3	0.304	0.821
16:1	4.5±0.5	4.5±0.3	4.3±0.3	0.987	0.226
17:1	0.3±0.1	0.3±0.02	0.3±0.03	0.693	0.867
18:1	19.3±2.0	20.6±1.3	16.8±1.5	0.119	0.0001
20:1	2.5±0.3	2.6±0.2	2.7±0.2	0.166	0.626
22:1	2.2±0.3	2.1±0.3	2.4±0.3	0.854	0.079
ΣMUFA	28.9±0.8	30.3±1.5	26.5±2.0	0.211	0.002
14 PUFA	0.2±0.2	0.1±0.02	0.2±0.1	0.100	0.436
16:2 *n*-4	0.3±0.04	0.3±0.04	0.3±0.03	0.683	0.670
16:3 *n*-4	0.3±0.1	0.3±0.04	0.3±0.03	0.639	0.632
16:4 *n*-1	0.4±0.1	0.4±0.1	0.4±0.1	0.655	0.619
18:2 *n*-6	4.5±0.4	4.7±0.2	7.3±0.3	0.369	0.000
18:3 *n*-6	0.2±0.02	0.2±0.01	0.2±0.01	0.672	0.004
20:2 *n*-6	0.3±0.02	0.3±0.03	0.4±0.03	0.912	0.000
20:3 *n*-6	0.2±0.1	0.3±0.04	0.2±0.1	0.173	0.030
20:4 *n*-6	0.8±0.1	0.8±0.1	0.8±0.1	0.878	0.425
Σ*n*-6	6.1±0.5	6.2±0.3	8.9±0.3	0.444	0.000
18:3 *n*-3	0.7±0.1	0.7±0.03	0.9±0.1	0.814	0.000
18:4 *n*-3	0.8±0.1	0.9±0.1	0.9±0.1	0.529	0.300
20:4 *n*-3	0.76±0.1	0.8±0.04	0.8±0.1	0.767	0.458
20:5 *n*-3	7.3±0.6	7.3±0.4	7.3±0.5	0.748	0.971
21:5 *n*-3	0.3±0.03	0.3±0.02	0.3±0.04	0.843	0.511
22:5 *n*-3	2.7±0.3	2.7±0.2	2.7±0.2	0.722	0.883
22:6 *n*-3	14.0±2.0	13.8±0.8	14.8±1.7	0.728	0.192
Σ*n*-3	26.6±2.7	26.4±1.3	27.8±2.2	0.843	0.224
PUFA	33.9±3.2	33.6±1.7	37.8±2.3	0.810	0.003
Sat/PUFA	1.0±0.1	0.9±0.1	0.8±0.1	0.670	0.029
n3/n6	4.4±0.3	4.3±0.2	3.1±0.2	0.217	0.000
Muscle total lipid					
% (WW)	4.0±1.0	3.8±0.72	3.5±0.6	0.682	0.413
% (DW)	16.1±3.4	15.4±2.5	14.7±2.4	0.595	0.645

Mean values and standard deviations (±SD) are presented for each parameter (n=9)
*P-value for starch type (ST) and protein level (PL) main effects

Table 7. Liver total lipid content (% wet weight, WW, or % dry weight, DW) and fatty acid composition (g/100 g total fatty acid) of blackspot seabream fed the different dietary treatments

	Diet			Main effects ANOVA *P*-value*	
	CS	GS	GS		
	45		35	ST	PL
Fatty acid (FA)					
10:0	1.1±0.1	1.1±0.6	1.6±0.3	0.855	0.153
14:0	2.6±0.3	2.5±0.3	1.7±0.1	0.772	0.009
16:0	19.7±1.2	19.3±1.4	15.7±0.9	0.746	0.011
17:0	0.3±0.03	0.3±0.03	0.4±0.04	0.560	0.002
18:0	10.9±0.9	10.1±0.7	9.7±0.5	0.237	0.530
20:0	0.3±0.01	0.3±0.04	0.3±0.01	0.800	0.551
ΣSaturates	35.0±1.4	33.6±2.7	29.6±1.4	0.424	0.043
16:1	3.4±0.2	3.4±0.2	3.1±0.2	0.950	0.098
17:1	0.3±0.03	0.3±0.1	0.3±0.04	0.444	0.4260
18:1	31.2±1.8	33.2±0.4	25.5±2.5	0.209	0.002
20:1	3.4±0.3	4.0±0.5	4.4±0.1	0.095	0.126
22:1	1.8±0.2	2.0±0.2	2.8±0.3	0.472	0.006
ΣMUFA	40.0±2.3	42.7±0.8	36.2±2.9	0.176	0.010
14 PUFA	0.2±0.1	0.3±0.1	0.3±0.1	0.413	0.832
16:2 *n*-4	0.1±0.01	0.1±0.01	0.1±0.01	0.026	0.184
16:3 *n*-4	0.1±0.02	0.1±0.01	0.1±0.1	0.015	0.199
16:4 *n*-1	0.1±0.01	0.1±0.01	0.1±0.00	0.001	0.568
18:2 *n*-6	2.4±0.4	2.4±0.4	5.4±0.3	0.865	0.0001
18:3 *n*-6	0.1±0.04	0.1±0.01	0.2±0.1	0.748	0.038
20:2 *n*-6	0.4±0.1	0.5±0.1	1.1±0.1	0.298	0.0001
20:3 *n*-6	0.3±0.1	0.3±0.1	0.5±0.1	0.782	0.108
20:4 *n*-6	0.6±0.1	0.5±0.04	0.6±0.1	0.373	0.103
Σ*n*-6	3.9±0.6	3.9±0.7	8.0±0.5	0.959	0.0001
18:3 *n*-3	0.3±0.04	0.3±0.1	0.5±0.02	0.427	0.001
18:4 *n*-3	0.3±0.03	0.2±0.03	0.2±0.02	0.042	0.166
20:3 *n*-3	0.1±0.01	0.1±0.1	0.2±0.01	0.521	0.009
20:4 *n*-3	0.7±0.1	0.6±0.1	1.0±0.1	0.789	0.003
20:5 *n*-3	3.2±0.3	2.6±0.1	3.2±0.4	0.056	0.070
21:5 *n*-3	0.2±0.1	0.2±0.02	0.3±0.01	0.951	0.007
22:5 *n*-3	2.4±0.5	2.4±0.3	4.4±0.4	0.880	0.001
22:6 *n*-3	7.8±0.9	6.7±0.3	9.5±1.4	0.221	0.013
Σ*n*-3	14.9±1.7	13.1±0.3	19.3±2.2	0.228	0.004
PUFA	19.3±2.2	17.5±1.1	27.8±2.7	0.336	0.001
Sat/PUFA	1.8±0.2	1.9±0.3	1.1±0.1	0.581	0.003
***n*3/*n*6**	3.9±0.5	3.4±0.5	2.4±0.2	0.259	0.022
Liver total lipid					
% (WW)	15.8±1.3	17.2±2.1	15.9±4.1	0.567	0.592
% (DW)	44.2±2.7	44.5±3.22	43.2±8.6	0.947	0.782

Mean values and standard deviations (±SD) are presented for each parameter (*n*=9)
*P-value for starch type (ST) and protein level (PL) main effects

Table 8. Effects of different dietary treatments on blackspot seabream hepatic lipogenic enzymes activities

	Diet			Main effects ANOVA *P*-value*	
	CS	GS	GS		
	45		35	ST	PL
ME					
IU / g liver	2.0±1.2	3.1±0.9	1.1±0.65	0.056	0.0003
mIU / mg protein	23.9±14.8	33.9±9.1	14.1±7.5	0.081	0.002
G6PD					
IU / g liver	12.4±1.8	12.0±2.12	7.4±1.9	0.652	0.0001
mIU / mg protein	114.1±18.8	124.8±26.4	82.9±18.3	0.336	0.001
FAS					
IU / g liver	2.7±1.0	2.8±0.5	1.3±0.6	0.780	0.0004
mIU / mg protein	25.2±9.7	29.7±7.3	14.6±6.0	0.270	0.001

Mean values and standard deviations (±SD) are presented for each parameter (*n*=9)
*P-value for starch type (ST) and protein level (PL) main effects

Hepatic lipogenic enzyme activities are presented in Table 8. Malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PD) and fatty acid synthetase (FAS) were unaffected by starch type, whereas the reduction of 10% on dietary protein level (45 to 35%) markedly depressed (> 40%) the activities of those enzymes.

Glycolytic and gluconeogenic hepatic enzyme activities remained almost constant up to 2 h after feeding which represent the control values. No effect of starch type (CS vs GS) was observed on the activities of glycolytic [hexokinase (HK), glucokinase (GK), pyruvate kinase (PK)] or gluconeogenic [glucose-6-phosphatase (G6Pase)] hepatic enzymes regardless of the time after feeding (2, 4 and 24 h) (Table 9). Nevertheless, the reduction of 10% dietary protein level (45 to 35%) significantly increased PK activities at each postprandial time. In addition, GK was also affected by protein level at 4 and 24 h after feeding, being stimulated at 4 h and depressed at 24 h by the low-protein diet.

Glucose, cholesterol (CHOL) and triacylglycerol (TAG) plasma levels varied significantly with time after feeding (Table 10). Although insulin and cortisol followed the same trend (Fig. 2), in fish fed the 35 GS diet, insulin and cortisol, as well as TAG, showed less postprandial differences. Glucose levels were highest 4 h after feeding and no significant difference was observed with regard to the diet fed, even though there was a slight tendency for glucose to increase in fish fed the GS diet. Dietary starch type did not

induced any differences in CHOL or TAG postprandial levels but significantly affected insulin levels at 2 h and cortisol levels at 24 h after feeding (P<0.05) (Fig. 2). On the other hand, the decrease in dietary protein level (45 vs 35%) negatively affected insulin (P=0.024) and TAG levels 2 and 4 h after feeding, respectively. In addition, cortisol increased in this same group of fish at each postprandial time analysed (P<0.05).

Table 9. Postprandial hepatic glycolytic (HK, GK, PK) and gluconeogenic (G6Pase) enzymes specific activity (mIU/mg protein) in blackspot seabream fed the different dietary treatments

		Diet			Main effects ANOVA *P*-value*	
		CS	GS	GS		
Time (h)	Enzyme	45		35	ST	PL
2	HK	0.2±0.2	0.3±0.1	0.2±0.2	0.452	0.293
	GK	0.9±0.5	1.1±0.7	1.1±0.5 †‡	0.449	0.931
	PK	153.8±32.6	185.7±38.6	367.3±83.8 †	0.304	0.00001
	G6Pase	11.3±2.7 †‡	11.2±3.2	12.4±2.4	0.328	0.977
4	HK	0.4±0.2	0.2±0.1	0.1±0.1	0.045	0.443
	GK	0.9±0.3	0.8±0.55	1.5±0.8†	0.814	0.036
	PK	148.1±20.0	161.3±44.8	243.6±87.0 ‡	0.673	0.016
	G6Pase	8.5±2.1 ‡	10.7±3.5	11.3±1.0	0.146	0.673
24	HK	0.4±0.1	0.4±0.2	0.4±0.4	0.959	0.966
	GK	0.7±0.3	1.1±0.4	0.5±0.3 ‡	0.140	0.011
	PK	166.1±40.8	191.7±62.1	327.1±42.2 †‡	0.391	0.0001
	G6Pase	13.4±3.6 †	11.6±3.3	11.6±2.1	0.954	0.493
Anova P-value	HK	0.175	0.311	0.337		
	GK	0.680	0.589	0.008		
	PK	0.617	0.488	0.018		
	G6Pase	0.033	0.905	0.623		

Mean values and standard deviations (±SD) are presented for each parameter (*n*=6)
*P-value for starch type (ST) and protein level (PL) main effects
†‡ Mean values within a column unlike superscript symbols indicates significantly differences between time within diets (*P*<0.05).

Discussion

The ADCs for protein, lipid and energy were slightly lower than those previously observed in blackspot seabream juveniles (Silva *et al.* 2006; Figueiredo-Silva *et al.* 2009a). In addition, in the present experiment, high starch ADCs were obtained for all diets fed, suggesting a good capacity of blackspot seabream to digest carbohydrate. Even at such

high digestibility coefficients, corn starch gelatinization was shown to additionally improve starch ADC (97 to 98-99%).

Table 10. Postprandial plasmatic glucose (mg/dl), total cholesterol and triacylglycerol (g/l) in blackspot seabream fed different dietary treatments

		Diet			Main effects ANOVA *P*-value*	
		CS	GS	GS		
		45		35	ST	PL
Time (h)	**Plasma metabolite**					
2	Glucose	60.9±11.4 ‡	61.9±7.8 ‡	62.6±2.9 ‡	0.788	0.999
	Cholesterol	2.0±0.3 ‡	1.9±0.6 ‡	2.3±0.6 ‡	0.907	0.258
	Triacylglycerol	2.6±0.58 ‡	3.2±1.2 ‡	3.4±0.7	0.176	0.903
4	Glucose	74.4±14.2 †	90.6±27.4 †	89.8±27.2 †	0.094	0.949
	Cholesterol	2.9±0.4 †	2.8±0.5 †	2.4±0.5 †‡	0.286	0.530
	Triacylglycerol	4.2±1.0 †	5.2±0.9 †	3.5±0.6	0.276	0.024
24	Glucose	52.0±8.2 ‡	61.0±20.5 ‡	55.0±15.5 ‡	0.502	0.168
	Cholesterol	2.8±0.3 †	2.9±0.7 †	3.1±0.6 †	0.377	0.581
	Triacylglycerol	3.2±0.7 ‡	3.2±0.8 ‡	3.2±1.1	0.441	0.934
Anova P-value	Glucose	0.0005	0.009	0.001		
	Cholesterol	0.000005	0.001	0.008		
	Triacylglycerol	0.0001	0.0005	0.803		

Mean values and standard deviations (±SD) are presented for each parameter (n=12)
*P-value for starch type (ST) and protein level (PL) main effects
†‡ Mean values within a column unlike superscript symbols indicates significantly differences between time within diets(P<0.05).

This high capacity to digest carbohydrate has previously been reported for blackspot seabream (Figueiredo-Silva *et al.* 2009a), as well as for other carnivorous species such as European sea bass (Enes *et al.* 2006a; Moreira *et al.* 2008) and gilthead seabream (Enes *et al.* 2008a). However, in European sea bass (Enes *et al.* 2006a) and gilthead seabream (Enes *et al.* 2008a) starch ADCs were shown to be independent of either dietary starch type (native vs waxy starch) or level (10 to 20%). The reduction of 10% in dietary protein level clearly depressed protein, lipid and starch ADCs. It should be noted that the reduction in dietary protein level (45 to 35%) was achieved through the addition of wheat bran at the expense of fish meal. Indigestible carbohydrate has been reported to reduce lipid digestibility by inhibiting micelle formation and lipid solubilisation (Levrat *et al.* 1996). The lower digestibility of wheat bran together with the high feed intake of fish fed the 35 GS diet, probably increased gastrointestinal evacuation time, resulting in reduced nutrient ADC values.

In fish, feed intake is regulated by the digestible energy content of the diet (Kaushik & Oliva-Teles 1985; Boujard & Médale 1994; Paspatis & Boujard 1996). In the present study, although the dietary DE was similar among the diets (16 kJ g^{-1} DM), lower voluntary feed intake (VFI) was observed in fish fed diets containing gelatinized starch (GS) compared with those fed diets containing crude starch (CS) as has been previously observed in rainbow trout (Kaushik & Oliva-Teles 1985) and European sea bass (Peres & Oliva-Teles 2002) fed diets with similar GS content (25 to 30%). This decrease in VFI observed in fish fed the 25% GS diet resulted in significantly reduced final body weigh (FBW) and daily growth index (DGI), but improved protein efficiency ratio (PER) compared to fish fed the CS diet.

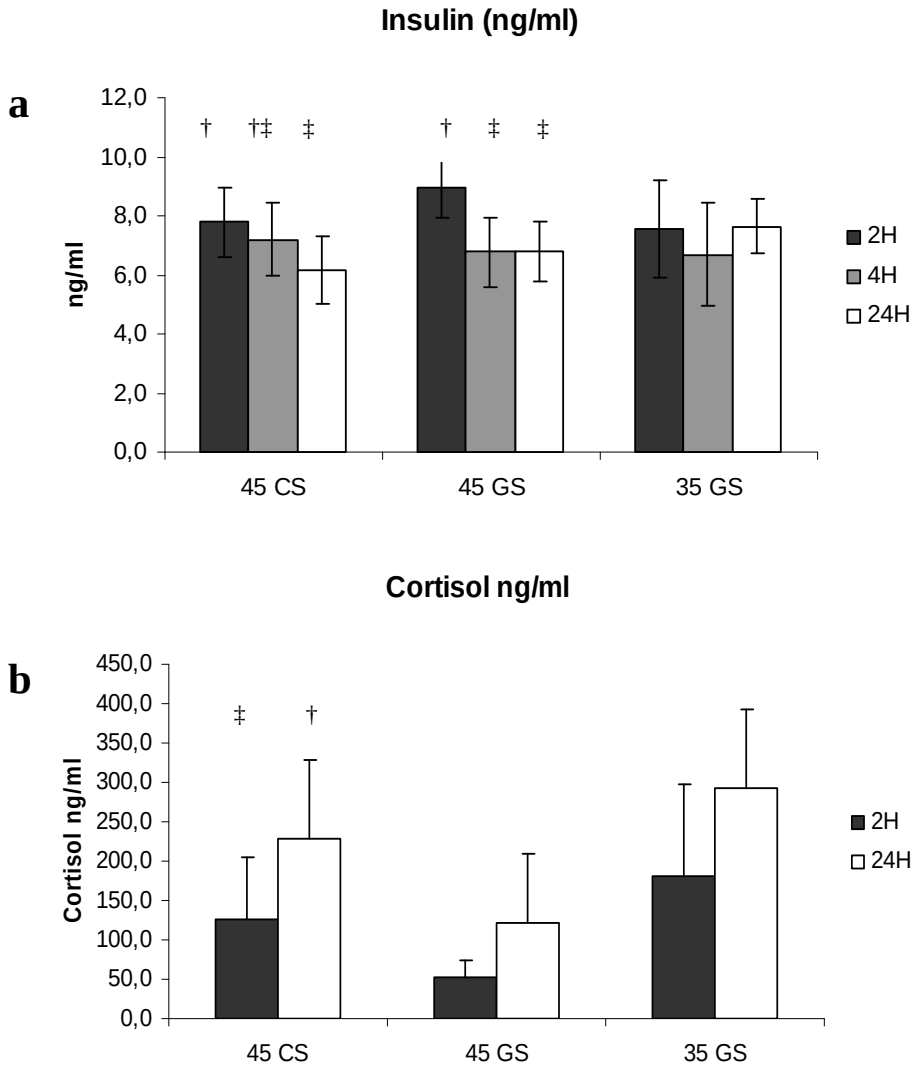

Figure 2. Dietary treatment effect on blackspot seabream postprandial plasmatic a) insulin and b) cortisol levels (ng / ml), *n*=12.
†,‡ superscript symbols indicate significantly differences between times within dietary treatments. (*P*<0.05).

This apparent negative effect of GS on growth may be due to an excess of dietary digestible carbohydrate (25%) because a maximum level of 20% dietary carbohydrate has, in general, been suggested for marine fish species (Wilson 1994). Although substantial growth improvement has been reported with diets containing 20 to 30% digestible starch in several fish species (Baños *et al.* 1998; Dias *et al.* 1998; Alvarez *et al.* 1999; Venou *et al.* 2003; Fernández *et al.* 2007), neither the level nor type of starch affected the growth performance of Senegalese sole (Dias *et al.* 2004), European sea bass (Enes *et al.* 2006a) or gilthead seabream (Enes *et al.* 2008a). This discrepancy among results concerning starch type affects on fish growth may be due to various factors affecting carbohydrate utilization such as the fish species, dietary carbohydrate level and digestibility (Wilson 1994; Hemre *et al.* 2002; Stone 2003). Thus, further studies are required to define the optimal dietary level of digestible carbohydrate for blackspot seabream.

The reduction of 10% dietary protein level (45 to 35%) markedly depressed blackspot seabream FBW and DGI, indicates that dietary incorporation of 25% digestible starch in a low-protein level (35%) was not adequate to meet this species energy and/or nutrient requirements. Indeed, the 35 GS diet had a lower DP (24.7) and subsequently a lower DP/DE ratio (16 mg kJ^{-1}) than the other test diets (22-23 mg kJ^{-1}) which probably tended to increase dry matter, lipid and energy intake. In spite of the tendency for the 35 GS diet to increase VFI, daily protein intake was less than the values observed for the high-protein diets (45%), which associated with the poorer feed conversion ratio (FCR: 1.8), resulted in lower growth performance in those fish.

Blackspot seabream nutrient gain and hence whole body composition (18% protein and 13% lipid, % ww), were not affected by corn starch type (CS vs GS) or dietary protein level (45 vs 35%). Our results are in agreement with previous observations in rainbow trout (Alvarez *et al.* 1999), European sea bass (Enes *et al.* 2006a) and gilthead seabream (Peres & Oliva-Teles 2002; Enes *et al.* 2008a) where no effect of dietary carbohydrate type on whole body composition was observed. Increasing dietary digestible energy coming from digestible carbohydrate has been associated with higher whole body lipid content in several studies (Kaushik & Oliva-Teles 1985; Grisdale-Helland & Helland 1997; Dias *et al.* 1998; Venou *et al.* 2003; Fernández *et al.* 2007). In the present work, the additional DE coming from dietary digestible carbohydrate incorporation was negligible due to the elevated starch ADCs obtained, resulting in similar final whole body composition.

Moreover, as has previously been reported for others species (Dias *et al.* 1998; Alvarez *et al.* 1999; Peres & Oliva-Teles 2002), digestible carbohydrate diets have contributed to the reduction of nitrogen losses and thus the enhancement of protein retention, when expressed as % digestible intake (31 vs 35%; data not presented). Even without any effect on final whole body lipid content, decreased lipid (90-101 to 70%) and a tendency for decreased energy retention (28-29 to 22%) was observed with the reduction of 10% dietary protein (45 to 35%), indicating a clear effect of dietary protein level on blackspot seabream lipid metabolism.

