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Impact of fish meal replacement by alternative and sustainable ingredients in diets for gilthead seabream (*Sparus aurata* Linnaeus, 1758) juveniles

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Master's in Aquaculture and Fisheries

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Dissertation for the attainment of the Master's degree in Aquaculture and Fisheries

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

Universidade do Algarve, 29 de Setembro de 2017

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Abstract

Aquaculture is the animal production sector that is growing at a faster rate in the world but in the last decades, as fish meal has been widely used as the main dietary protein source, the industry became highly dependent on this ingredient. Fish meal prices are predicted to increase in the next 15 years, and aquaculture cannot be dependent on this protein source if the same growth registered in the past is maintained. Based on this fact, alternatives to fish meal that offer the same or even better results must be found. In the past years, research towards finding new protein sources have increased and plant-based proteins and terrestrial animal proteins have shown a great potential to replace the widely used fish meal in fish diets. Therefore, the aim of this Thesis was to evaluate growth performance, body composition and nutrient balance in gilthead seabream juveniles fed diets with high levels of fish meal replacement by alternative and sustainable ingredients. Three experimental diets were formulated including processed animal proteins (PAP), plant proteins (PLANT) or a mixture of micro/macro algae, insects and yeast (EMERG) as main protein sources. The performance of fish fed with these three diets was compared with a control (CTRL) diet, formulated to be similar to a commercial feed used nowadays in gilthead seabream culture. At the end of the experiment, fish fed with the PAP and PLANT diets showed improved growth performance, more efficient dietary nutrient utilization and lower environmental impact compared with fish fed with the CTRL diet. Performance of fish fed with the EMERG diet was, in general, negatively affected. The results obtained in the present study show that the gilthead seabream culture can be improved and can even be more environmentally sustainable using PAP and PLANT diets that contained only 5% of marine-derived ingredients.

Keywords: fish meal replacement, plant proteins, processed animal proteins, insect meal, nutrient balance, environmental impact.

Resumo

A dourada (*Sparus aurata*) é um peixe carnívoro, sendo uma das espécies mais importantes e mais produzidas no Mediterraneo, e tendo atingido uma produção de 166 794 toneladas em 2015. A aquacultura é o sector da produção animal que mais tem crescido a nível mundial, mas nas últimas décadas tornou-se extremamente depende de uma fonte de proteína, a farinha de peixe, um ingrediente de excelente qualidade, com elevado teor proteico (60-75%), excelente perfil de aminoácidos, boa digestibilidade de nutrientes e ausência de antinutrientes. A FAO prevê que os preços da farinha de peixe venham a duplicar nos próximos 15 anos, valores esses que não são compatíveis com o natural desenvolvimento desta indústria. Deste modo, têm de ser encontradas alternativas que ofereçam os mesmos, ou até mesmo melhores resultados, que os obtidos com a farinha de peixe. Só desta forma é que o sector poderá continuar a apresentar os níveis de crescimento verificados em anos anteriores, recorrendo a ingredientes mais sustentáveis e com preços mais baixos e deixando assim de ser dependente de uma fonte de proteína com preços tão variáveis e que depende da pesca de stocks selvagens. Nos últimos anos, a investigação de novas fontes de proteína para a alimentação dos peixes tem aumentado, tendo as proteínas de plantas e as farinhas proteicas de animais terrestres demostrado um grande potencial para substituir a farinha de peixe nas rações utilizadas hoje em dia. Assim sendo, o objetivo desta Tese foi avaliar a performance de crescimento, composição corporal e balanço de nutrientes em juvenis de dourada alimentados com dietas com uma elevada substituição de farinha de peixe por ingredientes alternativos e mais sustentáveis. Para isso, três dietas experimentais foram formuladas, incluindo como principais fontes de proteína, farinhas proteicas de animais terrestres (PAP), concentrados proteicos de plantas (PLANT) ou uma mistura de micro/macro algas, farinha de insetos e leveduras (EMERG). A performance dos peixes alimentados com estas três dietas experimentais foi comparada com a de uma dieta controlo (CTRL), com uma formulação similar a

uma ração comercial utilizada hoje em dia em aquacultura de dourada. No fim da experiência, os peixes que foram alimentados com as rações PAP e PLANT apresentaram uma melhor performance de crescimento, uma utilização mais eficiente dos nutrientes e um menor impacto ambiental do que que os peixes alimentados com a ração CTRL. De uma forma geral, a ração EMERG afetou negativamente a performance dos peixes. Os resultados obtidos no presente estudo demostram que as dietas PAP e PLANT, que continham apenas 5% de farinha de peixe, podem não só melhorar o crescimento da dourada como tornar o seu cultivo mais sustentável do ponto de vista ambiental.

Palavras-chave: substituição de farinha de peixe, proteínas vegetais, farinhas proteicas de animais terrestres, farinhas de insetos, balanço de nutrientes, impacto ambiental.

Abbreviations

ABW – average body weight

ADC – apparent digestibility coefficient

ANF – antinutritional factor

ANOVA – analysis of variance

BSE - bovine spongiform encephalopathy

cm - centimeter

Cr_2O_3 – chromium oxide

DAA – dispensable amino acid

DGI – daily growth index

DM – dry matter

EU – European Union

FAO – Food and Agriculture Organization

FBW – final body weight

FCR – feed conversion ratio

g – grams

h - hours

HCl – hydrochloric acid

HSI – hepatosomatic index

IAA – indispensable amino acid

IBW – initial body weight

kDa - kilodalton

kg – kilogram

L - liter

M - molar

m^3 – cubic meter

min - minute

MJ – megajoule

mL- milliliter

mm – millimeter

N – nitrogen

ºC – degrees Celsius

P - phosphorus

PAP – processed animal protein

PER – protein efficiency ratio

Tt – thousand tones

UN – United Nations

UPLC - ultra-high-performance liquid chromatography

µM - micromolar

VFI – voluntary feed intake

VSI – viscerosomatic index

WG – weight gain

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Introduction

General Overview of Aquaculture

According to the latest UN Food and Agriculture Organization report, aquaculture is the food animal-producing sector that is growing at a faster rate (FAO, 2016). Aquaculture production, in 2014, accounted for 44.1% of total production from capture fisheries and aquaculture (not including marine plants), generating a harvest of 73.8 million tonnes, with an estimated value of US$160.2 billion, from which 49.8 million tonnes of finfish (US$99.2 billion), 16.1 million tonnes of molluscs (US$19 billion), 6.9 million tonnes of crustaceans (US$36.2 billion) and 7.3 million tonnes of other aquatic animals (US$3.7 billion) (FAO, 2016). This trend is likely to increase even more in the next years due to the fishing pressure of the wild fish stocks (Fig.1).

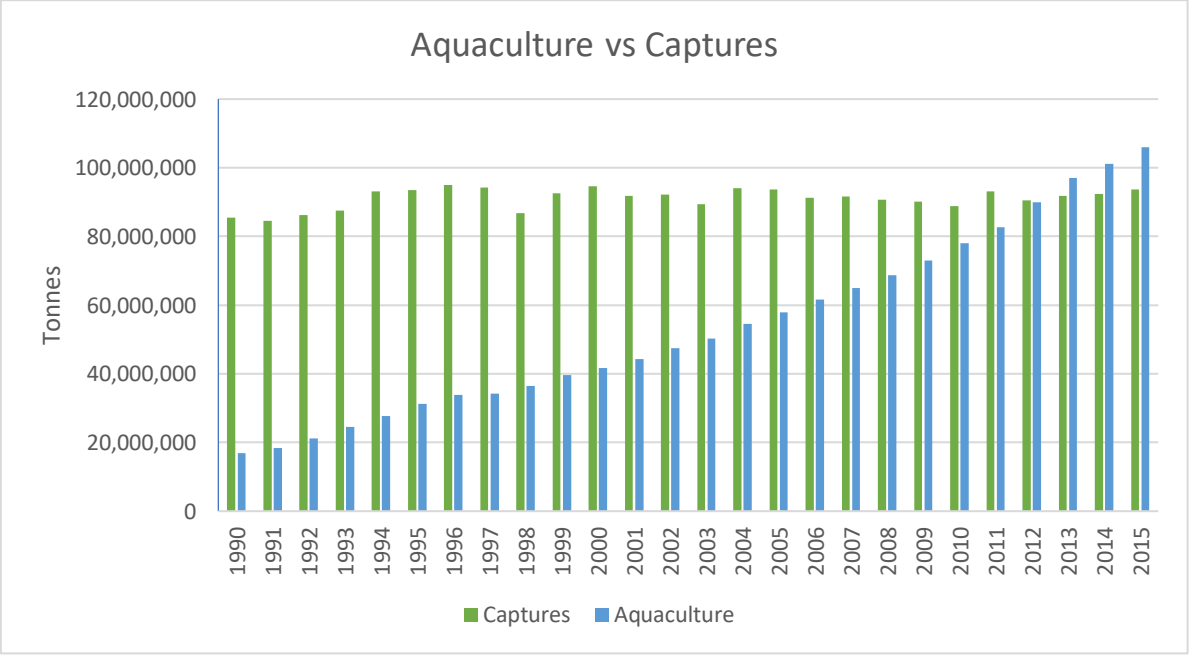
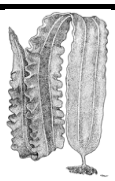
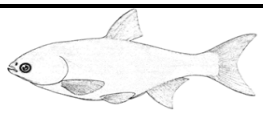
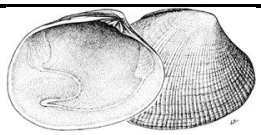
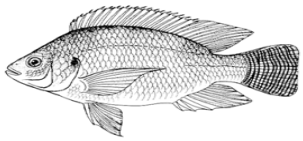
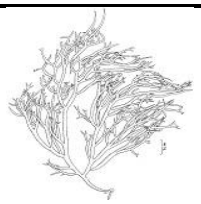
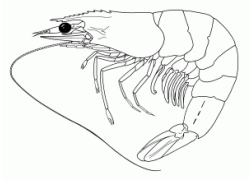
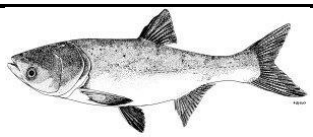
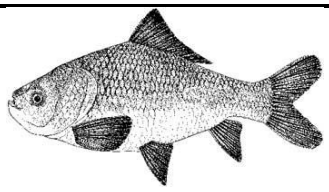
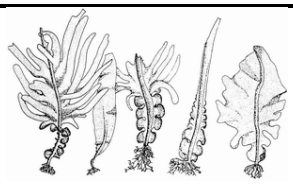
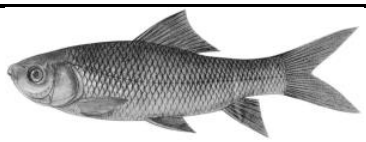
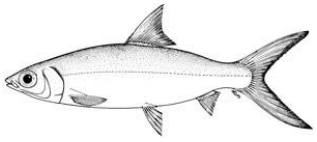
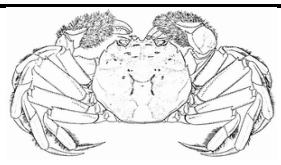


Figure 1 - Evolution of aquaculture production and capture fisheries in the past 25 years. Source: FAO (Available in: http://www.fao.org/fishery/en).