Viscerosomatic index (VSI) was unaffected by the degree of starch gelatinization while the hepatosomatic index (HSI) was significantly increased in fish fed the high digestible starch diet containing the lower protein level (35 GS). Indeed, feeding high digestible carbohydrate diets are known to result in increased liver glycogen content and consequently higher HSI (Peres & Oliva-Teles 2002; Novoa *et al.* 2004; Enes *et al.* 2006a, 2008a). Furthermore, glucose coming from high digestible carbohydrate diets has been shown to up-regulate hepatic lipogenesis and consequently enhance liver lipid content (Brauge *et al.* 1995; Dias *et al.* 1998; Fernández *et al.* 2007). Considering the similarity among the liver lipid content in the current study regardless of the diet fed, indicates that the higher HSI in fish fed the 35 GS diet probably resulted from increased liver glycogen content.

In both, muscle and liver, FA percentages were essentially unaffected by starch type but strongly affected by dietary protein level. Moreover, the dietary treatments significantly affected the saturated FA fraction in liver, but not in muscle. Since FA synthesis mainly occurs in the liver, it was expected that any dietary effects on lipid metabolism should be revealed to a greater extend in liver rather than in muscle tissue. In fish, the main newly-synthesized FAs should be palmitate (16:0), stearate (18:0) and myristate (14:0) acid (Corraze 2001; Sargent *et al.* 2002). In blackspot seabream, 14:0 and 16:0 FA levels were significantly higher in the liver of fish fed the high-protein diets, indicating an important stimulation of *de novo* synthesis, particularly for 16:0. This was corroborated by the elevated lipid retention and FAS activity found in this group compared to those fed the low-protein diet. Blackspot seabream fed the 35 GS diet had the lowest MUFA percentages, in both tissues, mainly due to the lower level of 18:1 (oleic acid). MUFA percentages were considerably higher in liver than in muscle regardless of the diet fed, probably due a higher bioconversion of saturated to mono-unsaturated FA, in the liver. Higher linoleic acid (18:2*n*-6) and subsequently *n*-6 PUFA percentages were found in muscle and liver of fish fed the 35 GS diet reflecting the higher content of these FAs in the

35 GS diet, due to the increase in wheat bran content (15 to 30%). Muscle *n*-3 PUFA contents were similar among the different dietary treatments, while in liver a significant increase in *n*-3 PUFA content was observed in fish fed the 35 GS diet due to higher 22:5 *n*-3 and 22:6 *n*-3 (DHA) levels**.** These higher muscle *n*-6 and liver *n*-3 and n-6 PUFA percentages, found in fish fed the 35 GS diet, resulted in higher PUFA tissue content but lower liver *n*-3/*n*-6 ratio.

Lipogenic enzyme activities observed in the present work correspond to those previously found on blackspot seabream (Figueiredo-Silva *et al.* 2009a). Glucose-6-phosphate dehydrogenase (G6PD) was about 5 times higher than that of malic enzyme (ME) suggesting that NADPH reducing equivalents required for lipogenesis are mainly provided by G6PD, as reported for other species (Hung & Storebakken 1994; Dias *et al.* 1998, 2004). Diets containing CS or GS, at the required protein level (45%), were fed to blackspot seabream in an attempt to understand the effect of carbohydrate on the lipogenic potential in this species. However, hepatic ME, G6PD and fatty acid synthetase (FAS) were unaffected by starch type, corroborating the extremely high but similar digestible starch intake of the fish, irrespectively of dietary starch type (CS vs GS). Similarly, in Senegalese sole, ME and FAS activities have been found to be little affected by changes in the nature of dietary carbohydrates (Dias *et al.* 2004), whereas the level of carbohydrate was shown to affect the lipogenic enzyme activities in other fish species (Likimani & Wilson 1982; Shimeno *et al.* 1993; Hung & Storebakken 1994; Dias *et al.* 1998; Fernández *et al.* 2007).

The pentose phosphate pathway is usually stimulated together with glycolysis in fish fed high carbohydrate/low-protein diets (Metón *et al.* 1999; Dias *et al.* 2004; Fernández *et al.* 2007) indicating an ability to utilize carbohydrate and thus spare protein. However, in the present work, G6PD was not stimulated in fish fed the high GS diet compared with those fed the CS diet. This may be related to the lower dietary DP/DE ratio and/or glucose availability to be derived as a substrate for pentose phosphate pathway.

Dietary protein *per si* is recognized as a potent regulator of lipid biosynthesis in higher vertebrates (Herzberg & Rogerson 1981; Rosebrough *et al.* 1996) as well as in fish (Henderson & Sargent 1981; Shikata & Shimeno 1997; Alvarez *et al.* 1999; Dias *et al.* 1998, 2003). Increased levels of dietary protein and available carbohydrate favoured rainbow trout lipogenesis and leaded to an increase in saturated FA synthesis (Alvarez *et al.* 1999). In blackspot seabream, the reduction of 10% dietary protein level (45 to 35%) markedly depressed (> 40%) the lipogenic enzyme activities. High-protein diets stimulated

lipogenesis which resulted in increased hepatic synthesized FA (mainly 16:0) content and higher lipid retention. Considering all this, the nutritional regulation of lipogenesis in blackspot seabream appears to be more related to dietary protein level and apparently not to starch type.

Blackspot seabream hexokinase (HK) and glucokinase (GK) activities were generally lower than the activities found in gilthead seabream (Panserat *et al.* 2000a; Enes *et al.* 2008a,b) and rainbow trout (Panserat *et al.* 2000a; Capilla *et al.* 2003; Kirchner *et al.* 2003a,b), but comparable to those previously observed in common carp (Panserat *et al.* 2000a; Capilla *et al.* 2004) and European sea bass (Enes *et al.* 2006a,b). On the other hand, pyruvate kinase (PK) activities were well within the range of those found in gilthead seabream (Metón *et al.* 1999, 2003; Fernández *et al.* 2007), showing a species-dependent regulation.

Hepatic GK activity has been shown to be stimulated by dietary carbohydrate (Panserat *et al.* 2000a; Capilla *et al.* 2003; Enes *et al.* 2008a,b), whereas HK seems to lack any nutritional regulation (Panserat *et al.* 2000a; Capilla *et al.* 2004; Enes *et al.* 2006a,b, 2008a,b). Starch type did not caused any affect on blackspot seabream postprandial HK, GK, PK (glycolysis) or glucose-6-phosphatase (G6Pase) (gluconeogenesis) hepatic activities, following the same pattern as the postprandial plasma glucose levels. There were no noticeable postprandial changes in most of the glycolytic or gluconeogenic hepatic activities. Nevertheless, PK activity varied significantly among postprandial times in fish fed the low-protein diet, but was not correlated with plasma glucose level. Moreover, the 10% reduction in dietary protein positively inducted PK activity and subsequently pyruvate production, at each postprandial time. Similarly, Kirchner *et al.* (2003b) previously reported a strong induction of hepatic PK activity in rainbow trout fed low dietary protein levels (28%). In fish, amino acids rather than glucose are preferentially used for energy proposes (Médale & Guillaume 2001). It seems, therefore, that the increased glycolysis observed in blackspot seabream fed a low-protein diet, and hence low DP/DE ratio, was a way of meeting this species energy demands. In spite of the increased PK activity, a gluconeogenesis (G6Pase) reduction was not verified. Contrarily, Kirchner *et al.* (2003b) observed a significant decrease on hepatic fructose-1,6-biphosphatase (FBPase) and G6Pase mRNA levels and activities in rainbow trout. However, this decrease was attained at a lower protein intake level (2.6 g/kg ABW/day) than that used in the present work. Moreover, recent studies have found that high dietary glucose supply alone was insufficient for depressing G6Pase activity (Panserat *et al.*

2001; Enes *et al.* 2006a,b, 2008a,b), and hence dietary gluconeogenic amino acids intake could be a limiting factor for hepatic glucose production (Kirchner *et al.* 2003a,b, 2005).

Glucose, cholesterol and triacylglycerols (TAG) levels were within the levels previously reported for this fish species (Figueiredo-Silva *et al.* 2009a) and were generally enhanced after a meal. In spite of a slight tendency for a glucose increase, plasma lipid levels were not significantly affected by starch type (CS vs GS). As previously discussed, the similar digestible carbohydrate content among the diets is probably the cause of these results. Fish fed the low-protein diet had, however, decreased TAG levels 4 h after feeding as has been observed in rainbow trout (Kirchner *et al.* 2003a,b).

Dietary starch type only significantly affected insulin levels 2 h after feeding. Capilla *et al.* (2003) reported similar postprandial insulin levels in rainbow trout fed different carbohydrate sources and levels, whereas in other species dietary carbohydrate levels stimulated postprandial insulin levels (Baños *et al.* 1998; Novoa *et al.* 2004). In fish fed the low-protein diet, insulin levels were constant over time, and significantly lower than those fed the high-protein diet at 2 h post feeding. This result together with the fact that the highest insulin levels occurred before the glucose load (2 vs 4 h), suggests that although glucose stimulates insulin release, it is not the main insulin secretagogue in blackspot seabream, at least under the present nutritional conditions. Glucose can initiate and maintain insulin output from fish Brockmann bodies (BBs), but other compounds, especially amino acids, tend to be much stronger secretagogues than glucose (Hemre *et al.* 2002; Navarro *et al.* 2002). In addition, the absence of noticeable postprandial changes in plasma insulin levels together with the strong increase in PK activities found in fish fed the low-protein diet, suggests that insulin could be involved but not be the major key of PK blackspot seabream regulation.

Plasma cortisol has been used as an important stress indicator (Mommsen *et al.* 1999; Barton 2002). Handling stress has been demonstrated to elevate blood cortisol, and ultimately blood glucose levels in fish (Pankhurst *et al.* 2008), but the present data does not support such a conclusion. The high cortisol levels observed with low-protein diet seems to relate with other factors rather than stress. Previous workers have suggested that cortisol mobilizes peripheral protein (Vijayan *et al.* 1993; Vijayan & Moon 1994) and lipid (Vijayan *et al.* 1991) for energy purposes. Considering this, PK together with increased cortisol levels seems to be a metabolic response to the low DP/DE content in low-protein diet rather than to a stress response *per si*.

Conclusion

In conclusion, the high starch ADCs of all diets failed to demonstrate a clear lipogenic potential in blackspot seabream hepatic metabolism. This work showed that regulation the lipogenic and glycolytic pathways is related more to dietary protein level than starch type. Further studies on the regulation of lipid metabolism by dietary protein and/or amino acid profile are warrant as they seem to be the major factors on species lipid storage capacity and retention.

CHAPTER 4

Modulation of blackspot seabream (*Pagellus bogaraveo*) intermediary metabolic pathways by dispensable amino acids.

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Abstract

The objective of the present work is to investigate the main metabolic routes by which dispensable amino acids (DAA) are diverted towards lipid formation in blackspot seabream. For that purpose, a control diet was formulated to contain 45% of protein (7.2 g N) mainly supplied by fish meal (45P). In two other diets, 22.2% of the dietary nitrogen (1.6 g N) was replaced by an equivalent amount of nitrogen provided by two different mixtures of DAA: alanine and serine (diet AS) or aspartic and glutamic acid (diet AG). A fourth diet (diet 35P) only containing 35% of protein (5.6 g N) was included in order to analyse the possible additive effects of DAA. Each diet was distributed by hand to apparent satiety to triplicate groups of fish (12 g initial body weight), three times a day over 80 days. Blackspot seabream juveniles have responded well to dietary crystalline supplementation showing the different role of DAA, alanine and serine (pyruvate precursors), and aspartic and glutamic acids (TCA intermediates), in terms of growth and intermediary metabolic pathways. Blackspot seabream juveniles appear to make an efficient use of the 1.6 g N provided by alanine and serine than that provided by aspartic and glutamic acids mix in terms of growth. Surplus dietary alanine and serine content appear to play a preponderant role in lipogenesis while aspartic and glutamic acids mix have in fact the ability to reduce the specific activity of fatty acid synthetase (FAS). Both, dietary nitrogen reduction (45P vs 35P) or its replacement by aspartic and glutamic acids mix (diet AG) shown to up-regulate PEPCK but without increasing plasma glucose levels. Dietary nitrogen level and nature seems to exert a complex regulation on energetic pathways trough the gluconeogenesis/TCA interaction. This study evidenced different roles of DAA on fish metabolic pathways showing that besides IAA/DAA ratio also DAA must be taken into attention when fish meal is being replaced by plant protein sources.

Keywords: Blackspot seabream; Dietary nitrogen replacement, DAA, Intermediary metabolism

Introduction

The dietary protein requirements for maximum growth of fish are higher than those of other terrestrial vertebrates (NRC 1993; Wilson 2002), with a major part of this nitrogen being used for energy supply (Cho & Kaushik 1985). Given the current constrains on fish meal availability and cost, much effort is devoted to the evaluation of alternative protein sources in aquaculture feeds (Gatlin *et al.* 2007; Kaushik & Hemre 2008; Tacon & Metian 2008). Although the importance of indispensable amino acids (IAA) profiles (Wilson 2002) and the IAA/dispensable amino acids (DAA) ratio (Akiyama *et al.* 1997; Green *et al.* 2002; Gómez-Requini *et al.* 2003; Rollin *et al.* 2003; Peres & Oliva-Teles 2006; Silva *et al*. 2009) have been well studied, DAA have received much less attention in fish nutrition. The specific effects of DAA to overall protein nutrition have been emphasized since long in mammals (Harper 1974; Jackson 1983; Laidlaw & Kopple 1987) and also in fish (Hughes 1985; Fauconneau 1988; Mambrini & Kaushik 1994; Schuhmacher *et al.* 1995). Diets completely lacking DAA leads to reduced growth rate in rats, chicks and fish (Frost & Sandy 1951; Stucki & Harper 1961; 1962; Allen & Baker 1974; Shuhmacher *et al.* 1995). DAA, such as alanine, serine, aspartic or glutamic acids, seem to have a sparing effect on the use of IAA (Cowey & Sargent 1979; Ronnestad *et al.* 2001; Abboudi *et al.* 2009). Hence, emerging developments in amino acid (AA) nutrition suggests that the traditional indispensable or dispensable AA classification should be revaluated in mammals (Reeds 2000) and fish (Li *et al.* 2009).

We have recently shown that lipid retention was high in blackspot seabream (*Pagellus bogaraveo*) at low dietary lipid levels (10%), suggesting the conversion of nutrients other than lipid (protein and/or carbohydrates) into body fat (Figueiredo-Silva *et al.* 2009a). We also demonstrated that in this species, lipogenic and glycolytic regulation appears to be more related to dietary protein level than to dietary starch type (Figueiredo-Silva *et al.* 2009b). In addition, the protein source strongly affected lipogenesis in this species, with excessive plant protein leading to up-regulation of fatty acid synthesis *de novo* and to high lipid retention (Figueiredo-Silva *et al.* 2009a). Thus, the dietary protein level and source seems to be a major key issue on lipid metabolism in this species. When too much protein and therefore AA is supplied in the diet, only part of it will be used to make new proteins, and the remainder will be deaminated and converted into energy compounds like fat or glucose (Wilson 2002). The modification of body lipid content as well as lipogenesis by protein nature has been demonstrated in other teleosts (Goméz-Requeni *et al.* 2003; Kaushik *et al.* 2004; Dias *et al.* 2005) as in higher vertebrates (Iritani *et al.* 1986, 1996;

Kayashita *et al.* 1996; Padmakumarannair *et al.* 1998). The mechanisms underlying the modulation of fatty acid synthesis *de novo* by protein/ AA source are ill-defined, but dietary AA composition has been cited as one of the majors factors (Herzberg 1991). In fish, the incorporation of ^{14}C-alanine or ^{14}C-glutamate into triacylglycerols was shown to be much higher than that coming from ^{14}C-glucose (Nagai & Ikeda 1972; Henderson & Sargent 1981; Shikata & Shimeno 1997). However, the knowledge on the dietary DAA involvement on intermediary metabolic pathways in fish is scarce and mainly devoted to glucose metabolic pathways. Since some DAA constitute important substrates for endogenous glucose synthesis (Moon & Foster 1995), a high level of dietary DAA was hypothesized as a possible explanation for the prolonged postprandial hyperglycaemia observed in rainbow trout. However, Kirchner *et al.* (2003a) failed to show any effect of a dietary DAA surplus on hepatic glucose metabolism in rainbow trout.

Recognizing the propensity of blackspot seabream to turn protein into lipid deposits, we hypothesized that dietary DAA content will up-regulate the lipogenic potential in this species. Our objective was also to investigate the main metabolic routes by which DAA are diverted into lipid formation. For that propose, dietary nitrogen was partially replaced (22%) by two different mixtures of DAA: alanine and serine (pyruvate precursors) or aspartic and glutamic acids (intermediates of the tricarboxylic cycle, TCA).

Material and methods

Diets

The basal diet was formulated to contain 45% of protein (7.2 g N) supplied by fish meal (48%), CPSP G (5%) and wheat gluten (2.5%) (diet 45P). In two other diets, 22.2% of dietary nitrogen (1.6 g N) was replaced by an equivalent amount of nitrogen provided by two different mixtures of DAA: alanine and serine (diet AS) or aspartic and glutamic acid (diet AG). DAA nitrogen was added to the diets at the expense of fish meal nitrogen and the proportions between the two DAA of each mixture kept similar to that found in fish meal. A fourth diet (diet 35P) containing only 35% of protein (5.6 g N) was also included to determine if any growth/physiologic effect detected was due to nitrogen replacement and not due to shortage of dietary fish meal nitrogen supply (e.g., IAA). The four diets were formulated to contain similar energy content (20 kJ g^{-1} DM). All ingredients were supplied by Sorgal S.A. (Ovar, Portugal) and were finely ground, mixed and dry pelleted through a 2.4 mm die at 50 ºC (California Pellet Mill, C-300 model). Ingredient and proximate

composition of the diets are presented in Table 1, dietary fatty acid profiles in Table 2 and the dietary amino acid profile in Table 3.

Table 1. Ingredient and proximate composition of the diets (% as feed)

	Diets			
	45P	**AS**	**AG**	**35P**
***Ingredients* (%)**				
Fish meal [a]	48.00	31.50	31.50	31.50
CPSP G [b]	5.00	5.00	5.00	5.00
Wheat gluten	2.50	4.25	5.00	2.50
Fish oil	4.00	5.00	5.00	5.00
Gelatinized corn starch [c]	25.00	23.25	22.50	25.00
Wheat bran	14.50	19.10	13.84	30.00
Vitamins and mineral mix [d]	0.25	0.25	0.25	0.25
Choline chloride [e]	0.10	0.10	0.10	0.10
Lutavin E50 [f]	0.05	0.05	0.05	0.05
Lutavin C35 [g]	0.05	0.05	0.05	0.05
Betafin S1 [h]	0.05	0.05	0.05	0.05
Europellin [i]	0.50	0.50	0.50	0.50
L-Alanine [j]		6.24		
L-Serine [j]		4.66		
L-Aspartic acid [j]			6.19	
L-Glutamic acid [j]			9.97	
Proximate composition				
Dry matter, DM (%)	90.4	91.8	91.9	89.6
Crude protein (% DM)	45.2	45.8	45.3	34.8
Nitrogen (% DM)	7.2	7.3	7.3	5.6
Crude lipids (% DM)	10.4	10.2	9.7	10.8
Ash (% DM)	9.6	6.7	6.6	7.9
Gross energy (kJ/g DM)	20.4	20.3	20.0	20.7

[a] Fish meal: Dry matter: 89.9 %;Protein: 74.2 % DM; Lipids: 9.5 % DM.
[b] CPSP G, Fish soluble concentrate protein
[c] Gelatinized starch, Cerestar, Barcelona Spain.: Dry matter: 91.9 %; Protein: 0.4 % DM; Lipids: 0.5 %DM; Starch: 88.3 %DM.
[d] Vitamins (mg or IU/kg diet): Vitamin A, 8000 IU; vitamin D3, 1700 IU; vitamin K3, 10 mg; vitamin B12, 0.02 mg; thiamin, 8 mg; riboflavin, 20 mg; vitamin B6, 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; nicotinic acid, 70 mg; pantothenic acid, 30 mg.
Minerals (g or mg/kg diet): Mn (manganese oxide), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc oxide) 30 mg; Se (sodium selenite) 0.3 mg; Fe (iron sulphate), 60 mg; Ca (calcium carbonate), 2.15 g; dibasic calcium phosphate, 5 g; KCl, 1 g; NaCl, 0.4 g.
[e] Choline chloride, 1000 mg kg^{-1} diet.
[f] Lutavin E50: vitamin E, 300 mg kg^{-1} diet.
[g] Lutavin C35: vitamin C, 500 mg kg^{-1} diet.
[h] Betafin S1: betain, 500mg/kg diet.
[i] Europelin: Binder.
[j] L-Alanine, L-Serine, L-Aspartic acid, L-Glutamic acid provided by Ajinomoto, Paris, France.