Aquaculture production is dominated by Asian countries being China the biggest producer by a long margin, with 58795.3 thousand tones (Tt) followed by Indonesia (14330.3 Tt), India (4884 Tt), Vietnam (3411.Tt), Philippines (2337.6 Tt), Bangladesh (1956.9 Tt), Republic of Korea (1567.4 Tt), Norway (1332.5 Tt), Chile (1227.4 Tt) and Egypt with a production of 1137.1 Tt (FAO, 2016). In table 1 are presented the 15 most produced species in aquaculture in 2015 with their respective scientific illustration.

Table 1 - Most produced species in aquaculture worldwide. *Source: FAO (Adapted from http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en).*

Common Name	**Scientific Name**	**Tonnes Produced**	**Scientific Illustration**
Japanese kelp	*Laminaria japonica*	8 026 782	
Grass carp	*Ctenopharyngodon idellus*	5 822 869	
Silver carp	*Hypophthalmichthys molitrix*	5 125 461	
Common carp	*Cyprinus carpio*	4 328 083	
Japanese carpet shell	*Ruditapes philippinarum*	4 049 541	
Nile tilapia	*Oreochromis niloticus*	3 930 579	

Gracilaria seaweeds	*Gracilaria spp.*	3 880 748	
Whiteleg shrimp	*Penaeus vannamei*	3 879 786	
Bighead carp	*Hypophthalmichthys nobilis*	3 402 870	
Catla	*Catla catla*	2 764 944	
Atlantic salmon	*Salmo salar*	2 381 576	
Wakame	*Undaria pinnatifida*	2 296 468	
Roho labeo	*Labeo rohita*	1 785 900	
Milkfish	*Chanos chanos*	1 115 095	
Chinese mitten crab	*Eriocheir sinensis*	823 416	

Gilthead Seabream

General Species Characterization

Gilthead seabream (Fig. 2) is a perciform fish that belongs to the family Sparidae, genus *Sparus*. In the nature, it is commonly found in the Atlantic coasts of Europe, Mediterranean Sea and Black Sea (rare) and is one of the most important species of fish produced in the Mediterranean aquaculture industry (Ballester-Moltó et al., 2016; Moretti et al., 1999). This species can be found in marine and brackishwater environments, such as coastal lagoons and estuarine areas, mainly in the beginning of their life cycle. It is primarily a carnivorous fish, but under certain circumstances can be an accessorily herbivorous. It is a protandrous hermaphrodite, meaning that these animals are functional males in the first two years of their life and turn into females when they reach around 30 cm of length. The ovarian development is asynchronous and it is a batch spawner (daily spawns for a period of ± 3 months) between October and December (in the nature). After spawning, the eggs are pelagic, spherical, transparent, with a diameter usually between 0.94 – 0.99 mm and present a single large oil droplet (Arabaci et al., 2010; Moretti et al., 1999). Hatching occurs roughly 48h after spawning, and the newly hatched larvae have 3 mm of length. The definitive morphology is attained 90 days after hatching with a length around 30 mm (Moretti et al., 1999).

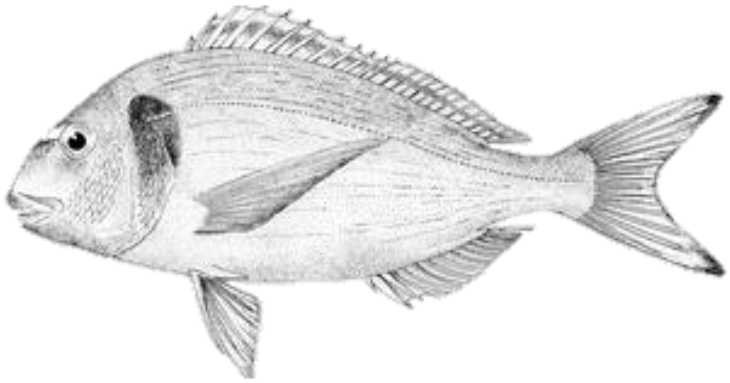


Figure 2 - Scientific illustration of gilthead seabream (*Sparus aurata*).
Source: FAO (Available in: http://www.fao.org/docrep/005/x3980e/x3980e05.htm).

Production Levels

Gilthead seabream can be reared in three different systems. Coastal ponds and lagoons are used in extensive and semi-intensive systems in densities of 0.0025 kg $m^{3\ -1}$ and 1 kg $m^{3\ -1}$ respectively. Sea cages are used in intensive systems in densities between 15-45 kg $m^{3\ -1}$ (Colloca & Cerasi, 2005). In Europe, most of the production occurs in the areas surrounding the Mediterranean Sea, with Turkey being the biggest producer (39%) in 2015, surpassing Greece (35%) which dominated the charts in the last 20 years. Spain (12%), Italy (5%), Cyprus and Croatia (3%), Malta (2%), France and Portugal (1%) are also an important part of the Mediterranean producers (Fig. 3). Gilthead seabream production is not constrained only to the European continent and African countries such as Egypt and Tunisia have considerable productions of this species, occupying respectively 10% and 6% of the world quota. In total 166 794 tonnes of this species were produced in 2015.

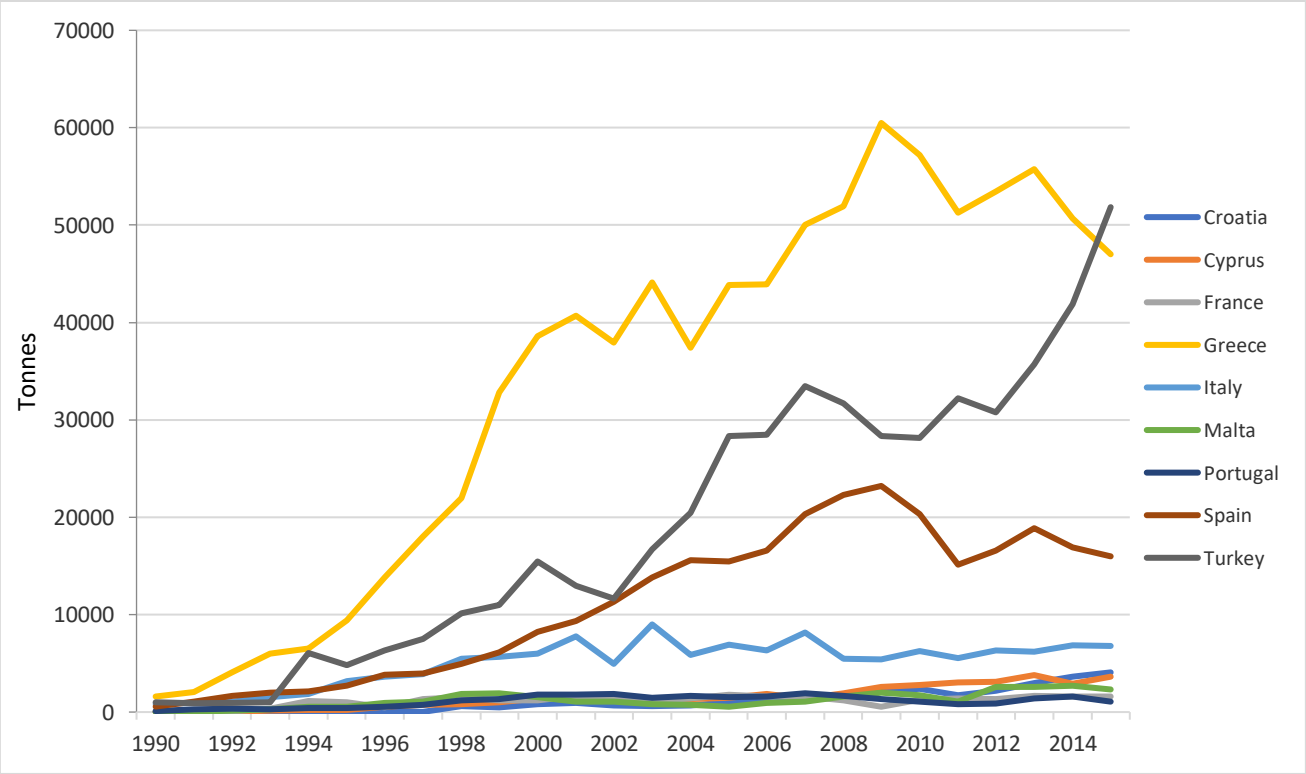


Figure 3 – European aquaculture production of gilthead seabream.
Source: FAO (Available in http://www.fao.org/fishery/aquaculture/en).

Importance of Protein and Phosphorus in Fish

Proteins are large organic, nitrogen-containing compounds comprising long chains of amino acids, which are required metabolic compounds used as either a major energy source or for protein synthesis, being an essential component on the diet for all animals (Bowyer et al., 2013; Jobling, 2001). Fish, like other animals, synthesize proteins from amino acids and these can be separated in essential (or indispensable, IAA) amino acids, which are those that animals cannot synthesize and the non-essential (or dispensable, DAA), amino acids that are synthesized from other compounds (Gatlin, 2010; Jobling, 2001).

From the 20 amino acids found within proteins, 10 are IAA (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine), 8 DAA (alanine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, proline, serine) and 2 are conditionally indispensable (cystine, tyrosine) for fish. Cystine and tyrosine are considered to be conditionally indispensable due to the fact that they can be synthesized exclusively from methionine and phenylalanine, respectively, meaning that a supply of these amino acids is not required if their precursors are present in the right quantities (Jobling, 2001).

Meeting a minimum dietary requirement of protein in fish is critical for a good growth and health. However, to provide excessive levels of protein in the diet is both economically and environmentally unreliable, due to the fact that protein is the most expensive dietary component and that its excess results in increased nitrogen excretion, therefore generating more waste (Gatlin, 2010).

Phosphorus is an important mineral, playing a vital role in bone mineralization. Fish can absorb minerals directly from the water where they live, but phosphorus is usually a limiting mineral, since it is available in relatively low quantities in water (Lim et al., 2001)

Utilization of Fish Meal in Aquafeeds

Fish meal is made from small, pelagic and oceanic fish namely menhaden, herring, anchovies and sardines. The fish are pulverized and the oil and water are removed. The residual solids are cooked, pulverized into a meal and the water is separated from the remaining liquid, providing fish oil as a byproduct of fish meal production (Boyd, 2015; Shepherd & Jackson, 2013).

The rapid expansion of aquaculture production lead also to a rapid development of aquafeed production, being fish meal in the past decades the preferred source of protein in aquafeeds (Fig. 4), due to their high protein content (usually 60-75%), excellent amino acid profile, high nutrient digestibility and lack of antinutrients (Gatlin et al., 2007; Jobling et al., 2001). As aquaculture increases in production numbers, it is only natural that the demand for fish meal will become even higher and according to the latest "The State of World Fisheries and Aquaculture" report (FAO, 2016), it is expected that during the 2010-2030 period, fish meal prices are going to escalate up to 90%. If aquaculture wants to maintain the expansion verified in the previous years, the industry cannot keep relying on a protein source that is known to have such a high variable cost and that relies on the harvest of wild stocks. With this in mind, it is essential to look for more sustainable and less expensive alternatives that can partially or even completely replace this protein source without harming the fish performance.