Table 2. Fatty acid composition of the diets (data expressed as g/100 g total fatty acid)

	Diets			
	45P	**AS**	**AG**	**35P**
14:0	5.2	5.1	5.0	4.8
15:0	0.5	0.5	0.5	0.5
16:0	17.6	17.3	17.2	16.9
17:0	0.4	0.4	0.4	0.4
18:0	3.6	3.4	3.5	3.3
20:0	0.3	0.3	0.3	0.3
Saturates	27.7	27.2	27.0	26.3
16:1	6.3	6.2	6.2	5.9
17:1	0.2	0.2	0.2	0.2
18:1	15.4	16.3	16.3	16.0
20:1	2.7	3.1	3.2	3.0
22:1	2.7	3.2	3.3	3.1
MUFA	27.5	29.1	29.3	28.3
14 PUFA	0.1	0.2	0.2	0.2
16:2 n-4	0.7	0.7	0.6	0.6
16:3 n-4	0.9	0.8	0.7	0.7
16:4 n-1	1.4	1.2	1.2	1.2
18:2 n-6	5.5	7.0	6.4	8.9
18:3 n-6	0.2	0.2	0.2	0.2
20:2 n-6	0.2	0.3	0.3	0.3
20:3 n-6	0.1	0.1	0.1	0.1
20:4 n-6	0.9	0.8	0.8	0.7
n-6	6.9	8.4	7.8	10.2
18:3 n-3	1.1	1.3	1.2	1.5
18:4 n-3	1.6	1.6	1.6	1.6
20:3 n-3	0.1	0.1	0.1	0.1
20:4 n-3	0.7	0.7	0.7	0.7
20:5 n-3	12.7	11.4	11.6	10.9
21:5 n-3	0.5	0.5	0.5	0.5
22:5 n-3	1.9	1.8	1.8	1.7
22:6 n-3	10.6	10.1	10.4	9.8
n-3	29.2	27.5	28.0	26.8
PUFA	39.4	38.7	38.5	39.7
Sat/PUFA	0.7	0.7	0.7	0.7
n-3/n-6	4.2	3.3	3.6	2.6

Growth trial

Experiments were directed by trained scientists (following FELASA category C recommendations) and were conducted according to the European Economic Community animal experimentation guidelines directive of 24 November 1986 (86/609/EEC). Juvenile

blackspot seabream were obtained from the Instituto Español de Oceanografía (IEO, Vigo, Spain) and transported to our facilities at CIIMAR, Porto, Portugal. After a quarantine and acclimatization period of 4 weeks, groups of 51 juveniles with an average body weight of 12 g (Table 4) were randomly distributed among 12 square fibre glass tanks (500 L), in a recirculating water system. . Each tank was supplied with filtered, heated (19 ± 1 ºC) saltwater (33 g L^{-1}) with dissolved oxygen content above 8 mg L^{-1} at a flow-rate of 2 L min^{-1}. The pH, ammonia, nitrites and nitrates in the water were monitored during the entire trial and maintained at levels compatible with marine species. Fish were exposed to natural photoperiod. Triplicate groups of fish for each treatment were fed by hand to apparent satiety, three times a day (09.30, 13.30 and 17.30 h) for 80 days

Fish were monthly bulk weighted under moderate anaesthesia (ethylene glycol monophenyl ether at 50 mg/L) and data on weight gain and distributed feed recorded. Prior to final tissue sampling, fish were fasted for 24 h and killed by a sharp blow on the head for enzyme determinations. At the beginning and at the end of the feeding trial a pooled sample of 12 fish per treatment were taken and stored at -20 ºC for subsequent whole body composition analyses. Muscle and liver tissues (9 fish per treatment) were collected for analysis of total lipid and fatty acids, lipogenic enzymes, glycolytic, gluconeogenic and amino acid catabolism (9 fish per treatment) enzymes. All samples were frozen in liquid nitrogen and stored at -80 ºC for subsequent analyses.

Blood collection was carried out in 12 fish per treatment at 4 h post feeding, and in less than three min at each sampling time to avoid any plasma metabolite response induced by handling stress. Samples were taken from the caudal vein, with syringes and collection tubes containing 15 to 20 µL of sodium fluoride and potassium oxalate (4% each). Plasma was obtained after centrifugation (6000 x *g* for 10 min at 4 ºC) and stored at -80 ºC for glucose, cholesterol (CHOL), triacylglycerol (TAG) and free amino acid profile analysis.

Feed and body composition analyses

Whole fish from each tank were pooled, ground, and moisture content determined. Fish were subsequently freeze-dried before further analysis. Feed and whole body samples were analyzed for dry matter (105 ºC for 24 h), ash by combustion in a muffle furnace (550 ℃ for 12 h), crude protein (micro-Kjeldahl; N x 6.25) after acid digestion, lipid content by petroleum ether extraction (at Soxhlet 40-60 ºC), and gross energy in an adiabatic bomb calorimeter (IKA, Werke C2000).

Table 3. Amino acid composition (g/16 g N) of the four diets

	Diets			
	45P	**AS**	**AG**	**35P**
Arginine	5.2	4.0	4.2	5.4
Histidine	2.9	2.1	2.1	2.9
Isoleucine	4.3	3.2	3.2	4.2
Leucine	7.1	5.4	5.3	7.2
Lysine	7.2	5.2	4.9	7.0
Methionine	3.1	2.3	2.4	3.1
Cystine	0.8	0.7	0.7	0.9
Phenylalanine	4.5	3.2	3.2	4.7
Tyrosine	3.1	2.0	2.4	3.0
Threonine	3.8	2.4	2.7	3.8
Valine	4.7	3.5	3.4	4.8
Alanine	5.7	**17.7**	4.1	5.9
Serine	3.2	**10.2**	2.4	3.3
Aspartic acid	8.4	6.1	**21.5**	8.4
Glutamic acid	13.9	11.9	**35.5**	15.4
Glycine	5.9	3.9	4.4	6.0
Proline	4.4	4.1	3.4	5.1
TOTAL AA	88.2	87.9	105.6	91.0
IAA	46.7	33.9	34.3	46.9
DAA	41.5	54.0	71.3	44.1
IAA/DAA	1.1	0.6	0.5	1.1

Tryptophan was not possible to determine.

Total lipid and fatty acids analyses

Total lipid was extracted and measured gravimetrically according to Folch *et al.* (1957) using dichloromethane instead of chloroform. Fatty acid methyl esters were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride methanol according to Santha & Ackman (1990) and analyzed as described in Figueiredo-Silva *et al*. (2009 a,b). Fatty acids were identified by comparison with known standard mixtures (Sigma 189-19, St Louis, MO, USA) and quantified using a Star computer package (Varian).

Amino acids analysis

AA composition was measured by ion exchange chromatography according to Moore & Stein (1951) and Stein & Moore (1954) by a certificated laboratory (AGROBIO, Rennes,

France)**.** Tryptophan is destroyed by the hydrolysis process, and is therefore not part of the evaluation of the amino acid profile.

Plasma metabolite assays

Plasma glucose, CHOL and TAG were determined using commercial kits: Glucose RTU (nº 61269); Cholesterol RTU (nº 61218) and Triglycérides Enzymatique PAP 150 (nº 61236) from Bio-Mérieux, Marcy-L'Etoile, France.

Enzyme activity

Liver samples for lipogenic enzyme assays were homogenised in three volumes of ice-cold buffer (0.02 mol/L Tris–HCl, 0.25 mol/L sucrose, 2 mmol/L EDTA, 0.1 mol/L NaF, 0.5 mmol phenylmethyl sulphonyl fluoride, 0.01 mol/L β-mercaptoethanol, pH 7.4) and centrifuged at 30 000 x *g*, at 4 ºC for 20 min. Selected lipogenic enzyme activities were assayed in the supernatant as described in Figueiredo-Silva *et al*. (2009 a,b).

Liver samples for glycolytic enzymes assays were homogenized in five volumes of ice-cold buffer (80 mM Tris; 5 mM EDTA; 2 mM DTT; 1 mM benzamidine; 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, pH 7.6) and centrifuged at 900 x *g* at 4 ºC for 10 min. Selected glycolytic enzyme activities were assayed in the supernatant as described in Figueiredo-Silva *et al*. (2009 a,b).

Liver samples for phosphoenolpyruvate carboxykinase activity (PEPCK, EC 4.1.1.32) assays were homogenized in ten volumes of ice-cold buffer (10 mmol/L HEPES; 0.25 mol/L Sacharose; 1 mmol/L DTT; pH 7.4). The homogenates were treated by ultrasound for 1 min (Pulse 1s, amplitude 70 W) and centrifuged at 900 x *g* at 4 ºC for 10 min, and the resultant supernatant was centrifuged at 10 000× *g* at 4 ºC for 20 m. Total (cytosolic and mitochondrial) PEPCK enzyme activities were assayed at 37 ºC as described previously by Scholz *et al.* (1998).

Liver samples for assaying activities of enzymes of amino acid catabolism were homogenized in ten volumes of ice-cold buffer (30 mM HEPES, 0.25 mM saccharose, 0.5 mM EDTA, 5mM K_2HPO_4, 1 mM DTT, pH 7.4) and then separated in two fractions, one for alanine aminotranferase (ALAT, EC 2.6.1.2) and aspartate aminotransferase (ASAT; EC 2.6.1.1) and one to measure glutamate dehydrogenase (GDH, EC 1.4.1.2) activities. Crude homogenate separated for GDH were firstly treated by ultrasound for 1 min (Pulse 1s, amplitude 50 W) and then all homogenates (ALAT, ASAT and GDH) firstly centrifuged at 1000× *g* at 4 ºC for 20 m and then at 15 000× *g* at 4 ºC for 20 m. ASAT and ALAT

activities were assayed at 37 ºC on the respective recuperated supernatants using commercial kits from Enzyline (ALAT/GPT, ref. 63313; ASAT/GOT, ref. 63213). GDH activities were assayed at 37 ºC on the respective recuperated supernatants adding 10mM of L-glutamic acid to the reaction mixture as described previously by Bergmeyer (1974).

For all enzymes, the activity (units IU), defined as µmoles of substrate converted to product, per min, at assay temperature, were expressed per mg of hepatic soluble protein (specific activity) or per g of tissue. Soluble protein content of tissues was determined on the supernatant fractions by the method of Bradford (1976) using bovine serum albumin (BSA) as standard (Sigma, St Louis, MO, USA).

Statistical analysis

Statistical analyses followed methods outlined by Zar (1996) and were determined using the STATISTICS 7.0 package (StatSoft, Inc., Tulsa, OK, USA). All data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Bartlett tests, and then submitted to a One-way ANOVA. When these tests showed significance ($P<0.05$), individual means were compared using Tukey test. Tank average values for feed intake, growth, body composition and nutrient accretion analysis were used as experimental units for statistical analyses. Correlation coefficients were obtained by the Pearson Product Moment Correlation Distribution and considered significant when $P<0.05$.

Results

In diets AS and AG, dispensable amino acids (DAA) content was increased by the incorporation of alanine and serine or aspartic and glutamic acids, respectively, changing the IAA/DAA ratio from 1.1 (diet 45P and 35P) to 0.6 (AS) or 0.5 (AG). Total alanine (8.2 g/100 g diet) and serine (4.7 g/100 g diet) contents in diet AS, and total aspartic (9.8 g/100 g diet) and glutamic (16.1 g/100 g diet) acid contents in diet AG comprise the amount supplemented and the basal content of those diets in these DAA. Plasma free amino acid (FAA) profile is presented in Table 4, and was positively correlated with the dietary amino acid (AA) profile (Pearson correlation= 0.48) (Table 3). Dietary nitrogen reduction from 7.2 to 5.6 g, (45P vs 35P diet) resulted in diminished plasma isoleucine and leucine concentrations, but these differences were not sufficient to significantly affect total plasma free IAA. The replacement of 1.6 g N by alanine and serine mixture significantly increased plasma alanine and serine levels and therefore the total free DAA concentrations.

Table 4. Plasma free amino acids (FAA) (µmol/ml), glucose (g/L), cholesterol and triacylglycerol (g/L) at 4 h postprandial in blackspot seabream fed the different diets

	Diets				ANOVA
	45P	**AS**	**AG**	**35P**	**P-value**
FAA					
Arginine	0.38±0.1 ^{a}	0.16±0.04 ^{b}	0.18±0.01 ^{b}	0.29±0.04 ^{ab}	0.017
Histidine	0.27±0.04 ^{ab}	0.33±0.04 ^{a}	0.22±0.04 ^{b}	0.24±0.01 ^{ab}	0.021
Isoleucine	0.49±0.02 ^{a}	0.41±0.02 ^{b}	0.28±0.03 ^{c}	0.36±0.02 ^{b}	0.0004
Leucine	0.46±0.01 ^{a}	0.40±0.02 ^{b}	0.26±0.03 ^{c}	0.34±0.01 ^{b}	0.0002
Lysine	0.42±0.02 ^{a}	0.24±0.04 ^{b}	0.22±0.02 ^{b}	0.32±0.03 ^{a}	0.002
Methionine	0.26±0.1 ^{a}	0.19±0.004 ^{ab}	0.13±0.03 ^{b}	0.20±0.03 ^{ab}	0.009
Phenylalanine	0.24±0.1 ^{a}	0.15±0.01 ^{b}	0.16±0.02 ^{b}	0.23±0.01 ^{a}	0.005
Tyrosine	0.17±0.03	0.10±0.01	0.12±0.02	0.17±0.04	0.039*
Threonine	0.37±0.1 ^{b}	0.41±0.1 ^{a}	0.18±0.3 ^{c}	0.28±0.02 ^{bc}	0.001
Valine	0.53±0.0 ^{a}	0.44±0.03 ^{a}	0.30±0.04 ^{b}	0.45±0.05 ^{a}	0.001
Alanine	0.48±0.03 ^{b}	1.58±0.37 ^{a}	0.61±0.30 ^{b}	0.53±0.05 ^{b}	0.0003
Serine	0.16±0.02 ^{b}	2.53±0.55 ^{a}	0.12±0.03 ^{b}	0.20±0.03 ^{b}	0.0000
Aspartic acid	0.04±0.00	0.05±0.00	0.04±0.00	0.04±0. 01	0.217
Glutamic acid	0.06±0.01 ^{b}	0.09±0.01 ^{a}	0.08±0.01 ^{a}	0.07±0.004 ^{b}	0.001
Glycine	0.30±0.03 ^{b}	1.11±0.15 ^{a}	0.28±0.03 ^{b}	0.47±0.1 ^{b}	0.0000
Proline	0.14±0.03 ^{a}	0.09±0.01 ^{c}	0.07±0.01 ^{c}	0.11±0.01 ^{bc}	0.002
Taurine	1.51±0.42	1.77±0.01	2.07±0.51	2.94±1.37	0.384
Glutamine	0.26±0.1 ^{b}	0.41±0.1 ^{a}	0.23±0.05 ^{b}	0.22±0.04 ^{b}	0.005
Asparagine	0.14±0.02 ^{b}	0.28±0.1 ^{a}	0.09±0.02 ^{b}	0.15±0.02 ^{b}	0.001
Hydroxyproline	0.04±0.01 ^{b}	0.09±0.02 ^{a}	0.05±0.00 ^{b}	0.06±0.01 ^{ab}	0.012
IAA	2.96±0.79	2.83±0.14	2.00±0.34	2.45±0.62	0.198
DAA	2.58±0.80 ^{b}	7.42±1.49 ^{a}	2.94±1.24 ^{b}	3.80±2.04 ^{ab}	0.013
Total FAA	5.54±1.58 ^{b}	10.26±1.54 ^{a}	4.94±0.94 ^{b}	6.25±2.58 ^{ab}	0.023
Glucose	0.94±0.12 ^{a}	0.88±0.08 ^{ab}	0.91±0.15 ^{a}	0.73±0.18 ^{b}	0.006
Cholesterol	1.8±0.3 ^{a}	1.8±0.3 ^{a}	1.3±0.2 ^{b}	1.6±0.2 ^{ab}	0.001
Triacylglycerol	2.6±0.9 ^{ab}	3.0±0.9 ^{a}	2.2±0.5 ^{ab}	2.1±0.6 ^{b}	0.026

Mean values and standard deviations (±SD) (n=3 for FAA and n=12 for glucose, cholesterol and triacylglycerol).
$^{a.b.c.}$ Mean values within a row unlike superscript letters were significantly different (P<0.05).
* Without significant differences with *Pos-hoc* analysis

On the other hand, aspartic acid concentration in plasma did not vary significantly among diets, whereas glutamic acid concentration was higher in fish fed both DAA mixtures. Glucose, cholesterol (CHOL) and plasma triacylglycerol (TAG) levels varied significantly among dietary treatments (Table 4). Glucose levels were higher in fish either fed 45P or AG diets than in those fed the 35P diet. Fish fed AS presented significantly higher plasma CHOL levels than those fed AG. Moreover, TAG levels were also the highest in fish fed AS but just differed significantly from the 35P fed fish.

At the end of the growth trial (80 days), all groups of blackspot seabream tripled their initial body weight regardless of dietary treatment (Table 5). Fish fed the 45P diet (7.2 g N) presented the highest final body weight (FBW) and daily growth index (DGI). Athough fish fed 35P (5.6 g N) increased feed intake, this increase was not enough to reach the same daily nitrogen intake observed in fish fed the 45P diet, resulting in reduced FBW, lower DGI and poor feed/weight gain ratio (FCR). The replacement of 1.6 g nitrogen from the fish meal by DAA reduced feed intake inducing lower DGI and FBW. Despite this reduction in FBW and DGI, fish fed AS or AG diets showed similar FCR and similar protein efficiency ratios (PER) when compared with the 45P fed group.

Table 5. Data on feed and nutrient intakes and growth performance of blackspot seabream fed the different diets over 80 days

	Diets				ANOVA
	45P	**AS**	**AG**	**35P**	**P-value**
Growth					
Initial body weight (g)	12.0 ± 0.1	12.0 ± 0.1	12.0 ± 0.1	12.0 ± 0.1	0.927
Final body weight (g)	41.0 ± 0.8 [a]	37.2 ± 1.1 [b]	35.1 ± 1.0 [bc]	34.5 ± 1.0 [c]	0.0006
FCR [a]	1.6 ± 0.1 [b]	1.6 ± 0.04 [b]	1.6 ± 0.02 [b]	1.9 ± 0.1 [a]	0.0002
DGI [b]	1.5 ± 0.03 [a]	1.3 ± 0.03 [b]	1.2 ± 0.03 [bc]	1.2 ± 0.04 [c]	0.0001
PER [c]	1.4 ± 0.1 [b]	1.4 ± 0.04 [ab]	1.4 ± 0.02 [b]	1.5 ± 0.1 [a]	0.005
Intake [d]					
(g or kJ/kg ABW/day)					
Dry matter	21.7 ± 0.6 [b]	20.6 ± 0.3 [c]	20.2 ± 0.1 [c]	22.8 ± 0.1 [a]	0.0001
Nitrogen	1.6 ± 0.04 [a]	1.5 ± 0.02 [ab]	1.5 ± 0.01 [b]	1.3 ± 0.01 [c]	0.000002
Fat	2.3 ± 0.1 [b]	2.1 ± 0.03 [c]	2.0 ± 0.01 [d]	2.5 ± 0.01 [a]	0.000001
Gross energy	443.3 ± 12.0 [b]	417.3 ± 6.0 [c]	405.3 ± 1.9 [c]	472.0 ± 2.4 [a]	0.00001

Mean values and standard deviations (±SD) are presented for each parameter (n=3)
[a,b,c,d] Mean values within a row unlike superscript letters were significantly different ($P<0.05$).
[a] FCR, Feed conversion ratio = dry feed intake/weight gain.
[b] DGI, Daily growth index = 100 x ((Final body weight)$^{1/3}$ - (Initial body weight)$^{1/3}$)/days
[c] PER, Protein efficiency ratio = weight gain/crude protein intake.
[d] Daily nutrient intake (g or kJ/kg ABW/day) = nutrient intake/((initial body weight+final body weight)/2)/days

Despite the differences observed on growth parameters and feed intake, blackspot seabream had similar whole body protein and energy contents (Table 6) irrespective of the dietary treatments. However, fish fed the AS diet had significantly higher whole body lipid content (12.9%) than the 35P fed group (10.7%). The reduced body lipid content observed in fish fed the low protein diet (diet 35P) correlates well with their reduced

viscerosomatic index (VSI). The hepatosomatic index (HSI) was unaffected by dietary treatments, although a high liver lipid content was found in fish fed AS comparatively to those fed diet 45P. Muscle total lipid content ranged between 5 to 6% of wet weight irrespective of the dietary treatment.

Table 6. Whole body composition (% or kJ g^{-1} of wet weight (ww)), nutrient gain and energy retention in blackspot seabream fed the different diets

	Diets				ANOVA
	45P	**AS**	**AG**	**35P**	**P-value**
Final body composition [1]					
Moisture %	65.3 ± 1.6	64.8 ± 1.3	66.5 ± 1.1	67.1 ± 0.9	0.172
Protein %	17.7 ± 0.7	17.9 ± 0.8	17.0 ± 0.6	17.1 ± 0.4	0.302
Lipid %	12.2 ± 0.4 [ab]	12.9 ± 1.1 [a]	11.5 ± 0.9 [ab]	10.7 ± 0.7 [b]	0.024
Energy kJ/g	8.4 ± 0.4	8.5 ± 0.5	8.2 ± 0.4	7.7 ± 0.1	0.136
HSI % [2]	1.4 ± 0.2	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.2	0.596
VSI % [3]	6.4 ± 1.3	6.5 ± 1.1	6.5 ± 1.1	5.0 ± 1.3	0.034*
Nitrogen gain (mg/ kg ABW [4]/d)	400.3 ± 25.6 [a]	383.2 ± 23.1 [ab]	343.8 ± 11.7 [b]	340.3 ± 16.2 [b]	0.015
Lipids gain (g/ kg ABW [4]/d)	1.8 ± 0.1 [a]	1.8 ± 0.2 [a]	1.5 ± 0.1 [ab]	1.3 ± 0.1 [b]	0.007
Retention (%) of intake					
Protein	25.5 ± 1.8	25.4 ± 1.9	23.4 ± 0.8	26.9 ± 1.3	0.126
Lipid	78.2 ± 5.9 [a]	85.9 ± 11.9 [a]	75.5 ± 3.7 [ab]	53.3 ± 4.2 [b]	0.003

Mean values and standard deviations (±SD) are presented for each parameter (n=3)
[a.b.c.d] Mean values within a row unlike superscript letters were significantly different (P<0.05).
[1] Initial body composition was: moisture: 68.4%; protein :16.3%ww; lipid :10.4%ww and energy : 7.0 kJ/g.
[2] HSI. Hepatosomatic index = 100 X liver weight/body weight
[3] VSI. Viscerosomatic index = 100 X weight of viscera/body weight
[4] ABW. Average body weight = (final body weight + initial body weight)/2
[*] Without significant differences after *pos-hoc* analysis

Overall, nitrogen gain varied according to differences in daily nitrogen intake, and consequently overall protein retention (% intake) was unaffected by dietary treatments so a different trend was observed with regard to lipid gain or retention, with fish fed 45P, AS or AG diets showing higher lipid gain and retention than those fed the lowest protein (35P) diet.