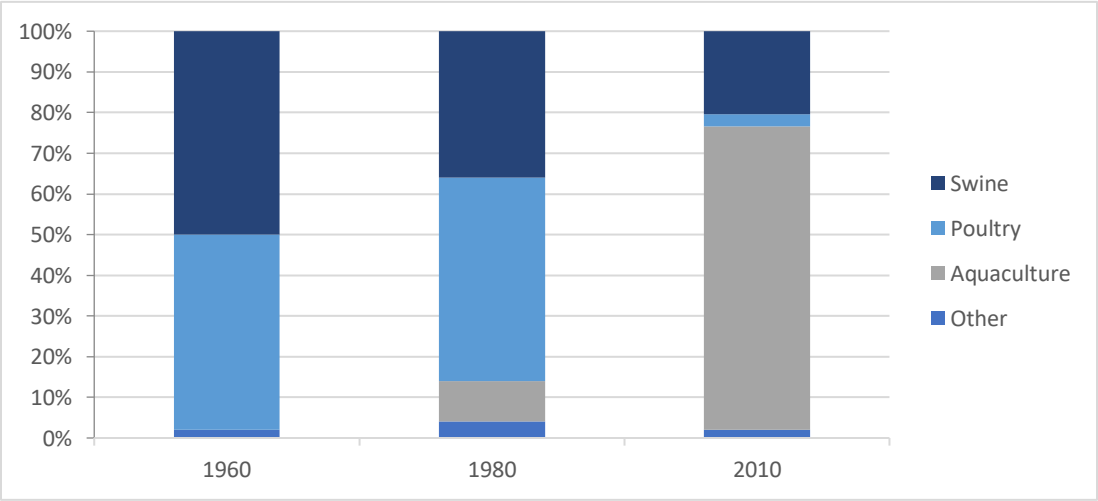


Figure 4 – Evolution of the fish meal utilization.
Source: Adapted from Shepherd & Jackson (2013).

Fish Meal Alternatives

The majority of fish species with economical value that are produced in aquaculture are almost pure carnivores, requiring a diet with a high protein content (Spinelli, 1980). Throughout the years, especially in the last two decades, a wide range of fish meal substitutes have been studied for the use in fish diets. Commercial feeds that are produced nowadays for carnivorous fish are usually based in a wide range of protein sources limiting the amount of fish meal used (Nogueira et al., 2012), being those substitutes from two main sources, namely plants and terrestrial animals (Glencross et al., 2007). In the past decades, several potential replacements for fish meal, such as plant proteins, have been evaluated and tested for suitability for use in aquaculture. The use of agricultural crops in the feeds lead to a competition between aquaculture and agriculture, and this can have effects on the availability and prices of these agricultural resources that are also used for human consumption, and because of this there is a need to look for feed sources that are not present in the human food chain (Olsen, 2011). Due to this fact, processed animal proteins (PAP), insects, one cell organisms and algae (micro and macroalgae) have been subjected to several experiments in the past years to assess their fittingness to be included in aquaculture feeds. Some ingredients used in these studies for fish meal replacement are presented in table 2, divided in five different classes: algae, one cell organisms, plant, insects and PAP.

Table 2 - Ingredients tested to replace fish meal in fish diets.

Ingredients	Class of Ingredient	Fish Species	References
Micro and macroalgae (*Porphyra* meal *Ascophulum* meal *Ulva* meal *Spirulina* meal *Gracilaria* meal *Tetraselmis* meal *Tisochrysis* meal)	Algae	Red seabream (*Pagrus major*); Sturgeon (*Acipenser baeri*); Nile tilapia; Gilthead seabream	(Mustafa et al., 1995; Palmegiano et al., 2005; Silva et al., 2015; Takeuchi et al., 2002; Vizcaíno et al., 2016; Vizcaíno et al., 2016)

Baker's yeast (*Saccharomyces cerevisiae*)	One cell organisms	Nile tilapia; European seabass (*Dicentrarchus labrax*)	(Abdel-Tawwab et al., 2008; Oliva-Teles & Gonçalves, 2001)
Blood meal	PAP	Australian silver perch (*Bidyanus bidyanus*); Nile tilapia; Rainbow trout (*Oncorhynchus mykiss*); Grouper (*Epinephelus coioides*); Gilthead seabream; Cuneate drum (*Nibea miichthioides*)	(Allan et al., 2000; El-Sayed, 1998; Lee et al., 2002; Lu et al., 2015; Millamena, 2002; Nogueira et al., 2012; Wang et al., 2006)
Blow fly (*Chrysomya megacephala*)	Insects	Nile tilapia	(Sing et al., 2014)
Canola meal	Plant	Australian silver perch; Ovate pompano (*Trachinotus ovatus*)	(Allan et al., 2000; Kou et al., 2015)
Carob (germ meal)	Plant	Meagre (*Argyrosomus regius*); Gilthead seabream	(Couto et al., 2016; Martínez-Llorens et al., 2007)
Common fly (*Lucilia sericata*)	Insects	Gilthead seabream	(de Haro et al., 2016)
Corn (gluten meal)	Plant	Australian silver perch; Senegalese sole (*Solea senegalensis*); Turbot (*Psetta maxima*); Rainbow trout; Gilthead seabream	(Allan et al., 2000; Cabral et al., 2013; Fournier et al., 2004; Gomes et al., 1995; Gómez-Requeni et al., 2004; Lu et al., 2015; Regost et al., 1999; Watanabe et al., 1993)
Cottonseed meal	Plant	Australian silver perch; Rainbow trout	(Allan et al., 2000; Lee et al., 2002)
Faba bean (protein concentrate)	Plant	Atlantic salmon (*Salmo salar*); Nile tilapia; Rainbow trout	(De Santis et al., 2015; Fontaínhas-Fernandes et al., 1999; Gomes et al., 1995)
Feather meal	PAP	Australia silver perch; Rainbow trout; Atlantic salmon; Gilthead seabream; Cuneate drum; European seabass	(Allan et al., 2000; Bureau et al., 2000; Campos et al., 2017; Hartviksen et al., 2014; Lee et al., 2002; Lu et al., 2015; Nogueira et al., 2012; Wang et al., 2006)
Grasshopper (*Zonocerus variegatus*)	Insects	African catfish (*Clarias gariepinus*)	(Alegbeleye et al., 2012)

Lupin (protein concentrate, sweet white)	Plant	Australian silver perch; Atlantic salmon; Nile tilapia; Turbot; Rainbow trout; Gilthead seabream	(Allan et al., 2000; Carter & Hauler, 2000; Fontaínhas-Fernandes et al., 1999; Fournier et al., 2004; Gomes et al., 1995; Gómez-Requeni et al., 2004; Robaina et al., 1995)
Meat and bone meal	PAP	Yellow croaker (*Pseudociaena crocea*); Australian silver perch; Rainbow trout; Nile tilapia; Spotted rose snapper (*Lutjanus guttatus*); Gilthead seabream; Cuneate drum; Snakehead (*Ophiocephalus argus*); Gibel carp (*Carassius auratus gibelio*)	(Ai et al., 2006; Allan et al., 2000; Bureau et al., 2000; El-Sayed, 1998; Hernández et al., 2016; Lee et al., 2002; Moutinho et al., 2017; Wang et al., 2006; Watanabe et al., 1993; Yu et al., 2015; Zhang et al., 2006)
Meat meal	PAP	Rainbow trout; Grouper	(Gomes et al., 1995; Millamena, 2002)
Pea (protein concentrate, extruded pea seed meal)	Plant	Australian silver perch; Milkfish; Senegalese sole; Atlantic salmon; Nile tilapia; Rainbow trout; Gilthead seabream	(Allan et al., 2000; Borlongan et al., 2003; Cabral et al., 2013; Carter & Hauler, 2000; Fontaínhas-Fernandes et al., 1999; Gomes et al., 1995; Gómez-Requeni et al., 2004; Hartviksen et al., 2014)
Potato (protein concentrate)	Plant	Senegalese sole; Rainbow trout	(Cabral et al., 2013; Tusche et al., 2012)
Poultry meal (by-products)	PAP	Australian silver perch; Nile tilapia; Atlantic salmon; Spotted rose snapper; Rainbow trout; Cuneate drum	(Allan et al., 2000; El-Sayed, 1998; Hartviksen et al., 2014; Hernández et al., 2014; Lee et al., 2002; Lu et al., 2015; Wang et al., 2006)
Rapeseed meal	Plant	Gilthead seabream	(Gómez-Requeni et al., 2004)
Soldier fly (*Hermetia illucens*)	Insect	Channel catfish (*Ictalurus punctatus*); Nile tilapia; Turbot; Rainbow trout; European seabass	(Bondari & Sheppard, 1981; Kroeckel et al., 2012; Magalhães et al., 2017; Stamer et al., 2014)
Sorghum	Plant	Australian silver perch	(Allan et al., 2000)

Soybean (soybean meal, protein concentrate, flour, full-fat, defatted soybean meal)	Plant	Australian silver perch; Atlantic salmon; Asian seabass (*Lates calcarifer*); Nile tilapia; African catfish; Rainbow trout; Sharpsnout (*Diplodus puntazzo*); Japanese flounder (*Paralichthys olivaceus*); Gilthead seabream; Cobia (*Rachycentron canadum*)	(Allan et al., 2000; Bjerkeng et al., 1997; Boonyaratpalin et al., 1998; Carter & Hauler, 2000; El-Saidy & Gaber, 2002; Fagbenro & Davies, 2001; Fontaínhas-Fernandes et al., 1999; Gomes et al., 1995; Hartviksen et al., 2014; Hernández et al., 2007; Kikuchi, 1999; Lee et al., 2002; Lu et al., 2015; Martínez-Llorens et al., 2007; Robaina et al., 1995; Suarez et al., 2013; Watanabe et al., 1993)
Sunflower (extracted sunflower meal)	Plant	Atlantic salmon; Gilthead seabream	(Hartviksen et al., 2014; Sánchez Lozano et al., 2007)
Super worm (*Zophobas morio*)	Insect	Red tilapia (*Oreochromis spp.*)	(Jabir et al., 2012)
Mealworm (*Tenebrio molitor*)	Insect	Rainbow trout: Nile tilapia	(Belforti et al., 2015; Sánchez-Muros et al., 2016)
Termite (*Macrotermes subhyalinus*)	Insect	Sampa (*Heterobranchus longifilis*)	(Sogbesan & Ugwumba, 2008)
Wheat (gluten meal, extruded whole wheat, wheat meal)	Plant	Australia silver perch; Senegalese sole, Turbot; Gilthead seabream; Rainbow trout	(Allan et al., 2000; Cabral et al., 2013; Fournier et al., 2004; Gómez-Requeni et al., 2004; Martínez-Llorens et al., 2007; Tusche et al., 2012)

Plant Proteins

Plant ingredients in the past years have been increasingly used in fish feeds. The complete replacement of fish meal by these plant protein sources has not been very successful, usually resulting in reduced growth and less efficient feed utilization. Many plant proteins are deficient in one or more IAA amino acids, so there is the need of supplementation of these amino acids to fit the requirements of the fish species, when a major amount of these ingredients are used (Jobling et al., 2001).

Soybean meal is considered to be the best available plant protein source being known to have a high content of available protein, a stable composition, a very reasonable price (comparing to fish meal) and a steady supply throughout the year (Hernández et al., 2007; Jobling et al., 2001). Most of the plant based sources are known to contain a wide variety of antinutritional factors (ANFs), and soybean is no exception. Although being widely used in aquafeeds, soybean meals usually present antinutrients. such as protease inhibitors and lectins that affect protein utilization and digestion, phytic acid that affects mineral utilization, saponins, phytoestrogens, antivitamins and allergens (Francis et al., 2001). Besides all these ANFs, the concentration of the 10 IAA, most importantly lysine, methionine, threonine and also tyrosine (a conditionally indispensable amino acid) are usually lower in soybean meals than in fish meal (Gatlin et al., 2007). Nowadays almost all soybean used in Europe is genetically modified, imported and therefore having a significant carbon footprint. Efforts must be done to find other sources of protein, with a lower carbon footprint, based in European products, thus boosting the local economy and resulting in more sustainable aquafeeds. With this in mind, other plant based ingredients such as rapeseed, pea, wheat, corn and carob, among others, appeared as possible replacements for fish meal.

Rapeseed is an oilseed, and the main product of its processing is rapeseed oil. After oil extraction, the resulting meal contains around 3.5% residual oil, 35% crude protein, 6% ash and 12% crude fiber, also containing 4% of phytic acid (ANF). From this product it is possible to obtain a protein concentrate with a protein content similar to high-quality fish meal (Gatlin et al., 2007).

Peas are a leguminous plant that is already commonly used in aquafeeds. Due to its nutrient profile, peas are a great candidate to replace a significant portion of fish meal (Gatlin et al., 2007). Albeit having a great potential for fish meal replacement, this leguminous plant has a few antinutritional factors such as protease inhibitors, lectins, tannins, cyanogens, phytic acid, saponins and antivitamins (Francis et al., 2001).

Wheat is a cereal widely produced around the world. Although being primarily milled for human consumption, nearly all the milling by-products can be used in animal diets. The gluten present in wheat is an effective binder for aquafeeds due to its strength and limited water solubility. This plant source has lower levels of protein (12%), lipids (1.7%) and ash (1.6%) when compared with other ingredients used for fish meal replacement and usually presents lower values of the IAA lysine, methionine and cystine (Gatlin et al., 2007). Because of its nutritional quality, this protein source has a limited potential for the use in diets and is usually combined with other sources with higher protein content (Tusche et al., 2012).

Corn gluten meal is the result of several milling processes that separate the corn kernel into its main components: fiber, germ, gluten and starch. After this process, gluten protein is concentrated, filtered and dried forming corn gluten meal. Refined corn gluten meal can have a crude protein content around 70-73%. This ingredient is widely used in aquafeeds, the protein is highly digestible but deficient in the IAA lysine (Gatlin et al., 2007).

Carob is a fruit pod obtained from the carob tree which grows throughout the Mediterranean region, namely in countries as Spain, Italy, Portugal and Morocco (Dakia et al., 2007). Carob germ meal is a by-product obtained from the germ of the carob seed after the separation of the gums and the fibrous coating of the seed and its protein content is high (45-50%). The ANF in this ingredient are tannins that reduce protein utilization and digestibility, which can be detrimental for fish growth (Martínez-Llorens et al., 2012).

Algae

In the past years, due to the deficiency in some amino acids of certain plant protein sources, algae appeared as a new potential element for inclusion in aquafeeds. Numerous species of micro and macroalgae started to emerge as a promising alternative, since they usually present a balanced amino acid profile (which varies from species to species), have a low lipid content, high levels of protein and are rich in minerals and vitamins. (Silva et al., 2015; Vizcaíno et al., 2016).

Processed Animal Proteins

In 2001, the use of terrestrial animal by-products in animal feeds was banned in the European Union (EU) countries, due to the problems associated with the bovine spongiform encephalopathy (BSE) that occurred in the late 1900´s. The following years after the prohibition, a few amendments were introduced that allowed slowly the re-introduction of some by-products in animal feeds (Nogueira et al., 2012). Only in 2013, in the EU Commission Regulation No 56/2013, processed animal proteins from the Category 3 were declared safe and were allowed for inclusion in aquaculture feeds. For this matter, research (within EU countries) in the utilization and incorporation of animal by-products in aquafeeds was stagnated for several years. The replacement of fish meal by terrestrial animal proteins is considered one of the approaches to reduce the amount of fish meal in fish diets. These animal proteins, such as poultry meal, blood meal, feather meal can be used in relatively high levels in aquafeeds (Wang et al., 2006). These protein sources have high protein levels, relatively balanced amino acid profiles, sometimes presenting deficiencies in the amounts of lysine, isoleucine and methionine (El-Sayed, 1998) and unlikely plant protein sources, these are free of ANFs (Nogueira et al., 2012).

Insects

Insects in the past years have also been considered as a new and renewable protein source for animal feed. According to Sánchez-Muros et al., (2014), there are around one million known species of insects and only 20% have been named and described showing the variety and the potential that these ingredients can have to replace fish meal. Insects are a natural food source for some species of fish, being rich in amino acids, lipids, vitamins and minerals (Henry et al., 2015). In the EU, the use of insects as aquaculture feed ingredient was not allowed until recently. In the 1st of July 2017, the EU Regulation 2017/893 was released, authorizing the use of insect proteins as fish feed, but only the ones derived from black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*).

One Cell Organisms

Yeasts are single cell proteins, and are a non-conventional protein source that can be used as a feed ingredient for fish diets due to their nutritional value, such as levels of protein, B-vitamins, pigments and complex carbohydrates (Oliva-Teles & Gonçalves, 2001).

Objective

The objective of this experiment was to evaluate the growth performance, nutrient balance and diet digestibility in gilthead seabream juveniles fed diets with high levels of fish meal replacement by alternative and more sustainable feed ingredients.

Methodology

Fish, Culture Conditions and Feeding Regime

The experiment was divided in two separate trials, a growth trial and a digestibility trial, both conducted at the Ramalhete Aquaculture field station of the University of Algarve, Faro, Portugal. The fish were obtained in the company Maresa (Huelva, Spain).

Growth Trial

Prior to the growth trial, fish were kept in a fiberglass tank and were fed with a commercial diet to maintain their weight. One week before the experiment, fish were individually sorted according to their weight (17.6 ± 2g $fish^{-1}$) and divided into 12 plastic tanks (3 per treatment) with a 110L capacity (Fig. 5), in a flow-through system with natural seawater, aeration and natural photoperiod. Initial density was nearly 5 kg m^{-3} (27 fish per tank). The number and weight of dead fish, oxygen and temperature were measured and registered daily. Water temperature was maintained with the aid of an exchanger and a heat pump to keep the temperature conditions (20 ± 2ºC) during the experiment.

Figure 5 – Growth trial experimental setup.

Before the start of the growth trial, fish were fed with a standard commercial diet for 5 days to aid in the acclimatization process. After this period, each diet was randomly assigned to triplicate tanks. Fish were fed for 12 weeks with the experimental feeds by hand, in small portions, until apparent satiation, two times a day (morning and afternoon), during six days (Monday to Saturday) and only once on Sundays (12h00). Prior to sampling days, fish were fasted during 24h.

Digestibility Trial

The fish used in the digestibility trial were the same fish subjected to the previous trial. One week before the start of the experiment, 180 fish were group weighted (63.56 ± 1.18g $fish^{-1}$) and transferred to 12 fiberglass cylindro-conical digestibility tanks (3 per treatment), with a 100L capacity, properly covered with a net to avoid any incidents (Fig. 6), in a semi-closed system with natural seawater, aeration and a 12h light/12h dark photoperiod, with the beginning of the light phase at 09h00. Water parameters such as temperature, oxygen and ammonia levels were measured and registered daily during the experiment. Water temperature was maintained around 20ºC with the aid of a heat resistance, and a 50% water change was performed every day after faeces collection, to ensure water quality.

Figure 6 - Digestibility trial experimental setup.

One week before the start of the experiment, fish were fed with the experimental feeds, to assure that all the feces collected were from the designated diet. The experimental diets were distributed in the tanks following the same order used in the previous experiment. Fish were fed with the experimental diets by hand in the morning (10h00), 12g per day, during 8 weeks from Monday to Saturday.

Experimental Diets

As presented in Table 3, a control (**CTRL**) diet was formulated, with a formulation similar to a commercial diet used nowadays in gilthead seabream production, with practical ingredients to contain 50% crude protein, 20% crude fat and 20 MJ kg^{-1} gross energy. The main protein sources consisted in fish meal (35%) and soy products (21%). Three other diets were formulated without any soy products and reducing the use of fish meal (by-products) to only 5%, to ensure the pellet palatability and therefore pellet acceptance by the fish. One diet was based in processed animal proteins, such as feather meal hydrolysates (4%), porcine blood meal (3.7%) and poultry meal (39%) as main protein sources (**PAP** diet). The second diet included plant-derived ingredients, such as pea protein concentrate (10%), wheat gluten (10%), corn gluten (14%) and carob germ (6.9%) as the main protein sources (**PLANT** diet). The third diet used a mixture of micro/macroalgae (*Spirulina*, *Chlorella* and *Scenedesmus*), insects (locust and tenebrio meal) and brewer's yeast as the main protein sources (**EMERG** diet). All experimental diets were formulated to be isoproteic and have similar levels of fat, energy and phosphorus. Table 4 presents the amino acid profile of each experimental diet. For the digestibility trial, the diets used had exactly the same formulation as the ones in the growth experiment, with the only difference being the incorporation of 1% of chromic oxide (Cr_2O_3), an inert marker which would allow to determine the apparent digestibility of nutrients and energy in each diet. All the experimental diets were formulated, manufactured and extruded at SPAROS, Lda. (Olhão, Portugal).

Table 3 - Formulation and proximate composition of experimental diets.

Ingredients	CTRL (%)	PAP (%)	PLANT (%)	EMERG (%)
Fish meal LT Diamante	30.0			
White fish meal (by-products)	5.0	5.0	5.0	5.0
Spirullina				7.0
Chlorella				15.0
Scenedesmus				15.0
Locust meal				5.0
Tenebrio meal				10.0
Feather meal hydrolysate		4.0		
Porcine blood meal		3.7		
Poultry meal 65		39.0		
Soy protein concentrate	11.0			
Pea protein concentrate			10.0	
Wheat gluten	3.0		10.0	4.5
Corn gluten	10.0	10.0	14.0	
Korfeed 60			14.0	5.0
Carob germ			6.9	
Soybean meal 48	10.0			
Rapeseed meal	5.0	5.0	5.0	
Wheat meal	7.0	15.5		
Wheat germ			5.0	5.7
Wheat DDGS			4.6	
Pea starch	3.0	3.0	3.0	
Fish oil	13.3	10.2	14	11.3
Vitamin & Mineral Premix PV01	1.0	1.0	1.0	1.0
Brewer's yeast				5.0
Soy lecithin – Powder	1.0	1.0	1.0	1.0
Binder (guar gum)	0.2	0.2	0.2	0.2
Macroalgae mix				5.0
Antioxidant powder	0.2	0.2	0.2	0.2
Sodium propionate	0.1	0.1	0.1	0.1
MCP		0.5	2.3	1.8
L-Histidine		0.1	0.4	0.4
L-Lysine		1.0	1.5	0.8
L-Threonine		0.2	0.7	0.2

L-Tryptophan			0.2	
DL-Methionine	0.2	0.3	0.9	0.8
Proximate composition				
Dry matter (DM), %	98.34	95.87	91.56	94.27
Crude protein, % DM	49.94	49.88	49.61	49.94
Crude fat, % DM	20.04	17.30	18.11	17.27
Ash, % DM	9.56	8.42	6.98	10.76
Phosphorus, % DM	1.14	1.18	1.15	1.19
Gross energy, MJ kg^{-1} DM	21.35	21.25	18.04	21.16

Table 4 - Amino acid profile (mg AA / g DW) of experimental diets.