Muscle and liver fatty acid (FA) compositions of blackspot seabream are shown in Tables 7 and 8, respectively. The different dietary treatments have significantly affected fatty acid (FA) composition in both tissues.

Table 7. Muscle total lipid content (% wet weight) and fatty acid composition (g/100 g total fatty acids) of blackspot seabream fed the different diets

	Diets				ANOVA
	45P	**AS**	**AG**	**35P**	**P-value**
14:0	3.1±0.3 [a]	2.9±0.3 [ab]	2.7±0.2 [b]	2.7±0.2 [b]	0.009
15:0	0.9±0.5	0.7±0.4	0.9±0.5	0.8±0.5	0.822
16:0	18.0±0.6 [a]	17.3±0.5 [a]	16.4±1.0 [b]	16.1±0.4 [b]	0.000
17:0	0.3±0.01	0.3±0.02	0.3±0.02	0.4±0.03	0.063
18:0	6.6±0.2 [a]	6.6±0.4 [a]	6.7±0.4 [a]	5.9±0.3 [b]	0.000
20:0	0.2±0.01 [a]	0.2±0.01 [ab]	0.2±0.01 [a]	0.2±0.01 [b]	0.013
Saturates	29.2±0.8 [a]	28.3±0.5 [ab]	27.3±1.7 [bc]	26.5±0.9 [c]	0.0001
16:1	4.8±0.4 [a]	4.5±0.3 [ab]	4.4±0.3 [b]	4.4±0.3 [b]	0.019
17:1	0.2±0.01	0.2±0.01	0.2±0.02	0.2±0.02	0.742
18:1	21.1±1.5 [a]	20.3±0.7 [a]	21.0±1.2 [a]	18.2±1.0 [b]	0.000
20:1	2.3±0.2	2.4±0.1	2.4±0.1	2.5±0.1	0.089
22:1	1.9±0.2 [b]	2.0±0.1 [ab]	2.0±0.1 [ab]	2.1±0.1 [a]	0.011
MUFA	30.3±1.9 [a]	29.4±0.6 [a]	30.0±1.6 [a]	27.4±1.4 [b]	0.001
16:2 n-4	0.4±0.04 [a]	0.3±0.04 [b]	0.3±0.03 [b]	0.4±0.04 [ab]	0.005
16:3 n-4	0.4±0.1 [a]	0.4±0.04 [b]	0.4±0.04 [b]	0.4±0.04 [b]	0.004
16:4 n-1	0.5±0.1 [a]	0.4±0.1 [b]	0.4±0.04 [b]	0.4±0.1 [b]	0.002
18:2 n-6	4.4±0.2 [c]	5.3±0.2 [b]	4.7±0.2 [c]	6.6±0.5 [a]	0.000
18:3 n-6	0.2±0.01	0.2±0.01	0.2±0.02	0.2±0.01	0.185
20:2 n-6	0.3±0.02 [b]	0.3±0.02 [b]	0.3±0.03 [b]	0.3±0.02 [a]	0.000
20:3 n-6	0.2±0.01 [b]	0.2±0.02 [a]	0.2±0.01 [a]	0.2±0.02 [b]	0.000
20:4 n-6	0.8±0.1 [ab]	0.8±0.1 [ab]	0.8±0.02 [b]	0.9±0.1 [a]	0.006
n-6	5.9±0.3 [c]	6.9±0.2 [b]	6.2±0.3 [c]	8.2±0.5 [a]	0.000
18:3 n-3	0.7±0.04 [b]	0.7±0.02 [b]	0.7±0.1 [b]	0.9±0.1 [a]	0.000
18:4 n-3	0.8±0.1	0.7±0.1	0.7±0.04	0.8±0.1	0.121
20:3 n-3	0.1±0.01 [b]	0.1±0.01 [b]	0.1±0.01 [ab]	0.1±0.02 [a]	0.005
20:4 n-3	0.8±0.04	0.8±0.04	0.8±0.05	0.8±0.06	0.318
20:5 n-3	9.0±0.4 [a]	8.4±0.3 [b]	7.8±0.2 [c]	8.5±0.4 [b]	0.000
21:5 n-3	0.4±0.02 [a]	0.3±0.04 [b]	0.4±0.02 [b]	0.4±0.03 [b]	0.0002
22:5 n-3	3.4±0.3	3.5±0.3	3.5±0.3	3.5±0.2	0.769
22:6 n-3	11.6±1.3 [b]	12.2±0.7 [ab]	11.1±0.4 [b]	13.2±1.1 [a]	0.0004
n-3	26.7±1.9 [ab]	26.8±1.3 [ab]	25.2±0.8 [b]	28.1±1.3 [a]	0.001
PUFA	34.0±2.1 [bc]	34.8±1.4 [b]	32.5±1.0 [c]	37.5±1.5 [a]	0.000
Sat/PUFA	0.9±0.1 [a]	0.8±0.04 [b]	0.8±0.1 [b]	0.7±0.04 [b]	0.000
n-3/n-6	4.5±0.2 [a]	3.9±0.1 [b]	4.1±0.2 [b]	3.4±0.2 [c]	0.000
Muscle total lipid %(wet weight)	5.6±0.7	4.9±0.7	5.5±0.5	5.4±0.8	0.096

Mean values and standard deviations (±SD) are presented for each parameter (n=9)
[a.b.c] Mean values within a row unlike superscript letters were significantly different (P<0.05).

Table 8. Liver total lipid content (% wet weight. WW) and fatty acid composition (g/100 g total fatty acids) of blackspot seabream fed the different diets

	Diets				ANOVA
	45P	**AS**	**AG**	**35P**	**P-value**
14:0	2.3±0.3 [a]	2.5±0.1 [a]	2.3±0.1 [a]	1.6±0.004 [b]	0.003
15:0	0.3±0.1	0.3±0.1	0.3±0.02	0.2±0.01	0.806
16:0	17.4±1.9	16.2±1.3	16.0±0.6	14.2±1.0	0.138
17:0	0.3±0.04	0.3±0.03	0.3±0.02	0.4±0.02	0.063
18:0	11.1±0.3	10.2±0.6	11.4±0.1	10.2±0.6	0.036*
20:0	0.3±0.01	0.3±0.02	0.3±0.01	0.3±0.02	0.369
Saturates	31.7±1.9	29.8±1.8	30.6±0.5	26.9±1.5	0.052
16:1	3.9±0.2	4.1±0.2	3.8±0.1	3.4±0.1	0.022*
17:1	0.3±0.02	0.3±0.01	0.3±0.02	0.3±0.01	0.255
18:1	32.3±1.7	31.3±1.4	31.0±1.2	28.2±1.1	0.076
20:1	3.0±0.4	3.0±0.1	3.2±0.1	3.7±0.2	0.071
22:1	1.4±0.2 [b]	1.6±0.1 [b]	1.6±0.1 [ab]	2.0±0.03 [a]	0.011
MUFA	40.9±1.6	40.3±1.1	40.0±1.0	37.5±1.2	0.102
14 PUFA	0.05±0.002	0.1±0.01	0.1±0.01	0.1±0.01	0.474
16:2 n-4	0.2±0.01	0.2±0.03	0.2±0.02	0.2±0.02	0.495
16:3 n-4	0.2±0.02	0.2±0.04	0.2±0.04	0.1±0.03	0.139
16:4 n-1	0.1±0.01	0.1±0.04	0.1±0.03	0.1±0.02	0.240
18:2 n-6	3.1±0.5 [c]	4.4±0.5 [ab]	3.6±0.3 [bc]	5.8±0.5 [a]	0.002
18:3 n-6	0.1±0.003	0.2±0.07	0.2±0.02	0.2±0.02	0.072
20:2 n-6	0.5±0.2 [b]	0.5±0.1 [b]	0.6±0.05 [b]	0.9±0.1 [a]	0.004
20:3 n-6	0.3±0.05	0.5±0.1	0.5±0.1	0.5±0.02	0.056
20:4 n-6	0.6±0.1	0.5±0.03	0.50±0.01	0.6±0.03	0.067
n-6	4.6±0.7 [c]	6.2±0.7 [b]	5.4±0.3 [bc]	8.1±0.5 [a]	0.002
18:3 n-3	0.4±0.1 [b]	0.5±0.1 [ab]	0.5±0.1 [ab]	0.7±0.1 [a]	0.046
18:4 n-3	0.2±0.02	0.3±0.1	0.3±0.1	0.2±0.1	0.134
20:3 n-3	0.1±0.1 [b]	0.2±0.02 [ab]	0.2±0.02 [ab]	0.2±0.02 [a]	0.030
20:4 n-3	0.9±0.2	1.1±0.2	1.0±0.1	1.2±0.04	0.220
20:5 n-3	3.9±0.2	3.6±0.3	3.3±0.3	3.7±0.7	0.242
21:5 n-3	0.3±0.1	0.3±0.1	0.3±0.04	0.4±0.02	0.292
22:5 n-3	4.1±1.3	4.2±0.6	4.4±0.5	6.0±0.1	0.123
22:6 n-3	6.5±0.6 [b]	6.4±0.4 [b]	6.4±0.3 [b]	8.0±0.5 [a]	0.026
n-3	16.5±1.7	16.6±1.6	16.3±1.3	20.5±1.3	0.060
PUFA	21.5±2.4 [b]	23.4±2.4 [ab]	22.4±1.7 [b]	29.0±1.8 [a]	0.029
Sat/PUFA	1.5±0.2	1.3±0.2	1.4±0.1	0.9±0.1	0.058
n-3/n-6	3.6±0.3 [a]	2.7±0.1 [b]	3.0±0.1 [b]	2.5±0.01 [b]	0.000
Liver total lipid %(WW)	24.2±2.3 [b]	33.0±2.8 [a]	26.8±2.8 [ab]	31.3±4.0 [ab]	0.027

Mean values and standard deviations (±SD) are presented for each parameter (3 pools of 3 fish each. n=3)
[a.b.c.] Mean values within a row unlike superscript letters were significantly different ($P<0.05$).
[*] Without significant differences after *pos-hoc* analysis

Muscle total saturated FAs were higher in fish fed both the 45P and the AS diet than in those fed the 35P, mainly due to an increase in 14:0 and 16:0 FAs. Liver 14:0 and 18:0 FA contents were higher in fish fed 45P, AS and AG than in those fed the low protein diet (35P). Nevertheless, these differences were not sufficient to significantly affect liver saturated FA fraction. Fish fed the 35P diets had the lowest MUFA content in both muscle and liver mainly due to the low level of 18:1 (oleic acid) content, but this difference was only significant in muscle. Higher proportions of linoleic (18:2n-6) and then n-6 PUFA were found in both muscle and liver of fish fed diet 35P resulting from the higher dietary content of these FAs (Table 2).

A trend to increased n-3 PUFA in liver and a significant increase in muscle was observed in fish fed diet 35P mainly due to the higher percentages of 22:6n-3. The higher muscle and liver n-6 FA content found in fish fed diet 35P consequently resulted in a lower n-3/n-6 FA ratio. Moreover, this ratio was lower in fish fed AS and AG diets than in those fed diet 45P, mainly due to the higher content of n-6 FAs in these diets (Table 2).

Data on the hepatic enzyme activities (lipogenic, glycolytic, gluconeogenic and amino acid catabolism) are presented in Table 9. Malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PD) and fatty acid synthetase (FAS) activities were markedly depressed by the dietary nitrogen reduction (45P vs 35P diet) but responded differently to the replacement of 1.6 g fish meal nitrogen by the two different DAA mixtures. Regarding protein nature, FAS specific activities (mIU/mg protein) were reduced in fish fed AG diet compared to fish fed 45P and AS diets.

Neither the reduction nor the nitrogen replacement by the two different DAA mixtures has affect hexokinase (HK) or glucokinase (GK) activities. The reduction of dietary nitrogen significantly increased pyruvate kinase (PK) activities. Phosphoenolpyruvate carboxykinase (PEPCK) specific activities were significantly lower in fish fed diet 45P than in those fed AG or 35P diet.

Activities of AA catabolic enzyme (alanine aminotranferase, ALAT; aspartate aminotransferase, ASAT; glutamate dehydrogenase, GDH) were higher in 45P than in AG or 35P fed groups, but when expressed as specific activities no significant differences were observed.

Table 9. Effects of different dietary treatments on blackspot seabream key hepatic enzymes activities

	Diets				ANOVA
	45P	**Ala+Ser**	**Asp+Glu**	**35P**	**P-value**
Lipogenic enzymes					
ME					
IU / g liver	3.6±1.0 [a]	2.6±0.7 [a]	2.6±0.1 [a]	0.7±0.2 [b]	0.000001
mIU / mg protein	49.5±12.2 [a]	45.9±13.2 [a]	39.9±17.0 [a]	13.9±5.5 [b]	0.00001
G6PD					
IU / g liver	9.7±2.2 [a]	9.2±2.2 [a]	9.0±1.2 [a]	5.4±1.9 [b]	0.0001
mIU / mg protein	96.6±19.3 [a]	108.6±15.1 [a]	94.6±14.8 [a]	57.4±11.7 [b]	0.000001
FAS					
IU / g liver	1.6±0.6 [a]	1.1±0.5 [ab]	0.9±0.6 [ab]	0.4±0.2 [b]	0.005
mIU / mg protein	15.2±5.3 [a]	13.6±5.9 [ab]	8.2±4.5 [bc]	4.5±2.7 [c]	0.001
Glycolytic enzymes					
HK					
IU / g liver	0.03±0.02	0.02±0.01	0.02±0.01	0.04±0.02	0.102
mIU / mg protein	0.3±0.2	0.2±0.1	0.3±0.2	0.5±0.2	0.064
GK					
IU / g liver	0.1±0.03	0.1±0.03	0.1±0.03	0.1±0.02	0.929
mIU / mg protein	0.7±0.4	0.8±0.5	0.9±0.3	0.7±0.3	0.897
PK					
IU / g liver	15.9±4.8 [b]	12.9±2.7 [b]	15.3±4.9 [b]	26.2±6.6 [a]	0.0001
mIU / mg protein	181.9±69.1 [b]	159.4±35.1 [b]	201.6±63.5 [b]	332.2±81.6 [a]	0.0001
Gluconeogenic enzymes					
PEPCK					
IU / g liver	1.7±0.3	2.1±0.7	2.4±0.4	2.1±0.5	0.071
mIU / mg protein	18.4±3.0 [b]	23.5±6.9 [ab]	30.0±5.9 [a]	26.4±7.2 [a]	0.011
Amino acid catabolism					
ALAT					
IU / g liver	116.9±35.0 [a]	87.6±18.9 [ab]	77.7±27.4 [b]	80.4±17.4 [b]	0.016
mIU / mg protein	1252.4±429.0	1205.0±279.0	1147.3±340.3	1016.8±278.0	0.528
ASAT					
IU / g liver	147.5±33.7 [a]	110.3±21.0 [b]	100.4±23.1 [b]	93.1±22.5 [b]	0.0006
mIU / mg protein	1587.1±406.5	1530.1±388.2	1413.5±318.0	1264.0±344.0	0.270
GDH					
IU / g liver	8.3±1.0 [a]	7.1±0.9 [ab]	5.9±0.9 [bc]	5.3±1.4 [c]	0.00001
mIU / mg protein	82.5±28.3	92.8±17.9	77.2±18.6	69.9±16.1	0.165

Mean values and standard deviations (±SD) are presented for each parameter (n=9)
[a.b.c.] Mean values within a row unlike superscript letters were significantly different (P<0.05).

Discussion

The growth parameters and feed efficiencies obtained for blackspot seabream juveniles are well within the range of values for this species (Figueiredo-Silva *et al.* 2009a,b). The reduction of dietary crude protein to levels below 45% level known to be optimal for this species (Silva *et al.* 2006), was previously shown to markedly depress growth rates (Figueiredo-Silva *et al.* 2009b). The correspondence observed between dietary and postprandial free amino acids (FAA) concentrations together with the 3 fold increase on initial body weight (IBW) attest the good acceptability of the different diets. Plasma alanine and serine concentrations were positively correlated (Pearson correlation = 0.97 and 0.99) with the respective dietary content, but the same was not verified for aspartic and glutamic acids (Pearson correlation = 0.47 and 0.17). The time-course of amino acid (AA) absorption is known to relate with AA form, free or protein bound, as well as with AA nature (Murai *et al.* 1987; Cowey & Walton 1988), which to some extent may explain this discrepancy. In addition, the time-course of the dispensable amino acids (DAA) plasma concentrations do not always demonstrate a direct influence of the AA composition of the test meal (Kaushik 1977).

Although fish growth responsiveness has been shown to depend on the DAA nature (Hughes 1985; Fauconneau 1988) and level (Mambrini & Kaushik 1994), the results are quite controversial. In rainbow trout, Fauconneau (1988) registered better growth rates with diets supplemented with aspartic acid than with alanine or glutamic acid. However, no effect of the DAA nature on fish growth performance was found when nitrogen was partially replaced by a single DAA (alanine, aspartic or glutamic acid) (Kirchner *et al.* 2003a) or by either a single (alanine, glutamate or glycine) or a mixture of DAA (alanine, aspartic and glutamic acid, glycine, proline and serine) (Mambrini & Kaushik 1994), as also observed here. It is generally accepted that reduced utilization of synthetic AA relative to protein-bound AA for anabolic purposes results from their rapid absorption (Cowey & Walton1988; Ambardekar *et al*. 2009). Thus, the inferior final body weight obtained with AG (<20%) than with AS diet (<13%) when compared to the 45P, may resulted from a faster or poorer absorption of aspartic and glutamic acids than of alanine and serine.

Fish body fat was shown to associate with dietary DAA nature pointing out the different individual DAA contribution as metabolic intermediate precursors (Mambrini & Kaushik 1994). The similarity of the data concerning lipid gain and body lipid content between 45P

and AS or AG fed blackspot seabream showed the ability of this fish to use these two different mixtures of DAA as precursors for fatty acid synthesis, with lipid gain being positively correlated with nitrogen intake (Pearson correlation = 0.73). Although not statistically different, fish fed the diet AS showed a tendency to increased daily lipid gain and body lipid content compared to those fed AG. This result may perhaps explain the superior weight gain found in fish fed AS diet. Compared with the low protein diet (35P), fish fed with diet AS or AG showed a higher or similar lipid gain and whole body lipid content, respectively. Considering the inexistence of significant differences in muscle lipid content, irrespectively of the dietary treatment, and the low contribution of liver lipid content for whole body lipid content, this accretion seems to mainly result from a higher visceral lipid deposition evidenced by the viscerosomatic index (VSI) found in fish fed both AS or AG diets compared to fish fed the 35P. An increased hepatosomatic index (HSI) has been reported in rainbow trout fed with DAA supplemented diets (Kirchner *et al.* 2003a). Date on HSI obtained in the present work do not support such a conclusion, although fish fed the DAA effectively showed higher liver total lipid contents compared with the 45P diet. The still elevated liver lipid contents of blackspot seabream, fed the 35P diet, have probably resulted from a higher liver uptake of circulating TAG to fuel β-oxidation. The decreased muscle MUFA content together with the reduced VSI of fish fed the 35P diet, suggests that FA are being moved to fuel β-oxidation, a process well established in fish in order to meet energy demands (Sargent *et al.* 2002). Although dietary treatments have to some extent altered some of the tissue FA composition, both muscle and liver FA profile were generally well within those previously found for this species fed a diet with similar FA composition (Figueiredo-Silva *et al.* 2009a,b). Higher linoleic acid (18:2n-6) and subsequently n-6 PUFA percentages were found in muscle and liver of fish fed the low protein diet reflecting the higher content of these FAs in diet 35P, due to the increase in wheat bran content (30%).

In the present work, the reduction of dietary nitrogen supply from 7.2 g to 5.6 g led to a reduction in all the lipogenic enzymatic activities (ME, G6PD and FAS). Dietary protein supply is recognized as a potent regulator of lipid biosynthesis in higher vertebrates (Herzberg & Rogerson 1981; Rosebrough *et al.* 1996) as well as in fish (Henderson & Sargent 1981; Shikata & Shimeno 1997; Alvarez *et al.* 1999; Dias *et al.* 1998, 2003), particularly in blackspot seabream (Figueiredo-Silva *et al*. 2009b). Moreover, the protein source has also been shown to modify body lipid content (Kaushik *et al.* 2004; Dias *et al.* 2005) and lipogenesis (Goméz-Requeni *et al.* 2003; Dias *et al.* 2005) in other fish species and in higher vertebrates (Iritani *et al.* 1986, 1996; Kayashita *et al.* 1996;

Padmakumarannair *et al.* 1998). The replacement of fish meal by high inclusions of corn gluten meal (Dias *et al*. 2005) or by corn and wheat gluten (Kaushik *et al*. 2004) appears to up-regulate FAS activities and increase body lipid content, but the effects of soy protein concentrates in hepatic lipogenesis showed to be high variably (Dias 1999). A significant interaction between P/L level and protein source (fish meal vs wheat gluten) was previously found to affect blackspot seabream lipid retention and lipogenesis (Figueiredo-Silva *et al.* 2009a). Accordingly, in the present study, a major role of dietary nitrogen source on blackspot seabream lipogenic pathways was evidenced by the ability of fish either fed 45P, AS or AG to show similar lipogenic enzyme activity levels and similar levels of 14:0 and 18:0 FA in the liver. These FA together with the 16:0 are the main newly-synthesized FA in fish (Corraze 2001; Sargent *et al.* 2002). Contrarily to fish fed AG, fish fed AS attained similar specific FAS activities to 45P fed fish, suggesting a further role on this lipogenic pathways. Hepatic lipogenic enzyme activities have been found to be down-regulated in gilthead seabream fed high dietary glutamic acid (Gómez-Requeni *et al.* 2003). Similarly, it was here demonstrated that the partial replacement of dietary fish meal nitrogen by a mixture of aspartic and glutamic acids mixture has in fact the ability to reduce the specific FAS.

Glycolytic pathways were unaffected by the surplus DAA dietary content as previously found on rainbow trout (Kirchner *et al.* 2003a) or gilthead seabream (Gómez-Requeni *et al.* 2003). The up-regulation of pyruvate kinase (PK) and reduction of plasma TAG levels with dietary nitrogen decrease attest the important role of dietary protein level on blackspot seabream (Figueiredo-Silva *et al.* 2009b) and rainbow trout (Kirchner *et al.* 2003b) glycolytic pathways regulation. The PK activation associated to a readily liver uptake of circulating TAG seems to result from a metabolic adaptation of fish fed 35P to a low dietary energy supply situation.

An excess of different individual DAA (alanine, aspartic or glutamic acid) failed to modify rainbow trout gluconeogenesis (Kirchner et al. 2003a), but as suggested by the authors, fish were fed protein-rich diets (53%) which in fact could possibly mask potential effects of dietary DAA on glucose metabolism since DAA are known to be synthesized in animal body, either from intermediates of cellular metabolism or by transamination of other AA (Li et al. 2009). Although, no distinction between cytosolic and mitochondrial PEPCK forms was made in our study, PEPCK total activities were well within values found for rainbow trout (Kirchner *et al*. 2003b), with the surplus dietary content on aspartic and glutamic acids shown to up-regulate PEPCK activities compared to 45P fed group. However, this

up-regulation did not result in a concomitant increase of plasma glucose levels. Moreover, at similar PEPCK activities, reduced glycaemia was verified on blackspot seabream fed 35P than in group fed the diet AG. Several works carried out in mammals suggests that PEPCK flux may interact with energy generation in the hepatic tricarboxylic cycle (TCA) (She *et al*. 2000; Burgess *et al*. 2004, 2007; Hakimi *et al*. 2005). PEPCK was indeed indentified as a critical enzyme for the synthesis of glycerol-3-phosphate required to re-esterify part of the FA coming from adipocytes hydrolyse, back to TAG (Forest *et al*. 2003; Chakravarty *et al*. 2005). Therefore, is seems that under unfavourable energy state and the concomitant FA β-oxidation activation, blackspot seabream up-regulate both PEPCK and PK. PEPCK produces phosphoenolpyruvate (PEP) and the PK converts it into pyruvate to uphold the pyruvate flux between mitochondria and cytosol in an attempt to generate energy through the TCA cycle, and not to produce glucose (gluconeogenesis). Furthermore, the augmented cortisol levels previously found in blackspot seabream fed a similar low protein diet (Figueiredo-Silva *et al*. 2009b) favour this hypothesis, given that amplified PEPCK activity and expression are found at high glcucocorticoids circulating levels (Gunn *et al*. 1975; Hanson & Reshef 1997). Hence, it would be of interest to study the PEPCK activity and expression responsiveness to different fish energy states.

The measurement of key hepatic enzymes activity involved in AA catabolism (ALAT, ASAT, GDH) has been pointed as an useful indicator of the metabolic utilization of dietary components by fish. However, the effect of dietary factors on the activity of these enzymes is often relatively contradictory within fish species (Cowey & Walton 1989). In blackspot seabream, the AA catabolic enzymes were generally within the activities found in species with alike protein requirements like gilthead seabream (Méton *et al.* 1999; Gómez-Requini *et al.* 2003, 2004; Enes *et al.* 2008a) or sea bass (Enes *et al.* 2006a). Nevertheless, in blackspot seabream the three main enzymes involved in AA catabolism displayed different responses to dietary nitrogen level and nature. Hepatic aminotransferases ALAT and ASAT and GDH deaminating enzyme showed higher activities in fish fed high protein diet (45P) than in those fed the low protein diet (35P), standing for the hypotheses that fish fed with higher levels of dietary protein use considerable amount of dietary AA for fatty acid synthesis or energy production. The up-regulation of ALAT by dietary protein level was also found in rainbow trout (Lupiañez *et al.* 1989), gilthead seabream (Méton *et al.* 1999) or in Nile tilapia (Gaye-Siessegger *et al.* 2006). Minor response of hepatic aminotranferases to dietary AA profile was noticed in gilthead seabream (Gómez-Requeni *et al.* 2003) or Nile tilapia (Gaye-Siessegger *et al.* 2007) while a reduction on the specific activity of GDH was observed in gilthead

seabream fed high dietary glutamic acid content (Gómez-Requeni *et al.* 2003). Conversely, high dietary levels of glutamic acid induced increased GDH activity in rainbow trout (Moyano *et al*. 1991). In blackspot seabream, AA catabolic enzymes have responded (IU/g liver) or showed a tendency (mIU/mg protein) to respond accordingly to dietary nitrogen nature, particularly to nitrogen provided by aspartic and glutamic acids mixture (AG). Further studies are needed to elucidate the role of DAA on these important AA catabolic enzymes.

Conclusion

Blackspot seabream juveniles have responded well to dietary crystalline supplementation showing the different role of DAA, alanine and serine (pyruvate precursors) and aspartic and glutamic acids (TCA intermediates), in terms of growth propose and intermediary metabolic pathways. The tendency of blackspot seabream juveniles to make more efficient use of the 1.6 g N provided by alanine and serine than that provided by aspartic and glutamic acid mixture in terms of growth. Although not statistically different, fish fed the diet AS showed a tendency to increased daily lipid gain and body lipid content compared to those fed AG diet, which probably has contributed to the superior gain of weight verified in those fish. Surplus dietary alanine and serine content appear to play a preponderant role in lipogenesis while aspartic and glutamic mix have in fact the ability to reduce the specific activity of FAS. Both, dietary nitrogen reduction (45P vs 35P) or its replacement by aspartic and glutamic acids mixture (diet AG) shown to up-regulate PEPCK activity but without increasing plasma glucose levels. Dietary nitrogen level and nature seems to exert a complex regulation on energetic pathways trough the gluconeogenesis/TCA interaction. This study evidenced different roles of DAA on fish metabolic pathways showing that besides IAA/DAA ratio also DAA must be taken into attention when fish meal is being replaced by plant protein sources.

CHAPTER 5

A same diet can be differently metabolized by blackspot seabream depending on the feeding method

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Abstract

Blackspot seabream growth and nutrient utilization was studied under self-feeding or hand feeding for 90 days. Groups of 31 fish with an initial body weight of 24 g were fed either by hand two times a day (09:00, and 18:00 h) to apparent satiety or by self-feeders. Final body weight and daily growth index were unaffected by the feeding method. However, the marked reduction in voluntary feed intake associated with similar nutrient gain on the self-fed group resulted in improved nutrient efficiency and in subsequent increased protein, lipid and energy retentions compared to fish hand-fed at set hours. The self-fed group displayed depressed malic (<62%) and fatty acid synthetase (<35%) activities as well as reduced triacylglycerol plasma levels, which correlated positively with feed intake and at some extent with fish lipid content. These results indicate the ability of blackspot seabream to adjust their lipid metabolism according to fish feeding rhythm. No effect of feeding method was however observed on glycolytic hepatic activities or on glucose, cholesterol and insulin plasma levels. Self-feeders led to similar growth but better efficiency, and hence, can be regarded as a helpful tool to optimize feed distribution according to this species natural rhythm. The maximal number of demands occurring between 20:00 and 21:00 h (dusk/sunset), together with the fact that 61% of the feed demands took place during the night, reveals a preferential crepuscular / nocturnal feeding pattern of this species.

Keywords: Diel feeding rhythm; Blackspot seabream; Feeding method; Glycolytic enzymes; Lipogenic enzymes; Nutrient utilization; Self-feeding

Introduction

Fish display daily cycles of feeding (Madrid *et al*. 2001), implying that the restriction of food availability to predefined day times could profoundly affect fish behaviour and physiology (Bolliet *et al*. 2001a) like it does in mammals (Froy 2007). Several studies have already demonstrated that feeding time affects fish growth performance (Boujard & Leatherland 1992a; Bolliet *et al*. 2001a; Bolliet *et al*. 2004; Velázquez *et al*. 2004) but the mechanisms underlying this process are far from being understood (Bolliet *et al*. 2001a; Madrid *et al*. 2001). In mammals, an endogenous circadian clock has been reported to regulate metabolism and energy homeostasis in the liver and peripheral tissues, by mediating the expression and/or activity of certain metabolic enzymes and transport systems (Hirota & Fukada 2004; Kohsaka & Bass 2007). Some examples are malic enzyme (ME), fatty acid synthetase (FAS) and glucose-6-phosphate dehydrogenase (G6PD) (Fukuda & Iritani 1991). Moreover, like in mammals, numerous hormones and metabolites involved in fish nutrient metabolism, showed daily fluctuations suggesting that fishes are not in the same physiological state throughout the day (Boujard & Leatherland 1992a; Spieler 1992; Boujard 2001). Plasma insulin levels are known to positively correlate with feed intake and ultimately to fish growth performances (Plisetskaya *et al*. 1991; Rungruangsak-Torrissen *et al*. 1999). Thus, given the feeding time implications on some physiological rhythms involved in nutrient metabolism, feeding fish in phase with their endocrine cycles might provide the best conditions to enhance growth (Boujard & Leatherland 1992a). Moreover, considering that feed represents the major aquaculture production cost (40-50%) (FAO 2007), the study of fish feeding rhythms would contribute for the reduction of feeding costs as well as the decrease in the waste from aquaculture systems. The self-feeding technique, that enable fish to feed on demand, has been largely used in the study of fish diel feeding pattern, including rainbow trout (Boujard & Leatherland 1992b; Sánchez-Vázquez & Tabata 1998; Bolliet *et al*. 2001b), European sea bass (Sánchez-Vázquez *et al*. 1995), turbot (Burel *et al*. 1997) and gilthead seabream (Sánchez-Muros *et al*. 2003), but was never used in blackspot seabream.

Blackspot seabream (*Pagellus bogaraveo*) has been considered a potential Mediterranean aquaculture species due to its high commercial value, excellent palatability and scarcity in the fishing grounds. A diet containing about 50% protein and 10% lipids was recently considered the most appropriated for blackspot seabream juveniles (Figueiredo-Silva *et al*. 2009a), but a suitable feeding strategy able to enhance growth and nutrient efficiency has never been evaluated in this species. Thus this work aims to evaluate two different feeding methods (hand feeding vs self-feeding) on blackspot seabream growth, nutrient utilization, plasma metabolites and hepatic lipogenic and glycolytic enzymes activities. In addition, the

depiction of the blackspot seabream diel feeding pattern will be of prime interest to fish farmers in order to establish species optimal feeding protocols.

Material and Methods

Experimental Diet

Table 1. Ingredient and proximate composition of the experimental diet

	Diet
Ingredients (%)	
Fish meal 67/10/15 Prime	48.0
Wheat Gluten	2.5
CPSP [a]	2.5
Squid meal	2.5
Gelatinized corn starch	25.0
Wheat bran	14.5
Fish oil	4.0
Vitamins and mineral mix [b]	0.25
Choline chloride [c]	0.1
Lutavin E50 [d]	0.05
Lutavin C35 [e]	0.05
Betafin S1 [f]	0.05
Europelin [g]	0.5
Proximate composition	
Dry matter (DM) (%)	91.1
Protein (%DM)	45.2
Lipids (%DM)	8.4
Energy (MJ/kg DM)	21.2
Starch (%DM)	23.6
Ash (%DM)	10.5

[a] Soluble fish protein concentrate.
[b] Vitamins (IU or mg kg^{-1} diet): Vitamin A, 8000 IU; vitamin D3, 1700 IU; vitamin K3, 10 mg; vitamin B12, 0.02 mg; vitamin B1, 8 mg; vitamin B2, 20 mg; vitamin B6, 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; nicotinic acid, 70 mg; pantothenic acid, 30 mg.
Minerals (g or mg kg^{-1} diet): Mn (manganese oxyde), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc oxide) 30 mg; Se (sodium selenite) 0.3 mg; Fe (iron sulphate), 60 mg; Ca (calcium carbonate), 2.15 g; dibasic calcium phosphate, 5 g; KCl, 1 g; NaCl, 0.4 g.
[c] Choline chloride, 1000 mg kg^{-1} diet.; [d] Vitamin E, 300 mg kg^{-1} diet.
[e] Vitamin C, 500 mg kg^{-1} diet; [f] Betain, 500 mg kg^{-1} diet; [g] Binder.

A diet containing about 50% protein and 10% lipids was recently considered the most appropriated for blackspot seabream juveniles (Figueiredo-Silva *et al.* 2009a). Hence, an

experimental diet was formulated to contain appropriated dietary protein/lipid levels for blackspot seabream juveniles, 45% protein and 10% lipids. All ingredients were supplied by Sorgal S.A. (Ovar, Portugal) and then finely grounded, mixed and dry pelleted through a 2.4-mm die at 50 ºC (CPM, C-300 model). Ingredients and proximate composition of the experimental diet is presented in Table 1.

Growth trial

Experiments were lead by trained scientists (following FELASA category C recommendations) and were conducted according to the European Economic Community animal experimentation guidelines, Directive of 24 November 1986 (86/609/EEC).

Blackspot seabream juveniles (*Pagellus bogaraveo*) were obtained from a fish farm (Grupo Isidro de la Cal, Valdoviño, Coruña, Spain), and acclimated to the experimental conditions for four weeks before the beginning of the trial. The growth trial was conducted at the experimental facilities of CIIMAR, Porto. Homogenous groups of 31 juveniles with an average initial body weight (IBW) of 24 g (Table 2) were randomly distributed among 6 square fibre glass tanks (500-l), in a close circulation water system. Triplicate groups of fish were fed either by hand two times a day (09:00 and 18:00 hours) to apparent satiety or by self-feeders during 90 days. Fish were considered satiated (apparent satiety) when the first unextruded pellets (high density pellets) reached the bottom of the tank. At that moment, feeding distribution was stopped in order to avoid feed waste. Three of the six tanks (self-feeding groups) were equipped with a self-feeder connected both to an individual control box and to a computer (Innovaqua, Sevilla, Spain). This equipment has permitted to monitor and record the demand feeding activity, over the entire trial. The feeder consisted on a rotating food container fitted with a cylindrical plastic trigger sensor (2-mm die). The sensor was located 3-cm below water surface and fish had to voluntary bite and pull the trigger sensor for activation to occur. Demand for food was satisfied on a 24 h basis and reward level was set to 1 g feed/kg fish, per trigger activation. The self-feeding groups were also automatically fed 1% of the fish biomass per tank per day (divided in two daily rations: 09:00 and 18:00 h) until they learned how to operate the sensor, that is, when and the request for food approached food intake displayed by hand (approximately two to seven days). Each day, all tanks (hand and self-fed) were monitored to be certain that all feed was eaten and hence, no feed lost was registered. When some uneaten food remained in the bottom of the tank, the reward level was accurately adjusted to avoid feed losses.

Each tank was supplied with filtered, heated (19 ± 1ºC) saltwater (33 g/l), with dissolved oxygen above 8 mg/l at a flow rate of 2 l/min. The pH, ammonia, nitrites and nitrates in the

water were monitored during the entire trial and maintained at levels compatible with marine species. Blackspot seabream juveniles were exposed to natural photoperiod (12L:12D; sunrise 06:00 h and sunset 18:00 h), and the experience has been carried out between February and April 2007.

Every four weeks, fish were bulk weighted under moderate anaesthesia (ethylene glycol monophenyl ether at 50 ppm) and data on weight gain and feed distribution were recorded. At the beginning and at the end of the feeding trial, a pooled sample of 9 fish per treatment were taken and stored at -20ºC for the subsequent whole body composition analyses. At the end of the experiment, the self-feeding equipment was turn off and both groups, those fed by hand or by self-feeders were starved for 24 h. A single meal was then distributed by hand to all groups of fish. Blood collection was carried out in 12 fish per treatment at three postprandial different times (2; 4 and 24 h), and in <3-min to avoid plasmatic metabolites response induced by handling. Samples were taken from the caudal vein, with syringes and collecting tubes containing 15-20 µl of sodium fluoride and potassium oxalate (4% each). Plasma was obtained after centrifugation (6000 x*g* for 10 min at 4ºC) and stored at -80ºC until further glucose, cholesterol, triacylglycerol, and insulin analysis. At the 24 h postprandial time, besides blood also muscle and liver tissues were collected for total lipid content (9 fish per treatment), lipogenic (9 fish per treatment) and glycolytic (6 fish per treatment) hepatic enzymes activities. Liver and viscera of those fish were weighted for calculation of hepatosomatic (HSI) and viscerosomatic index (VSI). All samples were immediately frozen in liquid nitrogen and stored at -80ºC for subsequent individual analyses.

Analytical methods

Feed and body composition analyses

Whole fish from each tank were pooled, ground and fresh moisture content was determined. Fish were subsequently freeze-dried before further analysis. Feed and whole body samples were analyzed for dry matter (105 ºC for 24 h), ash by combustion in a muffle furnace (550 ℃ for 12 h), crude protein (Micro-Kjeldahl; N x 6.25) after acid digestion, lipid content by petroleum ether extraction (at Soxhlet 40-60 ºC), and gross energy in an adiabatic bomb calorimeter (IKA, Werke C2000). Dietary starch content was determined according to Thivend *et al.* (1972). Total lipids were extracted from muscle and liver samples and measured gravimetrically according to Folch *et al.* (1957), using dichloromethane instead of chloroform.

Enzymatic analyses

Liver samples for lipogenic activity measurement were homogenised in three volumes of ice-cold buffer (0.02 mol/L Tris–HCl, 0.25 mol/L sucrose, 2 mmol/L EDTA, 0.1 mol/L NaF, 0.5 mmol phenylmethyl sulphonyl fluoride, 0.01 mol/L β-mercaptoethanol, pH 7.4) and centrifuged at 30 000 x*g*, at 4 ºC for 20 min. Selected lipogenic enzyme activities were assayed on supernatant: glucose-6-phosphate dehydrogenase G6PD (EC 1.1.1.49) according to Bautista *et al.* (1988), malic enzyme (ME, EC 1.1.1.40) according to Ochoa (1955), and fatty acid synthetase (FAS, EC 2.3.1.38) according to the methodology of Chang *et al.* (1967) and modified by Chakrabarty & Leveille (1969).

Liver samples for glycolytic enzymes measurements were homogenized in five volumes of ice-cold buffer (80mM Tris; 5mM EDTA; 2mM DTT; 1mM benzamidine; 1mM 4-(2-aminoethyl) benzenesulfonyl fluoride, pH 7.6) and centrifuged at 900 x*g* at 4 ºC for 10 min. The resultant supernatant was separated in two fractions, a first one for hexokinase (HK; EC 2.7.1.1) and glucokinase (GK; EC 2.7.1.2) activity determinations and a second one to measure L-type pyruvate kinase (PK; EC 2.7.1.40) activity. The HK (low K*m* HKs) and GK (high K*m* HK or HK IV) activities were measured at 37 ºC, using 0.5mM and 100mM of glucose, respectively, as previously described (Tranulis *et al.* 1996; Panserat *et al.* 2000a). The assay for measuring GK activity on frozen samples needs correction by measuring glucose dehydrogenase (EC 1.1.1.47) activity as described by Tranulis *et al..* (1996). PK activities were assayed in the supernatant resulting from a second centrifugation at 10 000 x*g* at 4 ºC for 20 min, according to Foster & Moon (1986).

Enzyme activity units IU, defined as µmoles of substrate converted to product, per min, at assay temperature, were expressed per mg of hepatic soluble protein (specific activity) or per gram of tissue. Soluble protein content of tissues was determined on supernatant by the method of Bradford (1976), using bovine serum albumin (BSA) as standard (Sigma, St Louis, MO, USA).

Plasma metabolites assays

Plasma glucose, cholesterol (CHOL) and triacylglycerol (TAG) were determined using enzymatic commercial kits: Glucose RTU (nº 61269); Cholesterol RTU (nº 61218) and Triglycérides Enzymatique PAP 150 (nº 61236) from Bio-Mérieux, Marcy-L'Etoile, France. Plasma insulin levels were measured by radioimmunoassay using bonito insulin as the standard and rabbit anti-bonito as antiserum, following the method described by Gutiérrez *et al.* (1984) previous validated for blackspot seabream (Figueiredo-Silva *et al.* 2009b).

Statistical analysis

Statistical analyses followed methods outlined by Zar (1996) and were determined using the STATISTICS 7.0 package (StatSoft, Inc., Tulsa, OK, USA). All data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov and Bartlett tests, and then submitted to a One-way ANOVA. When these tests showed significance (P<0.05), individual means were compared using Duncan´s test. Correlation coefficients were obtained by the Pearson Product Moment Correlation Distribution and considered significant when P<0.05. Tank average values for feed intake, growth, body composition, nutrient accretion analysis were used as experimental units for statistical analyses.