Amino acid	CTRL	PAP	PLANT	EMERG
Arginine	32.4	31.9	35.8	32.2
Histidine	10.4	11.1	13.0	13.7
Lysine	27.6	29.7	26.3	28.6
Threonine	17.5	17.5	17.9	19.1
Isoleucine	19.0	16.1	14.7	16.9
Leucine	38.6	36.2	32.2	29.4
Valine	22.4	23.4	18.2	25.2
Methionine	13.1	11.9	14.4	15.0
Phenylalanine	22.0	20.1	19.8	18.7
Cystine	2.3	2.2	2.3	2.1
Tyrosine	18.6	15.8	16.8	21.8
Aspartic acid + Asparagine	41.0	31.9	30.4	35.1
Glutamic acid + Glutamine	51.2	59.4	82.5	55.1
Alanine	16.4	25.6	19.0	29.8
Glycine	28.7	34.3	20.4	29.2
Proline	26.8	29.9	26.7	25.4
Serine	21.9	32.1	19.0	19.6
Taurine	2.9	2.7	0.7	0.7

Sampling

Growth Trial

Twelve whole-fish were sampled from the initial stock for body composition analysis. During the experimental period, the amount of feed provided to each tank was monitored and fish were sampled two times. In these intermediate samplings, fish were counted and weighted (group weighting), in order to calculate growth indexes and feed conversion ratio.

The final sampling (third sampling) was separated in two days. In the first day, fish were counted and the final weight of each group recorded. Five fish from each tank were sacrificed (overdose with 2-phenoxyethanol), individually weighed and frozen for posterior body composition analysis (Fig. 7). Five additional fish from each tank were also sacrificed, individually weighted and a necropsy was made to remove the liver and viscera (including perivisceral fat), to obtain through their weight, the hepatosomatic (HSI) and viscerosomatic index (VSI).

Figure 7 – Frozen sampled fish before processing for proximate composition analysis

In the second sampling day, five fish from each tank were sacrificed, individually weighted and blood sampled one hour after feeding to better understand the amino acid absorption kinetics of the diets. The blood was centrifuged (3 min, 10 000 × *g*, 4ºC), the plasma separated from the cells was collected, snap-frozen in liquid nitrogen and stored at -80ºC until plasma amino acid analysis.

Digestibility Trial

Faeces were allowed to settle (around 22h) in 120mL collector cups (Fig. 8) after the tanks were thoroughly cleaned to ensure the removal of any uneaten feed or any accumulated waste. Faecal samples were collected every morning (09h30) prior to feeding, the maximum amount of water removed and stored in a freezer at -20ºC. Daily faecal samples from each tank were pooled over the course of the trial, until the collector cups were filled.

Figure 8 - Faecal samples.

Analytical Methods

Amino Acid Analysis

Amino acid analysis was made as described in Aragão *et al.* (2014) in plasma (postprandial), diets and faeces samples. Samples of the experimental diets were finely ground before the analysis. Samples of faeces were grinded and using a sieve, debris such as scales and other unwanted components were separated from the faeces.

Samples from experimental diets and faeces were hydrolyzed (6M HCl at 116ºC over 48h in nitrogen-flushed glass vials) before total amino acid analyses. Plasma samples were deproteinized by centrifugal ultrafiltration (10kDa cut-off, 2500 x *g,* 20 min, 4ºC) and then analyzed for free amino acid content. All samples were then pre-column derivatized with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Taq method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters reversed-phase amino acid analysis system, using norvaline as an internal standard. The resultant peaks were analyzed with EMPOWER software (Waters, USA).

Proximate Composition

Proximate composition analysis was made as described in Aragão *et al.*, (2014) in whole-fish (from the beginning of the experiment and from the end of the experiment), experimental diets and faeces samples. Dry matter was performed by drying at 105ºC for a 24h period. After this procedure, the samples were weighted and putted into a muffle furnace at 550ºC for 12h to access ash contents. Total nitrogen content of dried samples was determined using a Vario EL II elemental analyzer (Elementar). Fat content was determined after petroleum ether extraction (40-60ºC) using the Soxhlet method, and gross energy in an adiabatic bomb calorimeter (IKA).

Determination of Liver Lipid Content

Total lipid determination in the liver was made with a protocol adapted from Bligh & Dyer (1959). In this method, liver tissue was homogenized in a mixture of distilled water, chloroform and methanol (0.9:1:1 v/v) with the aid of an Ultrathurrax. After centrifugation (2000 x *g*, 10 min, 20ºC), the dilution with chloroform and water separated the homogenate in two layers, the chloroform layer, which contained the lipid fraction and the methanol layer that contained the non-lipid fraction (Fig. 9). A lipid extract was obtained, carefully isolating the chloroform layer using a Pasteur pipette. After isolation, a known volume (0.5 to 1.2 ml) of the lipid extract was placed into pre-weighted tubes and placed in a dry bath overnight for chloroform evaporation. The tubes were weighted after cooling and the lipid contents determined.

Figure 9 - Sample homogenate with the chloroform (below) and methanol layer (above)

Chromium and Phosphorus Analysis

Chromic oxide in diets and faeces was determined according to the method described in Bolin et al., (1952) after a digestion with an oxidant reagent based in sodium molybdate and perchloric acid. Determination of total phosphorus content in diets, fish and faeces was performed according to the norm AFNOR V 04-406 using the samples digested previously.

Data Analysis

Growth indexes were calculated as follows:

Daily growth index (DGI): 100 × ($FBW^{1/3}$ – $IBW^{1/3}$) x $days^{-1}$, where FBW and IBW are the final and initial mean weight in grams, respectively.

Weight gain (WG, %IBW): 100 × (final biomass – initial biomass) x initial $biomass^{-1}$.

Other indexes were calculated as follows:

Feed conversion ratio (FCR): feed intake (g) x wet weight $gain^{-1}$ (g), where wet weight gain is: (FBW – IBW).

Voluntary feed intake (VFI, %IBW day^{-1}): 100 x crude feed intake x IBW x $days^{-1}$.

Protein efficiency ratio (PER): wet weight gain (g) × crude protein intake $(g)^{-1}$.

Hepatosomatic index (HSI): 100 × liver weight (g) × whole body weight $(g)^{-1}$

Viscerosomatic index (VSI) as: 100 × viscera weight (g) × whole body weight $(g)^{-1}$.

Nutrient retention (% intake, dry matter basis): (FBW x final carcass nutrient – IBW x initial carcass nutrient) / (nutrient intake) x 100

Apparent digestibility coefficients (ADC) were calculated as: 100 × (1 – (dietary Cr_2O_3 level / faeces Cr_2O_3 level) × (faeces nutrient or energy level / dietary nutrient or energy level)).

Nitrogen (N) and phosphorus (P) balance in gilthead seabream fed the experimental diets were calculated as follows:

N gain (mg N kg^{-1} fish day^{-1}): (carcass final N content - carcass initial N content) / ABW / days, where ABW = (IBW + FBW) / 2.

Metabolic N losses (mg N kg^{-1} fish day^{-1}): digestible N intake – N gain, where digestible N intake (mg N kg^{-1} fish day^{-1}) was calculated as: N intake x protein ADC.

Faecal N losses (mg N kg^{-1} fish day^{-1}): N intake x (100 – protein ADC%).

P gain (mg P kg^{-1} fish day^{-1}): (carcass final P content - carcass initial P content) / ABW / days.

Metabolic P losses (mg P kg^{-1} fish day^{-1}): digestible P intake – P gain, where digestible P intake (mg P kg^{-1} fish day^{-1}) was calculated as: P intake x phosphorus ADC.

Faecal P losses (mg P kg^{-1} fish day^{-1}): P intake x (100 – phosphorus ADC%).

Statistical Analysis

All percentage data were arsine transformed prior to analysis (Ennos, 2007). Homogeneity of variances was checked by performing Levene's test and when homogeneity was verified, data was compared by one-way analysis of variance (ANOVA) and, if appropriate, means were compared using the post-hoc Tukey's honest significant difference test (Tukey HSD test). When homogeneity of the samples was not verified, a Kruskal-Wallis test was performed and the post-hoc Games-Howell was made to access the differences between groups. Data analysis was performed with the software program SPSS 24.0 (SPSS Inc., Chicago, IL, USA). A significance level of 5% was used for all comparisons.

Results

Growth Performance

The performance of fish fed with the various experimental diets during the growth trial is presented on figures 10 to 14.

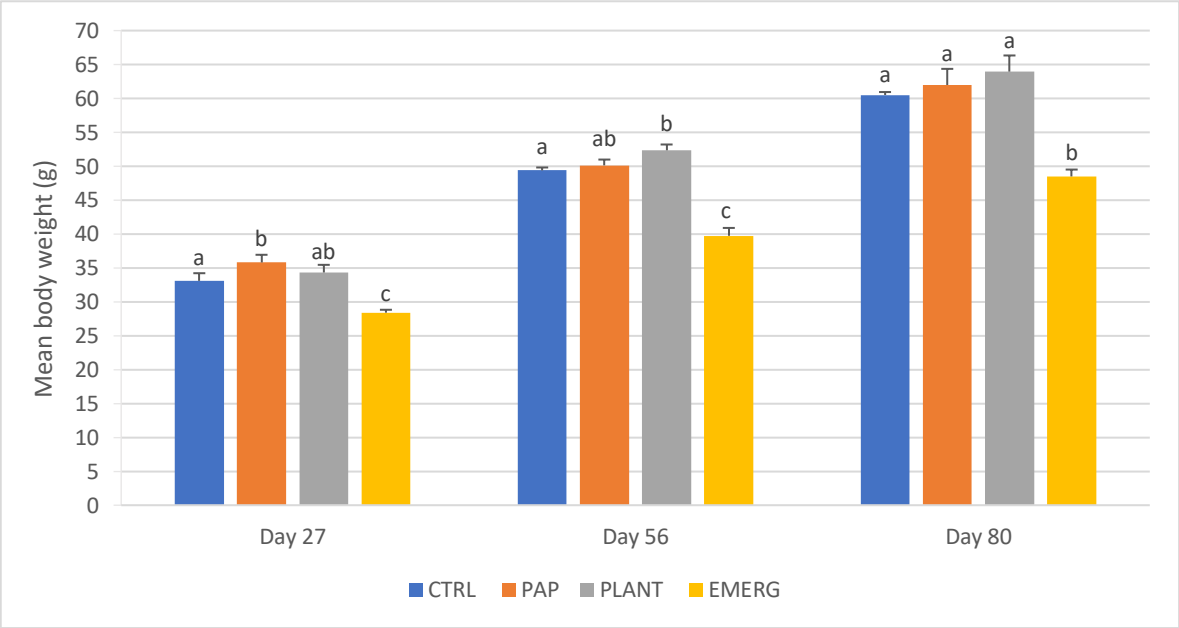


Figure 10 - Body weight during the experimental period. Data presented in this figure is divided in the 3 samplings that occurred during the experimental trial. Values are means ± standard deviation. Within the same sampling day, data represented in bars with different superscript letters differ significantly (P<0.05).

The effects of the dietary treatments can be seen in figure 10, which displays the wet weight over the 80-day trial. Fish grew from an initial mean weight of 17.6 ± 2g to a final weight of 60.50 ± 0.44g in fish fed with the CTRL diet, 61.98 ± 2.38g for fish fed with the PAP diet, 63.95 ± 2.38g for fish fed with PLANT diet and 48.49 ± 1.01g for fish fed with the EMERG diet. Final wet weight in fish from the EMERG treatment was significantly lower than in all other treatments.

During the experimental period, by day 27 fish fed with the EMERG diet showed a body weight significantly lower (P<0.05) compared with all the other experimental diets. Body weight of fish fed with PAP was significantly higher

(P<0.05) than fish fed the CTRL diet but not significantly different from the PLANT diet. On day 56, fish fed with the PLANT diet showed a body weight significantly higher (P<0.05) than the CTRL and EMERG treatments, but not significantly different from PAP. Fish fed with the EMERG diet ended the experiment with a significantly lower weight (P<0.05) than the other three experimental diets. Albeit no significant differences were verified between the remaining experimental diets, it is worth noticing that fish fed with the PLANT diet ended the experiment with the highest mean weight followed by PAP and CTRL diets.