Results

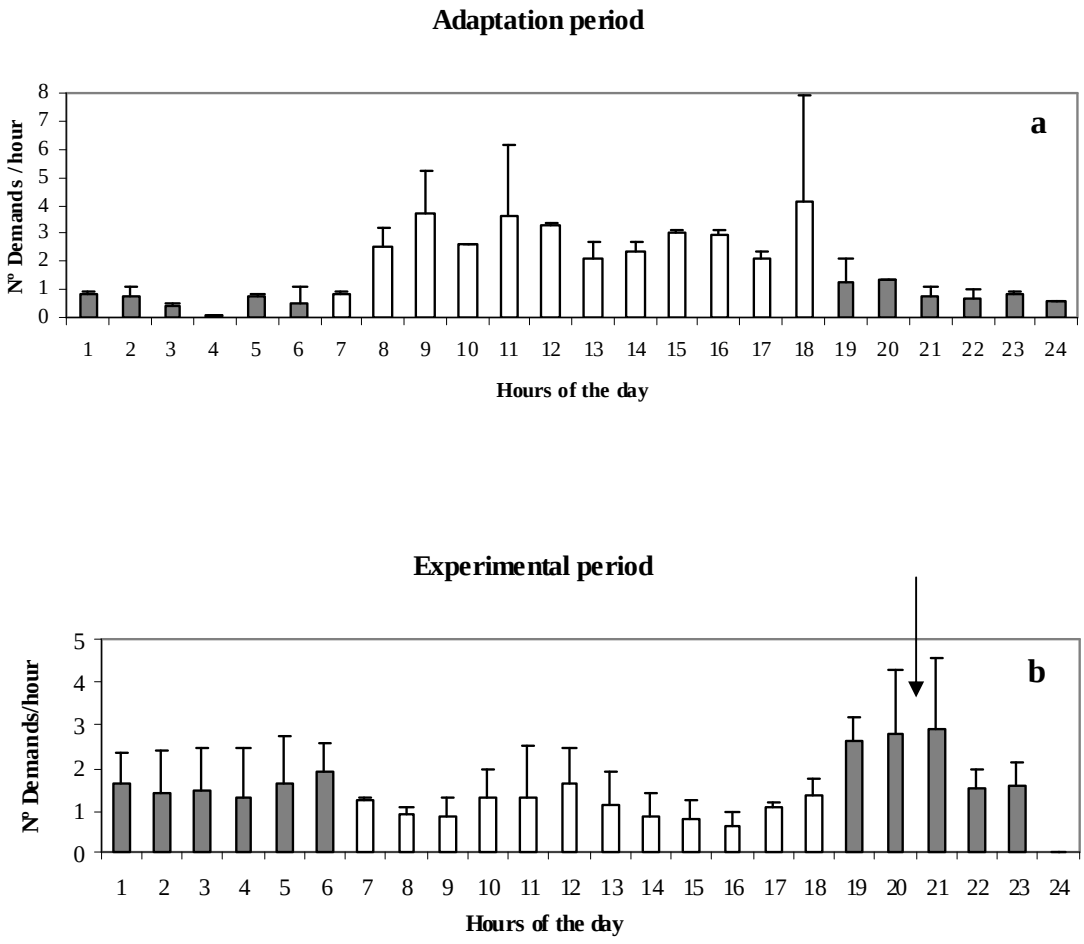

Figure 1. Blackspot seabream feeding rhythm along the **a**) adaptation period (30 days) and the **b**) following 60 days of experiment.
Values are means ± standard deviation (n=3).
The black and white bars represent nocturnal and diurnal demands, respectively.
The maximum demands period is signalled by an arrow in Fig.1-b
To make clear the figure, statistical differences are displayed in Table 6.

Blackspot seabream juveniles have rapidly learned (two to seven days) how to operate the self-feeding system described in the Material and Methods section. Nevertheless, a 30 days period was required until fish presented a constant number of demands per day which was hence considered the adaptation period. Feed released was rapidly consumed by fish without, as far as we could observe, any feed waste. During the adaptation period the maximum number of demands (Table 2) has been verified near the sunset (18:00 h), but fish have predominantly showed a diurnal feeding rhythm (Fig. 1-a) with 69% of the demands taking place during the day. Nevertheless, fish have progressively phase-shift their feeding pattern (Fig. 2-a) and data recorded during the experimental period showed that 61% of the feed demands became nocturnal (Fig. 1-b and 2-b). In general terms, the number of demands per hour decreased after the sun rise, showed a slight peak at midday, decreasing once more during the afternoon. The maximum number of demands verified after sunset, between 20:00 and 21:00 h, was significantly different from those verified on the period that goes from 6:00 to 11:00 h and from 12:00 to 18:00 h (P=0.017, Table 2). In the self-feeding groups, the daily number of feed demands increased linearly with time without significant differences among triplicates (P=0.133).

The growth performance (Table 3) of the fish was generally good for this species (fish practically triplicate their body weight over 90 days) and no mortality was recorded over the experimental period. At the end of the growth trial, the variation in fish size among tanks fed by hand or under self-feeders were found to be similar (P=0.638). No significant effect of the feeding method was observed on final body weigh (FBW, 72-73 g) or daily growth index (DGI, 1.4-1.5). However, fish fed by self-feeders displayed a significantly lower voluntary feed intake (VFI, 1.1 *vs* 1.7) and reduced feed conversion rate (FCR, 1.0 *vs* 1.5) compared to fish fed by hand. This reduced FCR indicates that all of demanded feed was ingested. Furthermore, the self-feeding method has clearly improved protein efficiency ratio (PER, 2.3 *vs* 1.5).

Blackspot seabream nutrient gain, whole body composition (18% protein; 13% lipid, %ww), hepatosomatic index (HSI, 1.0%), viscerosomatic index (VSI) (7.5 to 7.9%), muscle and liver lipid content (4 and 14 to 17%ww, respectively) were not significantly affected by feeding method (Table 4). Although fish fed by self-feeders presented significantly lower nitrogen, lipid and starch intakes, the similarity observed for nutrient gain has resulted in a noticeably enhancement of nutrient retentions.

The effects of feeding method (hand feeding vs self-feeding) on blackspot seabream hepatic lipogenic and glycolytic enzymes activities are presented in Table 5. Blackspot

seabream juveniles fed by self-feeders markedly depressed malic enzyme (ME, 62%) and fatty acid synthetase (FAS, 35%) activities while no significant effect of feeding method was observed on glucose-6-phosphate dehydrogenase (G6PD). In addition, ME and FAS activities were positively correlated with VFI and negatively correlated with PER data (Table 7). On the other hand, no effect of feeding method was observed on hexokinase, glucokinase or pyruvate kinase (HK, GK, PK) hepatic activities (Table 5). In addition, similar glucose, cholesterol and insulin plasma levels were obtained irrespective of the feeding method (Table 6). However, in fish fed by self-feeders significantly lower triacylglycerol levels were found at 4 h postfeeding, when compared with hand fed ones. Like ME activities, the triacylglycerol levels verified at 4 h post feeding were positively correlated with VFI (Table 7).

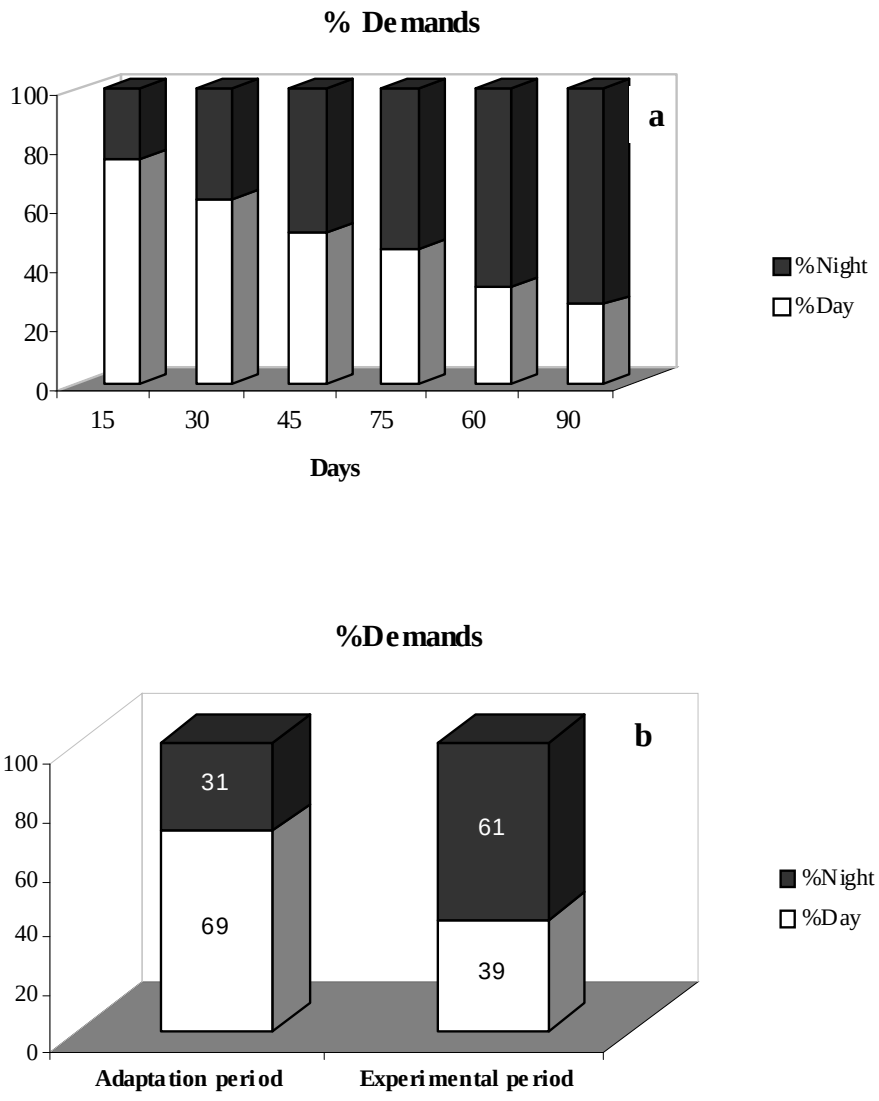

Figure 2. Day and night percentage (%) of feed demands a) at each 15 days of the entire feeding period and b) for the adaptation and experimental period independently.

In addition, ME and FAS activities were positively correlated with VFI and negatively correlated with PER data (Table 7). On the other hand, no effect of feeding method was observed on hexokinase, glucokinase or pyruvate kinase (HK, GK, PK) hepatic activities (Table 5). In addition, similar glucose, cholesterol and insulin plasma levels were obtained irrespective of the feeding method (Table 6). However, in fish fed by self-feeders significantly lower triacylglycerol levels were found at 4 h postfeeding, when compared with hand fed ones. Like ME activities, the triacylglycerol levels verified at 4 h post feeding were positively correlated with VFI (Table 7).

Table 2. Number of feeding demands per hour made by blackspot seabream during the adaptation period (30 days) and the following 60 days of experiment.

	Nº Demands/hour	
	Adaptation period	**Experimental period**
Hour		
1	0.8±0.1 ^{cde}	1.7±0.7 ^{abcd}
2	0.8±0.4 ^{cde}	1.4±1.0 ^{abcde}
3	0.5±0.1 ^{de}	1.5±0.9 ^{abcde}
4	0.1±0.1 ^{e}	1.3±1.1 ^{bcde}
5	0.8±0.1 ^{cde}	1.6±1.1 ^{abcd}
6	0.5±0.6 ^{de}	1.9±0.7 ^{abcd}
7	0.9±0.1 ^{cde}	1.3±0.1 ^{bcde}
8	2.5±0.7 ^{abcde}	0.9±0.2 ^{de}
9	3.7±1.6 ^{ab}	0.9±0.5 ^{de}
10	2.6±0.0 ^{abcde}	1.3±0.6 ^{bcde}
11	3.7±2.5 ^{ab}	1.3±1.2 ^{bcde}
12	3.3±0.1 ^{abc}	1.6±0.8 ^{abcd}
13	2.1±0.6 ^{abcde}	1.1±0.8 ^{cde}
14	2.3±0.4 ^{abcde}	0.9±0.5 ^{de}
15	3.0±0.1 ^{abc}	0.8±0.5 ^{de}
16	3.0±0.2 ^{abcd}	0.7±0.3 ^{de}
17	2.2±0.2 ^{abcde}	1.1±0.1 ^{cde}
18	4.1 ± 3.8 ^{a}	1.4±0.4 ^{abcde}
19	1.3±0.9 ^{bcde}	2.6±0.6 ^{abc}
20	1.4±0.0 ^{bcde}	2.8±1.4 ^{ab}
21	0.8±0.4 ^{cde}	2.9±1.6 ^{a}
22	0.7±0.3 ^{abcde}	1.5±0.5 ^{abcde}
23	0.9±0.1 ^{cde}	1.6±0.6 ^{abcde}
24	0.6±0.0 ^{cde}	0.0±0.0 ^{e}
ANOVA P-value	0.008	0.017

Values are means ± standard deviation (*n*=3). Each one of the self-fed group was constituted by 31 blackspot juveniles.

^{a,b,c,d,e} Mean values within a column unlike superscript symbols indicates significantly differences between time within treatment (P<0.05).

Discussion

Blackspot seabream juveniles showed a high capacity to use self-feeders like has already been demonstrated for rainbow trout (Boujard & Leatherland 1992b), gilthead seabream (Sánchez-Muros *et al*. 2003) and European sea bass (Sánchez-Vázquez *et al*. 1995). The maximal number of demands occurring between 20:00 and 21:00 h (dusk/sunset), together with the fact that 61% of the feed demands took place during the night, reveals a preferential crepuscular / nocturnal feeding pattern of this species, at least during this period of the year (February-April). Moreover, this feeding behaviour is similar to that previously reported for gilthead seabream (same family: *Sparidae*) at similar photoperiod conditions (February to March) (Sánchez-Muros *et al*. 2003). In fish, diel feeding activity is highly flexible and can 'phase shift' according to numerous factors such as food availability, fish ability to operate the feeder, reward level, photoperiod, water temperature, stocking density and social dominance (Madrid *et al*. 2001; Valente *et al*. 2001; Reebs 2002). Species like gilthead seabream (Sánchez-Muros *et al*. 2003), European sea bass (Sánchez-Vázquez *et al*. 1998; Boujard *et al*. 1996; Azzaydi *et al*. 2000), salmon (Smith *et al*. 1993) and yellowtail (Kohbara *et al*. 2003) exhibits flexibility in their feeding rhythms, being predominantly diurnal in summer and nocturnal in winter.

Table 3. Effect of feeding method (hand-feeding vs self-feeding) on blackspot seabream final growth performance

	Feeding method		ANOVA
	Hand-feeding	**Self-feeding**	**P-value**
Growth			
Initial body weight (g)	23.8±0.2	23.8±0.1	0.947
Final body weight (g)	73.3±1.9	71.8±3.4	0.550
FCR [a]	1.5±0.03	1.0±0.1	0.004
DGI [b]	1.5±0.03	1.4±0.1	0.541
PER [c]	1.5±0.03	2.3±0.3	0.013
VFI [d]	1.7±0.01	1.1±0.2	0.006

Values are means ± standard deviation (n = 3).
[a,b] Mean values within a row with different superscript letters are significantly different (P<0.05).
[a] FCR, Feed conversion ratio = dry feed intake/weight gain.
[b] DGI, Daily growth index = 100 x ((Final body weight)$^{1/3}$ - (Initial body weight)$^{1/3}$)/days
[c] PER, Protein efficiency ratio = weight gain/crude protein intake.
[d] VFI, Voluntary feed intake = 100 x crude feed intake/average body weight/day

Therefore, future studies regarding species ability to 'phase shift' their feeding activity accordingly to different environmental conditions should be considered.

Table 4. Whole body composition (% wet weight (ww) or MJ/kg ww), nutrient intake, gain and retention, in blackspot seabream fed by two different feeding methods (hand-feeding vs self-feeding)

	Feeding method		ANOVA
	Hand-feeding	**Self-feeding**	**P-value**
Final body composition [a]			
Moisture (%)	64.1±1.8	65.1±1.7	0.486
Protein (%)	17.8±0.8	17.5±1.0	0.686
Lipid (%)	13.4±1.3	12.7±1.2	0.527
Energy MJ/kg	9.1±0.7	8.7±0.7	0.549
Muscle total lipids			
(%)ww	3.8±0.7	3.8±1.3	0.957
Liver total lipids			
(%)ww	17.2±2.1	14.1±2.9	0.209
HSI % [b]	1.1±0.1	1.2±0.3	0.204
VSI % [c]	7.5±2.0	7.9±1.2	0.662
Intake (g/Kg ABW [d]/day)			
Nitrogen	1.2±0.01	0.8±0.2	0.006
Lipids	1.4±0.01	0.9±0.2	0.006
Starch	4.0±0.02	2.5±0.5	0.006
Gain (mg (g)/Kg ABW [d]/day)			
Nitrogen (mg)	335.6±22.0	322.0±18.2	0.457
Lipids (g)	1.4±0.2	1.3±0.2	0.475
Retention % of intake			
Protein	27.6±2.0	42.5±8.0	0.036
Lipid	101.3±16.8	148.4±14.3	0.030
Energy	28.5±3.7	41.6±5.1	0.022

Values are means ± standard deviation (n = 3).
[a,b] Mean values within a row with different superscript letters are significantly different (P<0.05).
[a] Initial body composition was: moisture 64.2 %; protein 16.3 % (DM); lipid 14.9 (DM) and energy 9.4 MJ/kg
[b] HSI, Hepatosomatic index = 100 X liver weight/body weight
[c] VSI, Viscerosomatic index = 100 X weight of viscera/body weight
[d] ABW, Average body weight = (final body weight + initial body weight)/2

When fish are fed using self-feeders, growth and feed conversion are expected to be improved because in such conditions fish are able to regulate feed intake accordingly to their energy demands (Kaushik & Médale 1994) and endogenous feeding rhythms (Boujard & Leatherland 1992a). In farmed fish, self-feeders lead to better feed conversion rates, and reduce the amount of uneaten feed pellets, being a useful tool to optimize feed distribution in several fish species (Alanärä 1992, 1996; Madrid *et al*. 1997; Paspatis *et al*. 2000; Sánchez-Muros *et al*. 2003). Similarly, the present study clearly showed that the self-feeding method improved blackspot seabream protein efficiency (PER) and feed conversion ratios compared to fish hand-fed at set hours. This improvement was mainly achieved by a reduced voluntary feed intake (VFI), and consequently a lower nitrogen and lipid intake. Moreover, the positive correlation obtained between VFI and malic enzyme (ME), fatty acid synthetase (FAS) and triacylglycerol levels at 4 h postfeeding suggest a regulation of feed intake according to fish endogenous feeding rhythms as previously suggested (Boujard & Leatherland 1992a), rejecting the idea of an overfeeding situation in fish fed by hand. Thus, and similarly to rainbow trout (Gélineau *et al*. 1998) and African catfish (Hossain *et al*. 2001), blackspot seabream apparently makes a more efficient use of dietary protein for growth when fed in phase with their natural feeding rhythm.

Table 5. Effects of feeding method (hand-feeding vs self-feeding) on blackspot seabream hepatic lipogenic and glycolytic enzymes activities (mIU/mg protein)

	Feeding method		ANOVA
	Hand-feeding	**Self-feeding**	**P-value**
Lipogenic enzymes			
ME	33.9±9.1	13.0±6.6	0.0001
G6PD	124.8±26.4	109.3±29.7	0.255
FAS	29.7±7.3	19.2±6.4	0.007
Glycolytic enzymes			
HK	0.4±0.2	0.2±0.2	0.226
GK	1.1±0.4	1.0±0.5	0.823
PK	191.7±62.1	187.9±41.6	0.900

Values are means ± standard deviation) (n=9 for lipogenic and n=6 for glycolytic enzymes)
[a,b] Mean values within a row with different superscript letters are significantly different (P<0.05).

Despite the better feed conversion rates, blackspot seabream growth rates were unaffected by the feeding method (hand feeding vs self-feeding). Therefore, the restriction of food availability to predefined day times (9.00 and 18:00 h), and hence the unadjustment of feeding times to fish natural biological rhythm, has not affected this

species growth performance. By contrast, feeding time has been reported to affect growth performances in several other fish species (Boujard & Leatherland 1992a; Bolliet *et al*. 2001a; Bolliet *et al*. 2004; Velázquez *et al*. 2004).

Table 6. Postprandial plasmatic glucose (mg/dL), insulin (ng/mL), total cholesterol and triacylglycerol (g/L) in blackspot seabream by two different feeding methods (hand-feeding vs self-feeding)

		Feeding method		ANOVA
Time (h)	Plasma metabolite	Hand-feeding	Self-feeding	P-value
2	Glucose	61.9±8.0‡	57.4±8.2‡	0.254
	Insulin	8.9±1.3 †	7.4±2.1	0.053
	Cholesterol	1.9 ± 0.7‡	2.5±0.4	0.053
	Triacylglycerol	3.2±1.2 ‡	2.1±1.1 ‡	0.047
4	Glucose	90.6±27.4 †	79.7±20.8 †	0.325
	Insulin	6.8±1.2‡	6.9±1.0	0.790
	Cholesterol	2.8±0.5†	2.5±0.7	0.273
	Triacylglycerol	5.2±0.9 †	2.8±0.7 †‡	0.00001
24	Glucose	61.0±20.5‡	61.5±18.2‡	0.955
	Insulin	6.8±1.8‡	5.9±1.7	0.217
	Cholesterol	2.9±0.7†	2.7±0.5	0.351
	Triacylglycerol	3.2±0.8‡	3.3±1.1†	0.884
Anova p-value	Glucose	0.009	0.007	
	Insulin	0.001	0.088	
	Cholesterol	0.001	0.294	
	Triacylglycerol	0.001	0.007	

Values are means ± standard deviation (n = 12).
^{a,b} Mean values within a row with different superscript letters are significantly different (P<0.05).
†‡ Mean values within a column unlike superscript symbols indicates significantly differences between time within diets(P<0.05).

Previous studies have reported that the feeding method has no effect on fish whole body composition (Azzaydi *et al*. 1998; Valente *et al*. 2001; Velázquez *et al*. 2006). Blackspot seabream whole body composition, nutrient gain, and tissue (muscle and liver) lipid contents were unaffected by the feeding method (hand-feeding vs self-feeders), but the marked feed intake reduction on self-fed group resulted in increased protein, lipid and energy retentions. It seems, therefore, that the adjustment of feeding times to match species natural rhythm has improved feed and nutritional conversion efficiency as previously observed in rainbow trout (Gélineau *et al*. 1998; Bolliet *et al*. 2001b), European

catfish (Bolliet *et al*. 2001b) and African catfish (Hossain *et al*. 2001). The distinct hepatic lipogenic enzymes activities displayed by blackspot seabream corroborate the suggestion that a same diet offered at different times of the day can be metabolized differently by the adjustment of digestive and/or metabolic enzymes (Madrid 1994; Carter *et al*. 2001). The decreased ME and FAS activities of fish fed by self-feeders, did not affect liver or body lipid content although a positive correlation between FAS and lipid body content and gain (Pearson correlation = 0.93 and 0.94, respectively) was found. The greater the amount of food eaten by fish, the greater will be the activity and concentrations of enzymes involved in cellular metabolism (Carter *et al*. 2001). Indeed, both ME and FAS were positively correlated with VFI and negatively with PER. Moreover, it was recently demonstrated that daily nitrogen intake has a preponderant role on the regulation of this species lipogenic and glycolytic pathways regulation (Figueiredo-Silva *et al*. 2009b). Nevertheless, the reason why feeding method has significantly reduced the lipogenic but not the glycolytic enzymatic activity warrants further clarification.

Table 7. Selected correlations obtained between nutrient retention, hepatic enzyme activities and plasma metabolites and voluntary feed intake (VFI),feed conversion ratio (FCR) and protein efficiency ratio (PER)

	VFI	FCR	PER
Nutrient retention %			
Protein	**-0.95**	**-0.93**	**0.95**
Lipid	-0.88	-0.85	0.83
Lipogenic enzymes			
ME	**0.90**	**0.93**	**-0.90**
G6PD	0.74	0.72	-0.69
FAS	0.86	**0.89**	-0.88
Glycolytic enzymes			
HK	0.67	0.64	-0.61
GK	0.52	0.47	-0.52
PK	-0.15	-0.11	0.13
Plasma metabolites (4h)			
Insulin	0.15	0.10	-0.21
Glucose	0.46	0.46	-0.47
Triacylglycerol	**0.96**	**0.96**	**-0.94**

Highlighted values refer to the most significant correlations found (Pearson Product Moment Correlation).

Numerous studies have evidenced that fish are not in a same physiological state throughout the day (Boujard & Leatherland 1992a; Boujard 2001). Moreover, the time of

the meals is considered one of the main synchronizers of hormonal fluctuations in teleosts (Boujard & Leatherland 1992a; Gélineau *et al*. 1996). Despite the differences implicit by feeding method on blackspot seabream feeding times (hand feeding vs self-feeding), similar glucose, cholesterol and insulin plasma levels were obtained among groups. On other hand, self-fed groups presented significantly lower triacylglycerol levels at 4 h postfeeding, which is probably related with the lower feed intake verified in those fish. The absence of an effect of feeding method on insulin corroborates glucose and glycolytic enzymes results.

The present work has described for the first time blackspot seabream diel feeding patterns. Even though, longer experiments (12 months) should be carried out to accurately determine the influences of seasonal changes, natural photoperiod and water temperature on blackspot seabream self-feeding behaviour. It has been demonstrated that self-feeders clearly improved protein, lipid and energy retentions and feed conversion ratios compared to fish hand-fed at set hours. The self-fed group displayed markedly depressed malic (<62%) and fatty acid synthetase (<35%) enzymes, and triacylglycerol plasma levels, which correlated positively with feed intake and at some extent with fish lipid content, suggesting a regulation of the lipid metabolism according to fish feeding rhythms. Nevertheless, the feeding method has not significantly affected the glycolytic enzymatic activity or on glucose, cholesterol and insulin plasma levels. This ability of fish to adapt their metabolism according to the feeding method could lead to notorious subsequent differences in growth if a longer-term study is considered. In the present study, the self-feeders led to similar growth but better efficiency than fish fed by hand, and hence, can be regarded as a helpful tool to optimize feed distribution according to this species natural rhythm. The maximal number of demands occurring between 20:00 and 21:00 h (dusk/sunset), together with the fact that 61% of the feed demands took place during the night, reveals a preferential crepuscular / nocturnal feeding pattern of this species.

CHAPTER 6

One of the most important requirements for the introduction of a new aquaculture species is the establishment of a specific feed formulation, able to meet the nutritional needs of a fish. A correct balance among protein, lipids and carbohydrates would result in their optimal use, with a consequent positive repercussion on growth, feed utilization and metabolic pathways. Taking into account that protein is the most expensive ingredient and incorporated at higher levels in diets for carnivorous species, a correct macronutrient balance should assure that dietary protein is being chiefly utilized for protein accretion and less for energy-production and/or fat deposition processes. Based on the available data, this Thesis aimed to give an insight into the nutritional regulation of the mechanisms beyond blackspot seabream energetic metabolism, as a first attempt to understand the basis for its excessive fat accumulation and slow growth performance. In this last Chapter, an overall and transversal approach of the main results was adopted to discuss the relative contribution of each macronutrient to this species lipid deposition and growth.

Nutritional assumptions behind dietary formulation options

The first studies carried out on blackspot seabream mostly refers to preliminary experiments conducted on individuals caught in the natural environment and have mainly dealt with reproduction and disease control, larvae and juveniles culture techniques (Chereguini *et al*. 1990; Peleteiro *et al.* 1994,1997, 2000; Genovese *et al.* 1998; Olmedo *et al.* 1998; Micale *et al.* 2002). The protein requirement for blackspot seabream maximal growth was estimated to be above 400 g Kg^{-1} of the diet (Silva *et al*. 2006), and thus well within values observed for seabass and seabream (Hidalgo & Alliot 1988; Santinha *et al.*1996; Vergara *et al.*1996). Moreover, the protein requirement calculated for blackspot seabream maintenance, 4.3 g kg^{-1} day^{-1} (Fig. 1; Silva *et al.* 2006), is quite higher than that reported for others carnivorous species like rainbow trout (2.6 g kg^{-1} day^{-1}) (Kaushik & Gomes 1988), gilthead seabream (0.86 kg^{-1} day^{-1}) (Lupatsch *et al.* 1998) or European seabass (2.0-2.8 g kg^{-1} day^{-1}) (Hidalgo & Alliot 1988; Ballestrazzi *et al*.1994). These results are in general accord with subsequent studies evidencing that blackspot seabream may grow slower than the other close-related species because a major part of its dietary protein energy is being used for lipid deposition (Ozório *et al.* 2009).

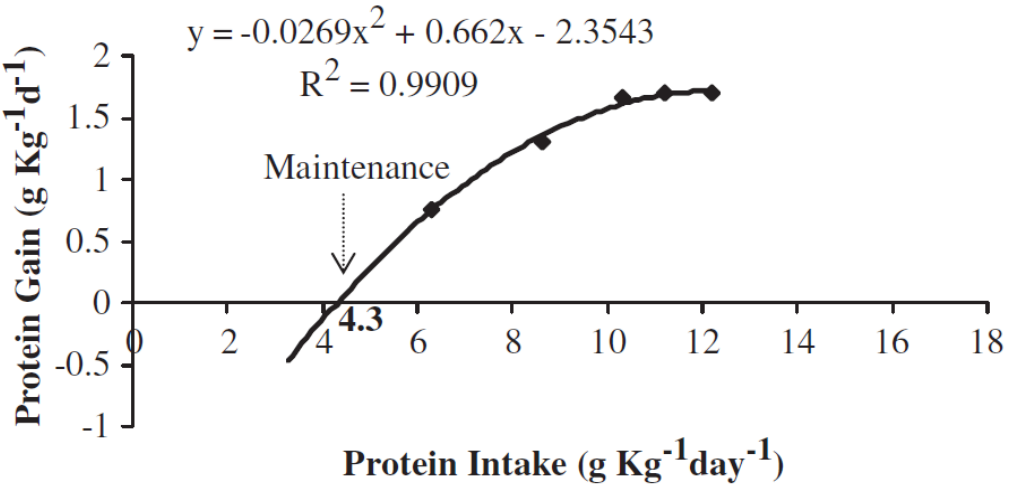

Figure 1. Relation between protein intake and protein gain in blackspot seabream, with an estimation of maintenance and growth needs by a polynomial model (Silva *et al.* 2006).

These reduced growth rates associated with the high propensity of this species to deposit fat (18-21% wet weight), even at a low dietary lipid incorporation level (12%), prompt us to start testing two high protein (50 or 60%)/ low lipid (6 or 10%) dietary level as an attempt to maximize this species growth whilst reducing its excessive fat accumulation. **As no improvement of blackspot seabream growth performances was accomplish,** and considering the major role of protein level as a regulator of lipid biosynthesis in fish (Chapter 3; Campbell *et al.* 1998; Walzem *et al.* 1991; Dias *et al.* 1998, 2003), **a dietary protein/lipid ratio of 45/10 was further considered as the most appropriate for this species.** An extreme diet containing a lower protein level (35%), but maintaining the dietary lipid level at 10%, was also formulated to clearly evidence the possible additive lipogenic effects of dietary protein.

Being the general goal of fish farmers to support the highest fish performance at the lowest cost, ingredients are usually chosen on the basis of cost, availability, chemical composition, and nutritional value (Hardy & Barrows 2002). In some cases, such is the research one, the selection of ingredients firstly depends on the purpose of the study. In the present Thesis, the selection of feedstuffs was based on their nutritional value and market availability rather than on feed cost. Diets were formulated to assure a high nutrient availability by selecting high quality and highly digestible feed ingredients which although more expensive were essential to stimulate feed intake and maximize fish growth rates.

Blackspot seabream n-3 HUFA requirement has not yet been defined, but in gilthead seabream it was estimated as 3% (Montero *et al*. 1996; Lanari *et al*. 1999). Hence, at dietary lipid incorporation levels as low as 6 or 10%, fish oil was selected as the most suitable dietary lipid source to satisfy this species essential fatty acid requirement. Dietary energy provided by carbohydrate rich cereal grains, like wheat and corn, has been extensively utilized in aquaculture feed manufacturing in order to minimize the amount of protein used. Nevertheless, in carnivorous fish species, the capacity to digest complex carbohydrates is limited and mainly depends on the carbohydrate source and type, on its dietary inclusion level and on the technological treatment applied (gelatinization or extrusion) (Wilson 1994; Hemre *et al*. 2002; Stone 2003). In view of their market availability, either as raw or gelatinized form, corn rather than other carbohydrate rich cereal grain has been included into blacktop seabream experimental formulas. A maximal inclusion level of 20% dietary digestible carbohydrate has been suggested for marine fish species (Wilson 1994), but a level up to 25% was tested in blackspot seabream as an attempt to detect any possible effect of carbohydrates on lipid metabolism without significantly compromise growth. Wheat sub-products such as micronized wheat or wheat bran have been included into some of the experimental formulas for their nutritive binder properties and at the same time enabling the adjustment of the dietary digestible protein to energy interaction (DP/DE).

High quality protein sources such as Prime fish meal (FM) (protein > 72% DM), fish soluble concentrate protein (CPSP) (protein > 75% DM) and squid meal (protein 90% DM) were selected to produce a diet with an amino acids (AA) balance and palatability able to stimulate blackspot seabream growth. Given the current constrains on FM availability and cost, much effort is devoted to the evaluation of alternative plant protein (PP) sources in aquaculture feeds (Gatlin *et al.* 2007; Kaushik & Hemre 2008; Tacon & Metian 2008). Palmegiano *et al.* (2007) has shown that rice protein concentrate (RPC) could replace FM up to 20% without affecting blackspot seabream growth or fillet quality. The feasibility of partial or even total FM replacement is usually done trough the incorporation of a mixture of plant protein (PP) sources supplemented with crystalline AA (Kaushik *et al.* 1995, 2004; Gómez-Requeni *et al.* 2004; Sitjá-Bobadilla *et al.* 2005; Dias *et al.* 2005; Silva *et al.* 2009), with the associated increase of costs. Gluten meal (protein > 80% DM) has been selected as a suitable vegetable source to replace 50% of the fish protein sources in diets for blackspot seabream due to its highly digestible protein content. The selected level was higher than that used by Palmegiano *et al.* (2007) using rice protein concentrate, and slightly beyond the incorporation level above which other fish species growth and

efficiency are reduced (40%) (Ballestrazzi *et al.* 1994; Robaina *et al.* 1995; Mambrini *et al.* 1999; Burel *et al.* 2000; Refstie *et al.* 2000), but was considered appropriate to test the feasibility of partial FM replacement at a metabolic level.

Despite the interest in the evaluation of protein-rich plant ingredients as alternatives to FM, few studies have dealt with the effects of dietary protein sources at a metabolic level. Several studies have been devoted to the importance of indispensable AA (IAA) (Wilson 2002) or to IAA/dispensable amino acids (DAA) ratio (Akiyama *et al.* 1997; Green *et al.* 2002; Gómez-Requini *et al.* 2003; Rollin *et al.* 2003; Peres & Oliva-Teles 2006), while the importance of the contribution of DAA to overall protein nutrition has been much less emphasized in fish. No effect on fish growth performance was found when up to 25% nitrogen was partially replaced by a single DAA (Kirchner *et al.* 2003a) or by either a single or a mixture of DAA (Mambrini & Kaushik 1994). When the dietary nitrogen replacement, by a mixture of DAA, increases up to 50%, growth is apparently reduced (50%) due to a subsequent deficiency in several IAA (Mambrini & Kaushik 1994). Considering all this, the effect of the dietary DAA nature was investigated through the partial replacement (22%) of dietary nitrogen by two different mixtures of DAA. The DAA included into the diets fed to blackpot seabream were chosen for their preferential connection with distinct metabolic pathways: the production of either pyruvate (alanine and serine) or tricarboxylic acid (aspartic and glutamic acid).

Nutritional implications on growth

The optimized feed formulation for blackspot seabream clearly improved both feed conversion rate (FCR, 1.3-1.5) and daily growth index (DGI, 1.0-1.5) compared to previous studies with juveniles (FCR, 1.6-2.0 and DGI, 0.5-1.4) (Peleteiro *et al*. 1994; Olmedo *et al*. 2000; Silva *et al*. 2006). Nonetheless, this growth improvement (Table 1) still remains slightly below other farmed Sparidae such as gilthead seabream (DGI, 2.0) (Santinha *et al.* 1996, 1999; Izquierdo *et al.* 2003; Gómez-Requeni *et al.* 2003, 2004; Sitjá-Bobadilla *et al.* 2005). The decrease of dietary protein level from 45 to 35% (Chapter 3 and 4) clearly depresses this species growth, confirming its high protein requirement. Silva *et al*. (2006) has previously estimated the protein requirement for blackspot seabream maximal growth to be above 400 g Kg^{-1} and in fact, no further improvement of blackspot seabream growth performances could be achieved with higher dietary protein levels. Fish optimal growth performance and feed efficiency are intimately associated with DP/DE (Cho & Kaushik

1990; Dias *et al*. 1998). Regarding the different diets fed to blackspot seabream (Table 1), the optimal growth performance was achieved at about 22 mg DP/kJ DE, with a 45P/10L diet. This value should be used as a reference, and not as a nutritional recommendation, once the experiences related to the current thesis were not designed to assess the optimal DP/DE ratio in blackspot seabream. **Considering all this we can conclude that the most cost effective diet able to induce good growth and high efficiency should contain a protein/lipid level of 45P/10L** (Chapter 2).

Table 1. General view of the effects of different dietary formulation on selected lipid deposition parameters.

P/L	60/6	50/10	45/10		35/10	
			A	B	A	B
DP	59.5	51.8	35.8	-	24.7	-
DE	17.9	18.9	16.4	-	15.5	-
DP/DE	33.3	27.5	21.9	-	16.0	-
Initial body weight (g)	37.7	37.6	23.8	12.0	23.8	12.0
Final body weight (g)	90.2	89.4	73.3	41.0	68.8	34.5
Feed conversion rate	1.3	1.6	1.5	1.6	1.8	1.9
Daily growth index	1.1	1.0	1.5	1.5	1.4	1.2
Initial whole body lipid content (%)	13.3	13.3	14.9	10.4	14.9	10.4
Final whole body lipid content (%)	11.7	14.7	13.4	12.2	12.7	10.7
Daily lipid gain g/Kg/day	0.9	1.3	1.4	1.8	1.3	1.3
Lipid Retention%	127.3	98.0	101.3	78.2	70.0	53.3
Muscle lipid content %	3.4	3.8	3.8	5.6	3.5	5.4
Liver lipid content %	12.2	14.1	17.2	24.2	15.9	31.3
Viscerosomatic index	5.0	5.7	7.5	6.4	7.1	5.0

60/6 and 50/10 FM diets refers to Chapter 2; 45/10 A and 35/10 A refers to Chapter 3; 45/10 B and 35/10 B refers to Chapter 4

Blackspot seabream juveniles showed a high capacity to digest carbohydrates (Chapter 2 and 3), as previously demonstrated for other carnivorous species such as European sea bass (Enes *et al.* 2006a; Moreira *et al.* 2008) and gilthead seabream (Enes *et al.* 2008a). As discussed in Chapter 3, the 25% GS diet resulted in slightly reduced FBW and DGI but improved FCR and protein efficiency ratio compared to fish fed the crude starch (CS) diet (Table1). Diet 45P/10L containing gelatinized starch rather than crude starch was further considered to mimic the physical properties of extruded diets commonly used in commercial aquaffeds. High digestible carbohydrate diets appear to contribute to the reduction of nitrogen losses and thus the enhancement of protein retention (31 vs 35% digestible intake, data not shown). However, this enhancement on fish protein retentions

was not accompanied by improved FBW or DGI (Table 1). Hence, and even without a specific study to define the most adequate digestible carbohydrate level, blackspot seabream seems to tolerate well distinct carbohydrates sources as confirmed with larger sized fish reared in cages (Valente *et al*. 2009a), but without major effects on growth performance. The pentose phosphate pathway is usually stimulated together with glycolysis in fish fed high carbohydrate/low-protein diets (Metón *et al.* 1999; Dias *et al.* 2004; Fernández *et al*. 2007) indicating an ability to utilize carbohydrate and thus spare protein. Different results were obtained in blackspot seabream, with pentose phosphate pathway and glycolysis enzymes lacking any nutritional regulation by the dietary starch type (Table 2) and thus discarding a protein sparing effect condition. **Thus, and overall, it seems that blackspot seabream has low ability to utilize carbohydrates as non-protein energy source and thus spare the utilization of other nutrients, such as protein and lipids, for energetic proposes.**

Several studies have shown that FM can be replaced at least up to 60-75% without significantly effects on growth of gilthead seabream (Gómez-Requeni *et al.* 2004; Sitjá-Bobadilla *et al.* 2005) or sea bass (Kaushik *et al.* 2004; Dias *et al.* 2005). Furthermore, almost total replacement of FM by a mixture of PP sources has also been shown to be possible in rainbow trout (Kaushik *et al.* 1995) or more recently in European sea bass (Kaushik *et al.* 2004) and gilthead seabream (Dias *et al.* 2009). However, in the large majority of these studies, vegetable diets were supplemented with IAA such as lysine and methionine which allowed PP to replace almost all FM without significant effects on growth. In blackspot seabream, the dietary inclusion of wheat gluten without AA supplementation significantly reduced FBW and DGI, when a protein/lipid level of 50P/10L was used, suggesting an IAA/DAA imbalance. In recent years the inclusion of several plant protein mixtures, rather than a single plant ingredient, was shown to be the best strategy to achieve an adequate dietary IAA/DAA balance resulting in good growth performances in several fish species (Dias *et al.* 2009; Kaushik *et al.* 2004). The AA profile of any protein source has a major influence on fish growth (Hughes 1985; Fauconneau 1988; Mambrini & Kaushik 1994; Schuhmacher *et al.* 1995). Some DAA seem to have a specific role with a sparing effect on the use of IAA (Cowey & Sargent 1979; Ronnestad *et al.* 2001; Abboudi *et al.* 2009).

Table 2. Specific activity (mIU/mg of soluble protein) of hepatic lipogenic, glycolytic, gluconeogenic and AA catabolic enzymes in some fish species.

Fish species	P[1]	L[2]	CH[3]	G6PD	ME	FAS	HK	GK	PK	G6Pase	PEPCK	ALAT	ASAT	GDH	Reference
Common Carp															
	33.6	12.2	22.1	-	-	-	1.8	5.0	-	-	-	-	-	-	Panserat *et al*. 2000a
Rainbow trout															
	39.5	16.6	20.4	-	-	-	55	22.5	-	-	-	-	-	-	Panserat *et al*. 2000a
	26.7	24.9	32.7	-	-	-	-	44	280	14	16	512	793	151	Kirchner *et al*. 2003b
	55.4	14.3	16.1	-	-	-	-	23	148	16	23	640	901	98	Kirchner *et al*. 2003b
European eel	-	-	-	149-296	18-34	3.7-19.2									Abraham *et al*. 1984
European sea bass															
	42.7	9.0	31.4	920	36	0.61	-	-	-	-	-	-	-	-	Dias *et al*. 1998
	52.0	9.3	19.8	900	42	0.73	-	-	-	-	-	-	-	-	Dias *et al*. 1998
	48.4	14	18.5	107	-	-	0.5	1.5	90.1	26	-	319	543	150	Enes *et al*.2006a
Gilthead seabream															
	47.6	12.5	17.1	201	-	-	-	-	513	-	-	1154	1693	-	Méton *et al*. 1999
	45.5	12.2	21.1	-	-	-	4	16	-	-	-	-	-	-	Panserat *et al*. 2000a
	52.7	15.7	-	202	20	0.1	-	-	-	29	-	874	1726	212	Gómez-Requeni *et al*. 2003
	46.1	16.5	-	-	-	-	-	-	-	-	-	830	2110	260	Gómez-Requeni *et al*. 2004
	47.6	15.1	17.5	50.3	-	-	0.3	13.6	46	-	-	350	673	90	Enes *et al*. 2008a
	53.9	14.4	19.6	177	-	-	-	-	463	-	-	959	799	-	Fernández *et al*.2007
Red sea bream															
	-	-	-	121	33	17.4	-	-	-	-	-	-	-	-	Iritani *et al*. 1984
Blackspot seabream															
	60	6	9.9-15.8	104-137	-	21-30	-	-	-	-	-	-	-	-	This Thesis
	50	12	15.4-18.7	95-108	-	17-22	-	-	-	-	-	-	-	-	This Thesis
	45	10	23.6	97-125	34-50	15-30	0.3-0.4	0.7-1.1	182-192	12	18	1252	1587	84	This Thesis
	35	10	26.5	57-83	14	5-15	0.4-0.5	0.5-0.7	327-332	12	26	1017	1264	70	This Thesis

[1,2,3] indicates dietary protein, lipid and carbohydrates level

Blackspot seabream appears to make different use of the dietary nitrogen (N) according to its source; with N provided by alanine and serine being more efficiently used for growth than that provided by aspartic and glutamic acids mixture (Chapter 4). **The industrial feasibility of FM replacement by plant feedstuffs in blackspot seabream diets should take into account both the dietary IAA/DAA balance and the absolute amount of DAA.**

Improving digestibility of diet formulations and optimizing feeding regimes can improve feed utilization efficiency in farmed fish (Cho & Bureau 2001). In order to optimize feeding systems, it is crucial to know when, how much and how fish should be fed, which in turn requires information on how fish behave under different feeding systems. Self-feeders had been used extensively to study feeding rhythms in fish, revealing that the adjustment of feeding times to match natural rhythm improves FCR, and reduce the amount of uneaten feed pellets in several fish species (Alanärä 1992, 1996; Madrid *et al*. 1997; Paspatis *et al*. 2000; Sánchez-Muros *et al*. 2003). The more efficient use of the optimized diet for blackspot seabream (dietary/lipid ratio of 45P/10L) was shown to occur when feed was distributed in phase with fish natural rhythm, compared to those hand-fed at set hours. Although no significant effect of the feeding method was observed on FBW (72-73 g) or DGI (1.4-1.5), alike rainbow trout (Gélineau *et al.* 1998) and African catfish (Hossain *et al.* 2001), blackspot seabream apparently makes a more efficient use of dietary protein for growth (PER, 2.3 *vs* 1.5) when fed in phase with their natural feeding rhythm. Moreover, fish fed by self-feeders displayed a significantly lower VFI (1.1 *vs* 1.7) and reduced FCR (FCR, 1.0 *vs* 1.5) compared to fish fed by hand. **Thus, self-feeders should be regarded as a helpful tool to optimize blackspot seabream feed distribution, maximizing nutrient efficiency and minimizing food waste.**

Nutritional implications on lipid deposition

Blackspot seabream reared with the various tested diets have always deposited significantly higher levels of lipid in muscle (3-6%), liver (12-24%) and particularly around viscera (around 65-70%; value estimated based on VSI and mesenteric fat index, and taking into account previous data on visceral lipid contents, Ozório *et al*. 2009; Valente *et al*. 2009b) than their wild counterparts (2, 11 and 12%, respectively) (Alvarez *et al*. 2009, Valente *et al.* 2009b). Nevertheless, this species whole body fat content ranged from 11 to 15% (wet weight basis) which is well within the values found for other cultured fish, such

as gilthead seabream (Santinha *et al.* 1996, 1999; Izquierdo *et al*. 2003) or European sea bass (Dias *et al.* 1998, 2005; Izquierdo *et al*. 2003; Kaushik *et al.* 2004).