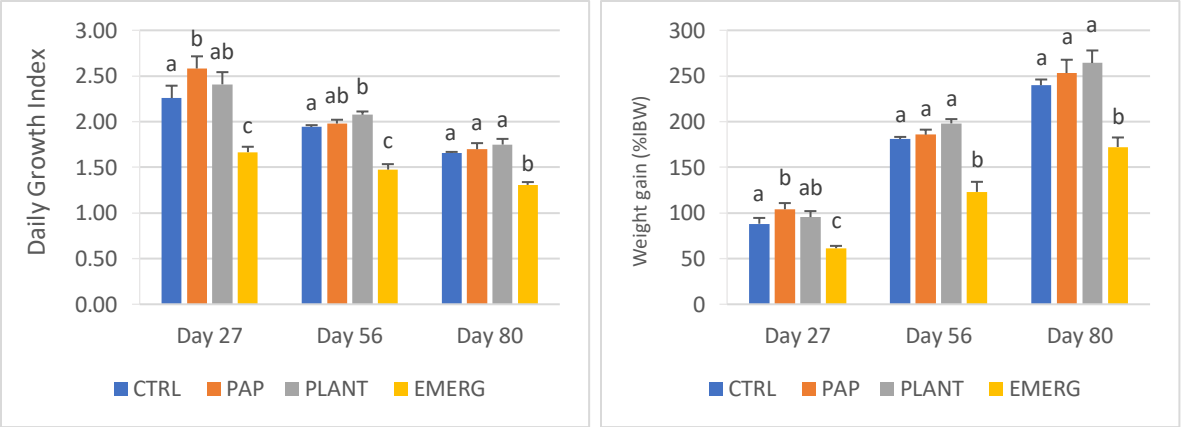


Figure 11 - Daily growth index and weight gain during the experimental period. Data presented in these figures is divided in the 3 samplings that occurred during the experimental trial. Values are means ± standard deviation. Within the same sampling day, data represented in bars with different superscript letters differ significantly (P<0.05).

Growth of gilthead seabream, expressed either as weight gain or daily growth index, on day 27, was significantly different (P<0.05) in the CTRL, PAP and EMERG treatments. No statistical differences were verified between PAP and CTRL or PLANT diets. On day 56, DGI from the CTRL, PLANT and EMERG treatments were significantly different from each other, and the PAP treatment only differed significantly from EMERG. Concerning WG by day 56, only the EMERG diet presented a significantly lower index than the other diets (P<0.05). At the end of the experiment DGI ranged between 1.31 and 1.75 and WG from 172 and 264%. EMERG presented significantly lower (P<0.05) values for both DGI and WG. Although no significant differences were observed within the other diets, it is worth verifying that the PLANT diet had highest value in both indexes.

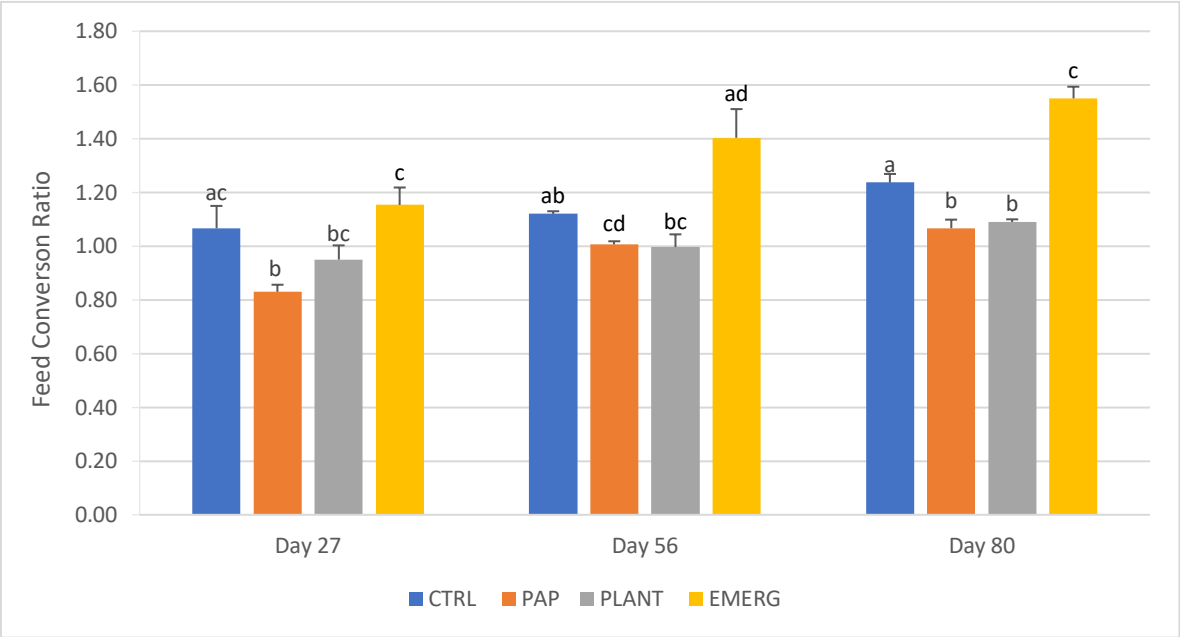


Figure 12 - Feed conversion ratio during the experimental period. Data presented in this figure is divided in the 3 samplings that occurred during the experimental trial. Values are means ± standard deviation. Within the same sampling day, data represented in bars with different superscript letters differ significantly (P<0.05).

Regarding the feed conversion ratio by day 27, fish fed with the PAP diet showed a significantly lower (P<0.05) FCR than fish fed with the CTRL and EMERG diets. On day 56, FCR was affected in all treatments (P<0.05) with PAP and PLANT showing lower values for this index. At the end of the experiment, FCR ranged from 1.07 to 1.55. Fish fed with PAP and PLANT diets, ended the experiment with a significantly lower (P<0.05) FCR than the other two experimental diets. Fish fed with the EMERG diet, had the worst FCR, ending the experiment with a FCR significantly higher (P<0.05) than the other experimental diets.

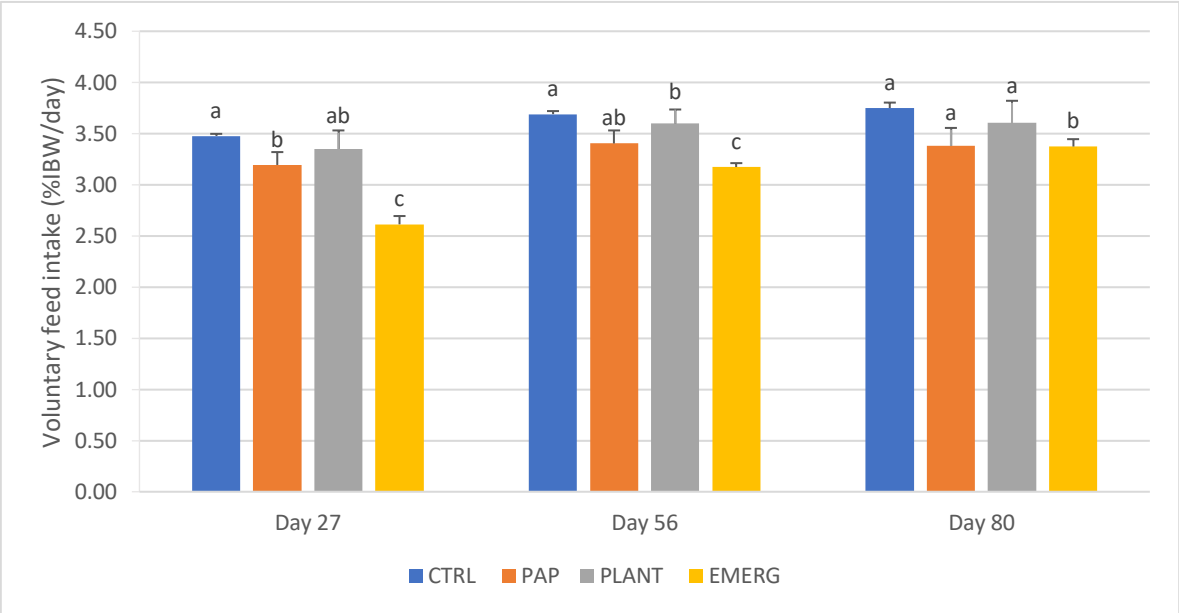


Figure 13 - Voluntary feed intake during the experimental period. Data presented in this figure is divided in the 3 samplings that occurred during the experimental trial. Values are means ± standard deviation. Within the same sampling day, data represented in bars with different superscript letters differ significantly (P<0.05).

Regarding the voluntary feed intake, by day 27, this index was significantly affected (P<0.05) in the various diets. The lowest value was verified in the EMERG diet and the highest in the CTRL diet. No differences were found between PLANT and CTRL or PAP diets. On day 56, as verified in the previous sampling, fish fed with the EMERG diet showed a significantly lower VFI (P<0.05). CTRL and PLANT treatments presented a significantly different VFI (P<0.05), with no significant differences between PAP and both of these treatments. At the end of the experiment VFI ranged between 3.38 and 3.75%IBW day^{-1}, with the EMERG treatment showing results significantly lower (P<0.05) from the other three diets.

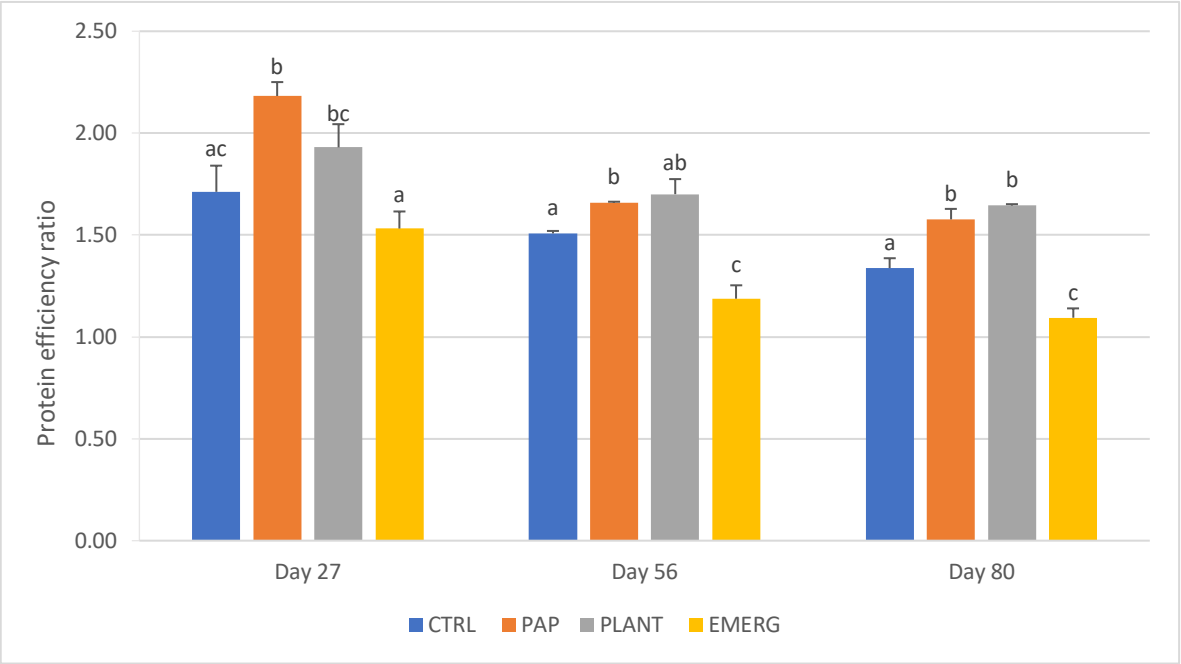


Figure 14 - Protein efficiency ratio during the experimental period. Data presented in this figure is divided in the 3 samplings that occurred during the experimental trial. Values are means ± standard deviation. Within the same sampling day, data represented in bars with different superscript letters differ significantly (P<0.05).