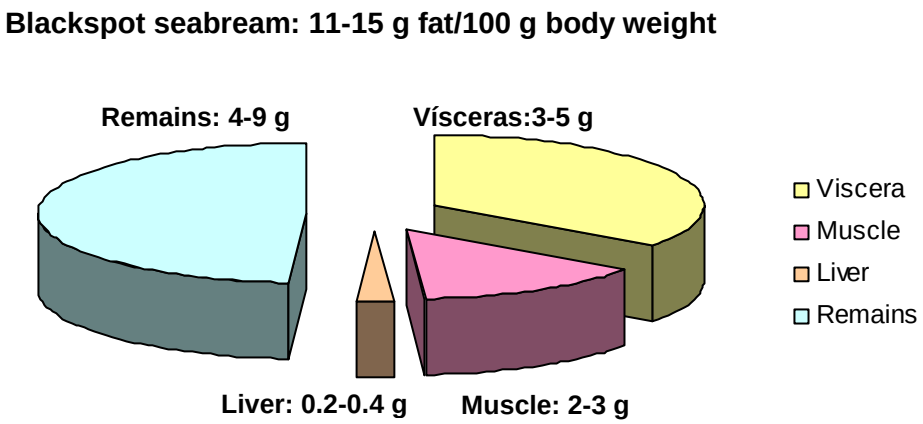

Figure 2. Blackspot seabream pattern of lipid storage

The contribution of muscle and visceral fat to blackspot seabream body weight was 2-3% and 3-5%, respectively, while liver has only accounted with 0.2-0.4% for blackspot seabream total body weight (Fig. 2). One point worth to be mentioned is the substantial fat contribution of other tissues than muscle, viscera or liver (remains: 4-9%) to total body weight. Similarly to flatfishes, such as turbot (Andersen & Alsted 1993), Atlantic Halibut (Berge & Storebakken 1991; Martins *et al.* 2007) and Senegalese sole (Borges *et al.* 2009), subcutaneous fat may comprise a significant contribution to body lipid content in blackspot seabream as previously observed in histological sections (Silva *et al*. 2009). Blackspot seabream, like the majority of pelagic marine species (Sheridan 1988; Corraze 2001; Tocher 2003), tends to deposit lipids preferentially in the visceral tissue and liver and to a lesser extend in muscle. As this fish species, in Portugal and Spain, is preferentially bought whole and not filleted, an excessive visceral fat content is a major issue under the consumer's point of view, with adverse effects on yield, product quality, and storage.

It has long been known that body lipid content can be modified by feeding regime manipulation (Shearer *et al.* 1997; Jobling *et al.* 1998; Johansson *et al.* 2000). Lipid storage in fish tissues mainly depends on the availability of circulating triacylglycerol (TAG) originated from both diet and endogenous formation (fatty acid synthesis *de novo* or lipogenesis). Indeed, energy intake and balance rather than dietary fat level appear to

influence whole body fat storage in fish (Shearer 1994), with surplus non-fat energy being converted into fat through lipogenic pathways (Hellerstein *et al.* 1996). The activities found for hepatic fatty acid synthetase enzyme (FAS) in blackspot seabream were higher than those found in gilthead seabream (Gómez-Requeni *et al.* 2003) or seabass (Dias *et al.* 1998, 2005), but well within the range of values observed in red seabream (Iritani *et al.* 1984) and European eel (Abraham *et al*. 1984) (Table 2). There is current evidence that the lipogenic capacity of a fish relates to a significant extent with the lipid-rich prey availability in their natural environment (Tocher 2003). Some species like European eel that in nature feeds on poor-lipid organisms have considerable lipogenic rates (Abraham *et al.* 1984). **Independently of the dietary formulation, the lipogenic rates found in blackspot seabream appear to assume, like in European eel, a considerable importance to this species lipid deposition.** This particular feature could be partly explained by species biological characteristics. Blackspot seabream is a demersal *Sparidae* species, found in depths from the inshore waters (juveniles) down to 400 m in the Mediterranean Sea and 700 m in the Atlantic (adults) (Bauchot & Hureau 1986). Thus, a high propensity to drive exogenous and endogenous *de novo* synthesize fatty acids into lipid deposition could have resulted from an evolutionary adaptation to reduced prey availability predictable at such profundities.

Whole body lipid content of blackspot seabream was, to some extent, positively correlated with the dietary P/L ratios (Chapter 2) and protein levels (Chapter 3 & 4), but apparently not with the dietary starch type. Since fat deposition mainly occurs in visceral adipose tissue, and to a lesser extent in muscle and liver, it is plausible to infer that differences in its whole body lipid content are mainly a result of visceral, and perhaps marginal body parts, fat modulation. Juveniles fed a 60P/6L FM based diet presented a lower lipid body content than those fed with 50P/10L, suggesting that **a 4% increase on dietary lipid level has a more preponderant effect on blackspot seabream fat deposition than the reduction of 10% dietary protein level.** The high propensity of this species to transform other nutrient than lipid into body fat has been confirmed in both Chapter 3 and 4, where an increase of 10% dietary protein level (35 to 45%) markedly enhanced (> 40%) hepatic activities of lipogenic enzymes (Table 2). **This 10% increase in dietary protein level has not resulted in a significant alteration of body lipid content, but the effective increase of 14:0 and 16:0 saturated fatty acids, the higher lipid gain and the better retention pointed out the important role of dietary protein level as a major nutritional regulator of blackspot seabream lipid deposition.**

High dietary digestible carbohydrate content resulted in increased fat deposition in rainbow trout (Kaushik & Oliva-Teles 1985), Atlantic salmon (Grisdale-Helland & Helland 1997), European sea bass (Dias *et al.* 1998) and gilthead seabream (Venou *et al.* 2003; Fernández *et al.* 2007). Other studies reported no effect of carbohydrate on whole body composition in rainbow trout (Alvarez *et al.* 1999), European sea bass (Enes *et al.* 2006a) or in gilthead seabream (Peres & Oliva-Teles 2002; Enes *et al.* 2008a). **Nutrient gain and whole body composition of blackspot seabream were not affected by starch type (CS vs GS)** (Table 1). **Starch did not induce any particular stimulation on blackspot seabream lipogenic pathways** (Table 2), though in several fish species, the dietary incorporation of gelatinized starch resulted in higher liver lipogenic activity (Shiau & Chen 1993; Shiau & Lin 1993; Shiau & Liang 1995; Hung *et al.* 1989; Arnesen *et al.* 1995; Robinson & Li 1995; Deng *et al.* 2000).

The modification of body lipid content as well as lipogenesis by protein nature has been demonstrated in teleosts (Goméz-Requeni *et al.* 2003; Kaushik *et al.* 2004; Dias *et al.* 2005) as in higher vertebrates (Iritani *et al.* 1986, 1996; Kayashita *et al.* 1996; Padmakumarannair *et al.* 1998). Palmegiano *et al.* (2007) has shown that rice protein concentrate (RPC) could replace FM up to 20% but the absence of data on whole body composition does not permit to infer about a possible effect on this species lipid content. In Chapter 2, a tendency (P=0.07) for the PP diets to increase fish body lipid content, was verified. PP and its interaction with protein level leaded to blackspot seabream fatty acid *de novo* up-regulation and extremely high lipid retention mainly due to increased liver lipid content, since muscle lipid content and VSI remained unaffected. Fish fed with high PP inclusion diets (60P/6L) have retained considerably more lipid than the ones fed low PP inclusion level (50P/10L). The replacement of fish meal by high inclusions of corn gluten meal (Dias *et al*. 2005) or by corn and wheat gluten (Kaushik *et al*. 2004) in European sea bass appears to up-regulate FAS activities and increase body lipid content, but the effects of soy protein concentrates in European sea bass lipogenesis were shown to be high variably (Dias 1999).

The present Thesis helps understanding the modulation of fish lipid content by the dietary protein source and thus by its respective AA profile. The activity of the AA catabolism enzymes (Table 1) were generally within values found in species with similar protein requirements like gilthead seabream (Méton *et al.* 1999; Gómez-Requini *et al.* 2003, 2004; Enes *et al.* 2008a) or sea bass (Enes *et al.* 2006a). Regardless of the dietary protein level or nature, the high activity of those enzymes in blackspot seabream (Table 2) suggests that this species uses considerable amount of AA for energy production and

fatty acid synthesis. In carp, *in vitro* fatty acid synthesis rate from ^{14}C -alanine and ^{14}C -glutamate was markedly higher than that from ^{14}C -glucose (Nagai & Ikeda 1972; Shikata & Shimeno 1997). In addition, Henderson & Sargent (1981) reported that the incorporation of ^{14}C -glucose into triacylglycerols was much lower than in ^{14}C-alanine incorporation in liver slices of rainbow trout. Alike, the dietary AA profile, in particular the dietary DAA content and nature was shown to play a preponderant role on this species lipogenic pathways corroborated by the increased 14:0 and 18:0 liver fatty acids (FA) content, which together with 16:0 are the main newly-synthesized FA in fish (Corraze 2001; Sargent *et al.* 2002). **Blackspot seabream body lipid content was shown to depend on dietary nitrogen nature, with high dietary PP inclusion levels tending to increase fat deposition (Chapter 2).** Thus, the excessive fat accumulation (18%) previously described for this species (Linares *et al.* 2000, 2001; Silva *et al.* 2006; Ozório *et al.* 2009) could have been an effect of dietary protein source used in those studies. **Individual DAA seem to activate distinct metabolic pathways, with surplus dietary alanine and serine playing a preponderant role in lipogenesis, while aspartic and glutamic acids mixture showing an ability to reduce the specific activity of FAS. The nature and level of DAA, such as alanine and serine, of a given PP feedstuff must be taken into consideration when FM is being replaced by PP.**

In several species, the recommendations relative to DP/DE ratio are currently under revision in view of protein economy and better energy efficiency. Although the present Thesis did not aim to evaluate the effects of dietary changes in terms of DP/DE levels on growth or fat deposition, the DP/DE ratio of the different regimes studied across this Thesis (Table 1) were to some extent related with daily fat gain (Fig.3). Blackspot seabream fed the highest DP/DE ratio (33 mg DP/kJ DE in diet 60P/6L) reduced daily fat gain and body lipid content (12% wet weight), but retained considerably more lipids (>100%) compared with 45P/10L (ratio) or 30P/10L (ratio). The highest DP/DE level was a result of the highest dietary DP level rather than lowest DE.

Dietary DE more than the DP/DE level may condition lipid deposition in fish (Cowey 1993; Kaushik 1997), with high DE resulting in increased lipid deposition with adverse effects on yield, product quality, and storage (Hillestad & Johnsen 1994; Jobling *et al*. 1998; Company *et al.* 1999a,b). On the other hand, the high level of DP in fish diets is associated with metabolic losses and nitrogen loading to the environment (Cho & Kaushik 1990) and with a higher susceptibility of fish muscle to lipid oxidation (Alvarez *et al*. 1998).

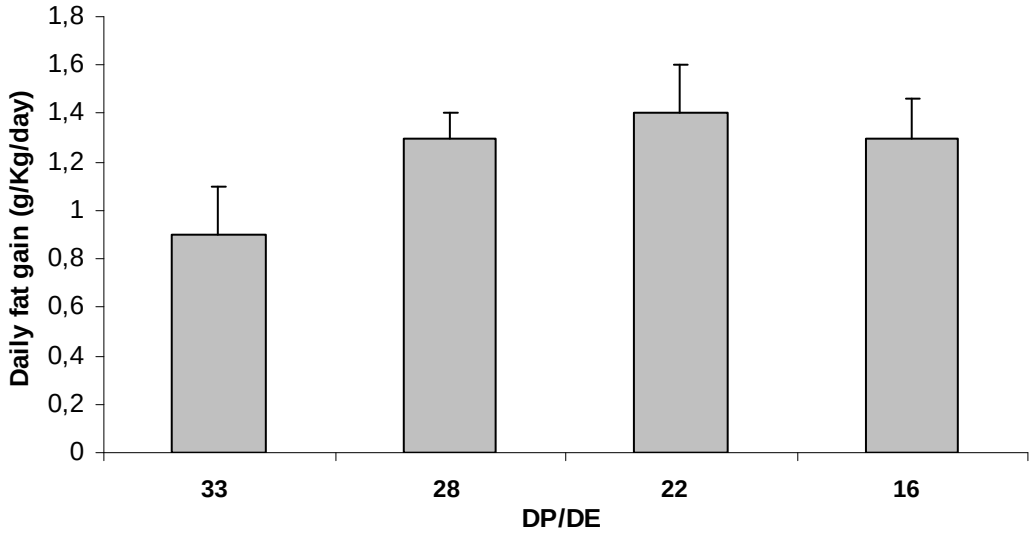

Figure 3. General overview of the different dietary DP/DE (mg/kg) used across the different Chapters and the respective daily fat gain (g/kg of average body weight/day).

Hence in blackspot seabream, the best growth performance combined with a suitable body lipid content (13%) is apparently achieved at a DP/DE ratio of 22 mg DP/kJ DE. Nevertheless, the present Thesis does not allow us to make a proper comparison of the different dietary DP/DE ratios and thus the evaluation of dietary changes in terms of DP/DE levels on blackspot seabream growth and lipid deposition, must be considered in future studies.

One point worth noting is that the preceding nutritional history should be taken into consideration when body lipid content of fish is being evaluated in respect to nutritional formulation (Table 1). It is interesting to mention that, when comparing the results between Chapter 3 and Chapter 4, different groups of blackspot seabream juveniles fed with the same P/L diet (45/10 or 35/10) had their body lipid content altered in a proportion that seems to be positively related with their initial body lipid content. In addition, the different genetic origin of fish stocks (Chapter 4 vs Chapter 2, 3 and 5) must be seriously considered, since genetic makeup is one of the numerous factors known to influence fish lipid content. That is even more relevant in species with a high genetic diversity, which is the case of blackspot seabream (Stockley *et al.* 2005; Pinera *et al.* 2007). Hence, besides nutritional factors, the genetic origins of fish stocks as well as the preceding nutritional history of those groups needs to be seriously taken into account when one regards the nutritional implications in lipid metabolism.

Physiological responses of feeding in fish are dependent upon the amount of food eaten and related with feeding times (Bolliet *et al.* 2001a; Carter *et al.* 2001). In a preliminary study (Chapter 5), the adjustment of feeding times to match blackspot seabream natural rhythm has not improved species growth performance, but resulted in superior feed and nutritional conversion efficiencies, compared to fish hand fed. In addition, this group of fish displayed markedly depressed ME (<62%) and FAS (<35%), enzymes which correlated positively with feed intake and lipid content. The distinct lipogenic enzyme activities displayed by blackspot seabream corroborate the suggestion that a same diet offered at different times of the day can be metabolized differently by the adjustment of digestive and/or metabolic enzymes (Madrid 1994; Carter *et al*. 2001), and thus could have important implications on species lipid metabolism.

Main Conclusions

The work carried out along this Thesis allowed us to understand and to some extent modulate the contribution of each macronutrient to blackspot seabream lipid deposition and growth. The following conclusions can be drawn from the results presented:

The use of high protein (50 or 60%)/ low lipid (6 to 10%) dietary levels failed to improve blackspot seabream growth performance, but confirmed the low dietary lipid tolerance of this species.

The decrease of dietary protein level from 45 to 35% points out protein level as a major nutritional regulator of blackspot seabream lipid deposition and clearly confirms its high dietary protein requirement.

Starch type does not induce any particular stimulation of blackspot seabream lipogenic pathways or shows the ability to spare the utilization of other nutrients like protein for growth.

The protein/AA source constitutes a major nutritional modulator of lipid deposition in blackspot seabream. High inclusion of dietary PP could significantly reduce this species growth performance and increase its fat deposition, depending on the dietary IAA/DAA balance but also on the absolute amount of DAA, such as alanine and serine.

At an optimal dietary/lipid ratio of 45P/10L, blackspot seabream improves nutrient utilization and reduces lipogenic propensity particularly when self-fed in phase with their natural rhythm.

Final considerations

The next steps concerning blackspot seabream nutrition point towards the search of alternative feedstuffs to marine sources (FM and fish oil) considering a dietary/lipid ratio of 45/10 as the most appropriate to maximize growth and reduce excessive fat. The ability of protein source to modulate blackspot seabream lipogenic pathways was evidenced, so new aquafeeds for this species must take into consideration dietary IAA/DAA ratio but also the absolute amounts DAA of a given PP feedstuff.

A significant portion (60–75%) of dietary fish oil can be substituted with alternative lipid sources without significantly affecting growth performance, feed efficiency and feed intake in almost all finfish species studied, provided the requirements in essential fatty acids (EFA) are met (Turchini e*t al*. 2009). However, if fish oil and FM are both substituted with alternative vegetable sources the risk of a net deficiency in EFA is likely to occur. In addition, the market prices of vegetable oils have exceed US$1000 per ton owing to rising global demands for edible oils and to the expanding bio-diesel industry, making vegetable oils presently less attractive for use in aquafeed formulations. The ability of blackspot seabream to be fed diets containing low levels or devoid of fish oil without compromising growth and even reducing its body lipid content may represent an advantage for the intensive production of this species by aquaculture industry. Future studies should determine the maximal replacement of FM by PP with simultaneous reduction of fish oil inclusion without compromising the supply of EFA required for blackspot seabream maximal growth. The effect of long term feeding diets combining PP and vegetable oils must also be evaluated in terms of growth performance, metabolism, flesh quality and health aspects of blackspot seabream. The potential of a finishing period based on a fish oil diet (wash-out or finishing diet) to restore the original FA profile in farmed species (Turchini e*t al*. 2009), should also be assessed in blacktop seabream.

Feeding fish in phase with their endocrine cycles provide the best conditions to enhance blackspot seabream growth and feed efficiency. Future studies should also consider whether a change in the fatty acid composition of the oil source or the AA acid profile of a determined plant source affects the feeding behaviour of this species. Such knowledge

might lead to specific adjustments of feeding strategies to the appetitive feeding behaviour of the blackspot seabream, and hence contribute for the reduction of feeding costs and the decrease of waste from aquaculture systems.

Selective breeding is an established aquaculture practice to improve profitable fish characteristics such as fecundity and growth rate (Gjoen & Bentsen 1997; Hulata 2001). Thus, its application to blackspot seabream might significantly contribute to reduce the time actually required to attain commercial size and to complete species production cycle.
Feed formulation clearly modifies blackspot seabream body lipid content but its effective regulation is likely to be under some genetic control. The genetic control of body composition and fatness is an alternative means of improving body and flesh characteristics. The lipid levels of individual salmon receiving a diet containing 28% lipid could vary from less than 5% to more than 18% of the wet weight of the flesh (Bell *et al.* 1998). In salmonids, heritabilities have been estimated for a number of traits related to body composition and carcass quality, such as carcass yield, fillet yields, body shape, flesh composition and colour (Elvingson & Johansson 1993; Kause *et al.* 2002, 2003, 2007; Quinton *et al*. 2005). Moreover, an interaction between genetic line and diet energy content has been demonstrated to modulate muscle fat contents in rainbow trout (Kolditz *et al.* 2008). Thus, future studies should be carried out to clarify to what extent environmental and/or genetic factors contribute to the observed biological variation in blackspot seabream lipid contents.

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Annex I. Muscle amino acid composition (g/100 g) of wild blackspot seabream.

Arginine	**6.89**
Histidine	**2.58**
Isoleucine	**4.49**
Leucine	**8.44**
Lysine	**10.06**
Methionine	**3.50**
Cysteine	**0.67**
Phenylalanine	**4.42**
Tyrosine	**3.84**
Threonine	**5.00**
Valine	**4.99**
Alanine	**6.41**
Aspartic acid	**10.84**
Glutamic acid	**15.54**
Serine	**4.74**
Glycine	**4.17**
Proline	**3.74**

Muscle samples were analyzed for total amino acid content by High Pressure Liquid Chromatography (HPLC) at the laboratoty of CCMAR, UALG, Algarve.