The protein efficiency ratio, by day 27, in the PAP diet, was significantly higher (P<0.05) than the CTRL and EMERG diets, but no significantly different from the PLANT diet. Concerning day 56, fish fed with the PLANT diet had higher values of PER, not being different from the ones obtained from CTRL and PAP diets. At the end of the experiment, PER in PAP and PLANT diets was significantly higher (P<0.05) than the in other two diets.

Fish Composition and Nutrient Retention

Comparing the proximal composition relative values of fish from the beginning of the experiment to the ones at the end (table 5), dry matter and gross energy values were higher in fish at the end of the experiment. Crude protein, ash and phosphorus by the end of the experiment showed a decrease in their relative values. Fish fed with the PAP diet were the only ones ending the experiment with a higher relative value of crude fat when compared with fish from the beginning of the experiment. At the end of the experiment, no significant differences were found among treatments.

Table 5 - Proximal composition of fish from the beginning and the end of the experiment.

	Initial	CTRL	PAP	PLANT	EMERG
Dry matter (DM), %	28.72 ± 0.53	34.19 ± 0.67	35.62 ± 0.23	34.87 ± 0.86	35.61 ± 1.70
Crude protein, % DM	56.36 ± 0.70	49.07 ± 2.32	48.40 ± 1.06	49.20 ± 4.77	48.98 ± 1.30
Crude fat, % DM	22.42 ± 0.33	21.87 ± 8.75	28.78 ± 1.88	20.38 ± 3.28	16.30 ± 1.08
Gross energy, MJ kg^{-1} DM	21.90 ± 0.07	23.29 ± 0.27	23.88 ± 0.15	23.19 ± 0.22	23.02 ± 0.54
Ash, % DM	12.66 ± 0.69	10.19 ± 1.65	9.35 ± 1.60	9.40 ± 0.98	11.51 ± 1.12
Phosphorus, % DM	2.46 ± 0.09	2.03 ± 0.08	1.87 ± 0.23	1.91 ± 0.10	2.22 ± 0.14

Values are means ± standard deviation.

The hepatosomatic index ranged from 1.54 to 1.89, with no significant statistical differences among treatments (table 6). Viscerosomatic index ranged from 4.42 to 5.04 and as the previous index, no significant differences were found among treatments. Fish fed with the PLANT diet had a liver lipid content significantly higher ($P<0.05$) than the ones fed with the EMERG diet.

Table 6 - Hepatosomatic index (HSI), viscerosomatic index (VSI) and lipid content in liver (% DW) of gilthead seabream fed with the experimental diets for 80 days.

	CTRL	PAP	PLANT	EMERG
HSI	1.54 ± 0.15	1.78 ± 0.19	1.64 ± 0.10	1.89 ± 0.18
VSI	4.85 ± 0.49	4.42 ± 0.48	4.65 ± 0.36	5.04 ± 0.26
Total lipid (% DW)				
Liver	29.62^{ab} ± 2.34	27.04^{ab} ± 3.87	35.09^{a} ± 4.13	25.02^{b} ± 3.06

Values are means ± standard deviation.

Within a row, means with different superscript differ significantly (P<0.05). Absence of superscripts indicates no significant difference between treatments.

Retention of ingested protein ranged from 23.63 to 33.10%. Protein retention was significantly lower (P<0.05) in fish fed with EMERG, when compared to fish fed with PAP and PLANT but no different from CTRL. Lipid retention in fish fed with the PAP diet was significantly higher (P<0.05) than fish fed with EMERG. No differences were verified amongst CTRL, PAP and PLANT diets. Phosphorus retention was not significantly affected (P>0.05) by the dietary treatments. Retention of energy varied between 27.91 and 40.55%, with seabream fed with PAP and PLANT diets showing a significantly higher energy retention than fish fed with the other two diets, while fish fed the EMERG diet showed the lowest energy retention.

Table 7 - Nutrient and energy retention (% intake) of gilthead seabream fed with the experimental diets over 80 days.

	CTRL	PAP	PLANT	EMERG
Protein	27.72^{ab} ± 2.15	33.10^{a} ± 1.71	32.07^{a} ± 3.27	23.63^{b} ± 0.75
Lipid	32.21^{ab} ± 17.92	54.83^{a} ± 3.32	33.72^{ab} ± 8.18	17.72^{b} ± 3.06
Phosphorus	49.22 ± 3.32	53.50 ± 10.87	52.14 ± 5.12	47.61 ± 4.46
Energy	32.50^{b} ± 1.19	40.55^{a} ± 0.79	37.11^{a}± 1.16	27.91^{c} ± 2.29

Values are means ± standard deviation.

Within a row, means with different superscript differ significantly (P<0.05). Absence of superscripts indicates no significant difference between treatments.

Apparent Digestibility of Nutrients

Data on the apparent digestibility coefficients of the experimental diets is presented in table 8.

Table 8 - Apparent digestibility coefficients (ADCs) of nutrients and energy of the experimental diets.

	CTRL	PAP	PLANT	EMERG
Protein	92.83 ± 0.74	91.11 ± 0.35	91.34 ± 4.93	69.79 ± 1.31
Lipid	94.45^{ab} ± 0.16	94.62^{a} ± 0.86	93.22^{b} ± 0.50	89.99^{c} ± 0.32
Energy	84.24^{a} ± 0.73	85.27^{a} ± 0.56	78.99^{b} ± 1.69	64.74^{c} ± 0.89
Phosphorus	58.71^{b} ± 3.90	58.04^{b} ± 2.47	67.75^{a} ± 0.42	62.45^{ab} ± 4.65

Values are means ± standard deviation

Within a row, means with different superscript differ significantly (P<0.05). Absence of superscripts indicates no significant difference between treatments.

No significant differences were observed in protein digestibility (P>0.05) among experimental diets (analysis on transformed data). Lipid digestibility in all treatments was high varying from 89.99 to 94.62%. Despite having high values, digestibility of lipids was significantly affected (P<0.05) in the various dietary treatments. Lipid ADC was significantly lower in fish fed with the EMERG diet and significantly higher in the PAP diet. Apparent digestibility of energy ranged from 64.74 to 85.27%, with CTRL and PAP treatments being significantly higher (P<0.05) than PLANT and EMERG, with the latter presenting the lowest value. Regarding phosphorus, availability was high (58.04 to 67.75%) in seabream fed with the experimental diets. Phosphorus ADC for CTRL and PAP treatments were significantly lower (P<0.05) than PLANT but no different from EMERG.

Data on the apparent digestibility of the amino acids is presented in table 9. Amino acid digestibility was affected in all experimental diets (P<0.05). IAA and DAA digestibility in general was high (above 85%) in the CTRL, PAP and PLANT diets with the exception of the amino acids valine, glycine and taurine in the PLANT diet. The EMERG diet presented values for digestibility bellow 85% in almost all amino acids with the exception of methionine and tyrosine. ADC of all amino acids but cystine were significantly lower in the EMERG diet than in the other three diets.

Table 9 - Amino acid apparent digestibility coefficients (%) of each experimental diet.

	CTRL	PAP	PLANT	EMERG
Arginine	$89.32^{b} \pm 0.29$	$94.03^{a} \pm 0.22$	$93.87^{a} \pm 0.27$	$81.64^{c} \pm 0.49$
Histidine	$94.87^{a} \pm 0.11$	$94.81^{a} \pm 0.05$	$95.24^{a} \pm 0.33$	$84.83^{b} \pm 0.63$
Lysine	$94.81^{a} \pm 0.11$	$95.15^{a} \pm 0.07$	$93.17^{b} \pm 0.13$	$82.32^{c} \pm 0.64$
Threonine	$88.13^{c} \pm 0.40$	$90.92^{a} \pm 0.20$	$89.30^{b} \pm 0.24$	$76.20^{d} \pm 0.77$
Isoleucine	$90.94^{a} \pm 0.33$	$89.17^{b} \pm 0.14$	$85.29^{c} \pm 0.29$	$73.98^{d} \pm 1.14$
Leucine	$91.04^{a} \pm 0.28$	$90.48^{a} \pm 0.24$	$86.33^{b} \pm 0.46$	$69.10^{c} \pm 1.05$
Valine	$86.82^{b} \pm 0.43$	$89.80^{a} \pm 0.27$	$83.37^{c} \pm 0.48$	$61.31^{d} \pm 3.71$
Methionine	$96.39^{a} \pm 0.12$	$95.54^{b} \pm 0.09$	$95.73^{b} \pm 0.22$	$93.50^{c} \pm 0.32$
Phenylalanine	$90.25^{a} \pm 0.40$	$88.83^{a} \pm 0.30$	$85.83^{b} \pm 0.35$	$76.26^{c} \pm 1.34$
Cystine	$94.70^{c} \pm 0.20$	$96.38^{a} \pm 0.05$	$95.59^{b} \pm 0.13$	$95.72^{abc} \pm 0.44$
Tyrosine	$90.42^{b} \pm 0.31$	$92.53^{a} \pm 0.12$	$91.16^{b} \pm 0.14$	$77.97^{c} \pm 0.65$
Aspartic acid + Asparagine	$91.16^{a} \pm 0.22$	$88.73^{b} \pm 0.35$	$85.28^{c} \pm 0.60$	$79.86^{d} \pm 0.67$
Glutamic acid + Glutamine	$91.21^{b} \pm 0.32$	$92.72^{a} \pm 0.18$	$93.51^{a} \pm 0.23$	$84.64^{c} \pm 0.67$
Alanine	$91.22^{a} \pm 0.15$	$92.06^{a} \pm 0.17$	$86.23^{b} \pm 0.61$	$68.08^{c} \pm 0.67$
Glycine	$87.78^{b} \pm 0.07$	$92.15^{a} \pm 0.20$	$83.15^{c} \pm 0.55$	$70.80^{d} \pm 1.20$
Proline	$91.00^{b} \pm 0.45$	$94.58^{a} \pm 0.04$	$92.36^{b} \pm 0.23$	$72.85^{c} \pm 1.27$
Serine	$91.01^{a} \pm 0.31$	$90.93^{a} \pm 0.31$	$87.28^{b} \pm 0.54$	$78.55^{c} \pm 0.95$
Taurine	$97.18^{a} \pm 0.27$	$95.65^{b} \pm 0.08$	$82.23^{c} \pm 0.30$	$78.68^{c} \pm 1.86$

Values are means ± standard deviation.

Within a row, means with different superscript differ significantly (P<0.05).

Nutrient Balance

Proximate carcass analysis combined with the data obtained on the ADC of the different diets, allowed the calculation of the nitrogen (Fig. 15) and phosphorus (Fig. 16) balance. Daily N gain (333 to 453 mg N kg^{-1} fish day^{-1}) was not significantly affected ($P>0.05$) within the dietary treatments. Faecal N losses ranged from 99 to 416 mg N kg^{-1} fish day^{-1}, with EMERG presenting significantly higher values ($P<0.05$) than the other three diets. Metabolic N losses varied between 585 and 872 mg N kg^{-1} fish day^{-1}. Fish fed with the CTRL diet had significantly higher ($P<0.05$) metabolic N losses that those fed with the other three diets.

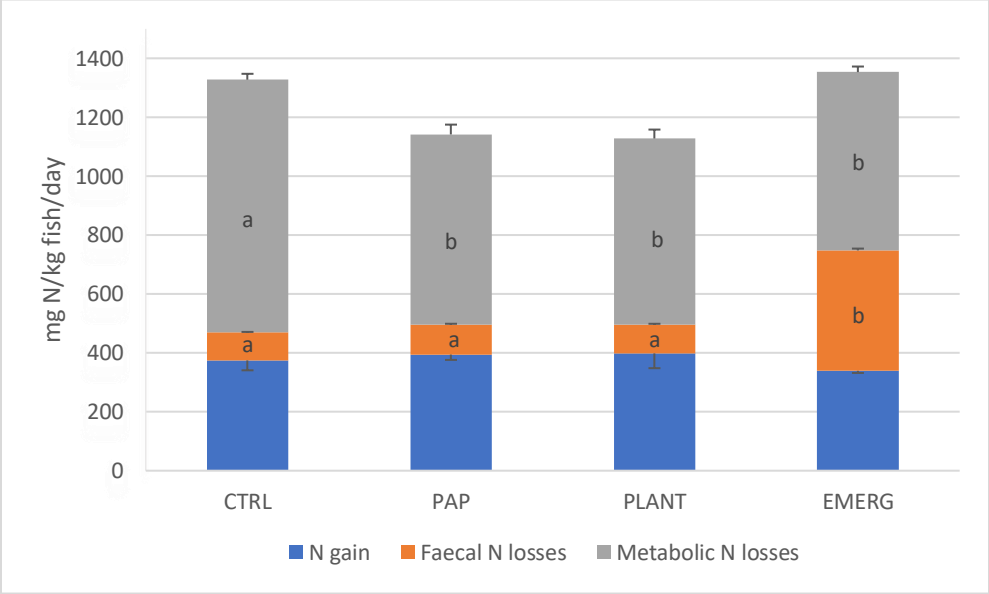


Figure 15 – Daily nitrogen balance in gilthead seabream fed with the various experimental diets. Values are means ± standard deviation. Data represented in bars with different superscript letters differ significantly ($P<0.05$). Absence of superscripts indicates no significant difference between treatments.

Daily P gain (80 to 110 mg P kg^{-1} fish day^{-1}) was not significantly affected (P>0.05) among the dietary treatments. Faecal P losses varied between 52 and 79 mg P kg^{-1} fish day^{-1}, with PAP and PLANT diets presenting values significantly lower (P<0.05) than the other two diets, being the values for PLANT diet significantly lower (P<0.05) than for PAP diet. Metabolic P losses ranged from 11 to 36 mg P kg^{-1} fish day^{-1} and no significant differences where verified amongst all diets.

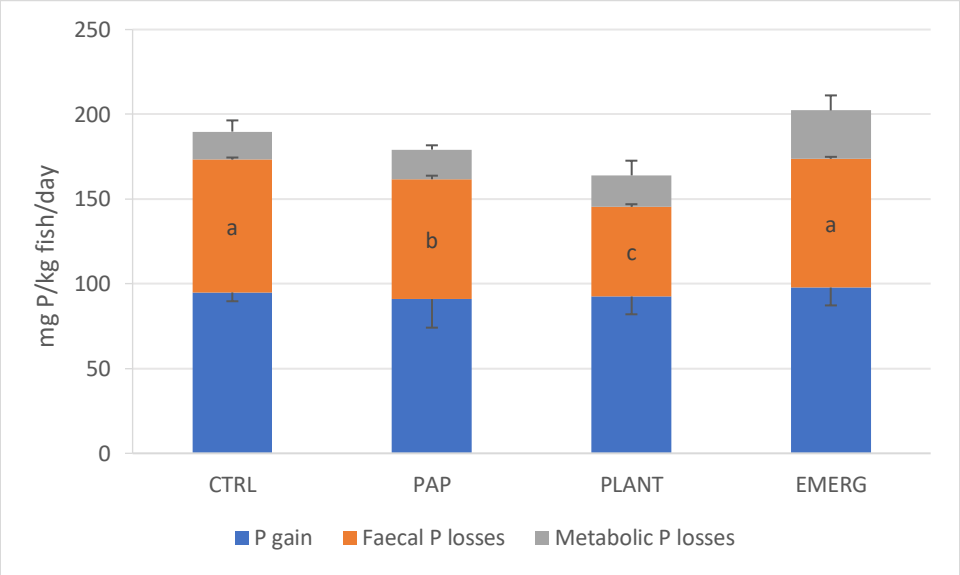


Figure 16 - Daily phosphorus balance in gilthead seabream fed with the various experimental diets. Values are means ± standard deviation. Data represented in bars with different superscript letters differ significantly (P<0.05). Absence of superscripts indicates no significant difference between treatments.

Plasma Free Amino Acid Analysis

Values of free amino acids in plasma collected 1h after feeding are presented in table 10.

Table 10 - Free amino acids (μM) in plasma of gilthead seabream collected 1h post-prandial.

	CTRL	PAP	PLANT	EMERG
Arginine	$34.52^{a} \pm 0.15$	$32.95^{b} \pm 0.39$	$37.25^{c} \pm 0.28$	$29.78^{d} \pm 0.29$
Histidine	$9.88^{a} \pm 0.11$	$10.35^{b} \pm 0.18$	$14.76^{c} \pm 0.19$	$11.05^{d} \pm 0.20$
Lysine	$39.12^{a} \pm 0.31$	$43.40^{b} \pm 0.57$	$52.94^{c} \pm 0.43$	$38.50^{a} \pm 0.08$
Threonine	$18.75^{a} \pm 0.12$	$21.70^{b} \pm 0.30$	$26.90^{c} \pm 0.24$	$17.65^{d} \pm 0.05$
Isoleucine	$15.66^{a} \pm 0.09$	$12.31^{b} \pm 0.19$	$13.86^{c} \pm 0.08$	$12.47^{b} \pm 0.05$
Leucine	$26.48^{a} \pm 0.14$	$26.20^{a} \pm 0.62$	$23.41^{b} \pm 0.29$	$20.14^{c} \pm 0.23$
Valine	$23.22^{a} \pm 0.15$	$23.25^{a} \pm 0.27$	$20.78^{b} \pm 0.62$	$21.59^{b} \pm 0.18$
Methionine	$15.09^{a} \pm 0.14$	$17.48^{b} \pm 0.32$	$26.00^{c} \pm 0.47$	$19.24^{d} \pm 0.22$
Tryptophan	$3.27^{a} \pm 0.10$	$2.96^{b} \pm 0.07$	$2.90^{b} \pm 0.06$	$3.17^{a} \pm 0.04$
Phenylalanine	$12.51^{a} \pm 0.18$	$12.85^{a} \pm 0.10$	$11.92^{a} \pm 0.74$	$9.12^{b} \pm 0.02$
Cystine	$2.88^{a} \pm 0.09$	$1.84^{b} \pm 0.02$	$1.77^{b} \pm 0.06$	$1.82^{b} \pm 0.06$
Tyrosine	$9.87^{a} \pm 0.12$	$9.47^{b} \pm 0.16$	$8.95^{c} \pm 0.08$	$9.45^{b} \pm 0.06$
Aspartic acid	$5.44^{a} \pm 0.13$	$4.74^{b} \pm 0.14$	$5.65^{a} \pm 0.01$	$5.04^{c} \pm 0.01$
Glutamic acid	$7.09^{a} \pm 0.09$	$7.32^{a} \pm 0.18$	$7.41^{a} \pm 0.14$	$8.86^{b} \pm 0.10$
Asparagine	$9.68^{a} \pm 0.08$	$10.67^{b} \pm 0.05$	$12.32^{c} \pm 0.15$	$8.62^{d} \pm 0.04$
Glutamine	$29.22^{a} \pm 0.47$	$28.53^{ab} \pm 0.38$	$27.65^{b} \pm 0.39$	$26.15^{c} \pm 0.24$
Alanine	$50.04^{a} \pm 0.43$	$70.44^{b} \pm 0.77$	$43.51^{c} \pm 0.52$	$38.72^{d} \pm 0.32$
Glycine	$25.69^{a} \pm 0.21$	$27.90^{b} \pm 0.32$	$26.95^{b} \pm 0.65$	$25.05^{a} \pm 0.22$
Proline	$17.13^{a} \pm 0.40$	$21.72^{b} \pm 0.51$	$19.87^{c} \pm 0.20$	$13.56^{d} \pm 0.2$
Serine	$21.46^{a} \pm 0.10$	$22.02^{a} \pm 0.33$	$17.32^{b} \pm 0.30$	$14.81^{c} \pm 0.25$
β-Alanine	$1.50^{a} \pm 0.01$	$1.50^{ab} \pm 0.04$	$1.56^{ab} \pm 0.04$	$1.34^{c} \pm 0.04$
Hydroxyproline	$9.52^{a} \pm 0.11$	$12.18^{b} \pm 0.19$	$7.76^{c} \pm 0.06$	$5.22^{d} \pm 0.12$
Ornithine	3.10 ± 0.16	2.86 ± 0.09	2.95 ± 0.03	2.89 ± 0.10
γ-Amino-n-butyric acid	$1.74^{a} \pm 0.10$	$1.58^{a} \pm 0.01$	$2.38^{b} \pm 0.10$	$1.60^{a} \pm 0.01$
Taurine	$36.07^{a} \pm 0.41$	$25.54^{b} \pm 0.32$	$24.48^{c} \pm 0.17$	$17.53^{d} \pm 0.22$

Values are means ± standard deviation.

Within a row, means with different superscript differ significantly (P<0.05). Absence of superscripts indicates no significant difference between treatments.

The concentration of free IAA and DAA in post-prandial plasma (1h after meal) was affected in all treatments (P<0.05) apart from the DAA ornithine (P>0.05).

The biggest differences in IAA concentrations between CTRL and the other three diets were mainly in isoleucine and cysteine. Availability of these amino acids was 12 to 21% lower than in CTRL for isoleucine and 36 to 39% for cysteine. Availability of the DAA taurine was much lower in the PAP, PLANT and EMERG diets than in CTRL with differences between 29 and 51%.

Discussion

A lot of research has been done towards decreasing the use of fish meal and fish oil in fish feeds. Regarding fish meal replacement, ingredients from plant origin have been the ones that got most of the attention from the scientific community in the past decades, but other protein sources as PAP, insects and algae are now emerging and showing a great potential to replace this widely used and finite ingredient.

Information about partial or total replacement of fish meal by plant protein sources is very scarce in gilthead seabream with the size-range used in this experiment. Martínez-Llorens et al. (2012) tested carob seed germ meal as a partial replacement for fish meal, reaching the conclusion that this ingredient can be included at levels up to 34% in the diets, in short term (less than 3 months), with no adverse effects either on growth or nutrient utilization. Data from that experiment also suggested that in a long-term feeding with this ingredient, negative effects could be seen in nutrient digestibility. In a previous study, Martínez-Llorens et al. (2007) tested soybean meal as a partial replacement for fish meal with results showing that this ingredient can be included at levels between 30 and 50% in diets without affecting growth. To the best of my knowledge, the studies made by Gómez-Requeni et al. (2004) and Kissil & Lupatsch (2004) are the only ones that attempted a complete replacement of fish meal by plant proteins in gilthead seabream. Gómez-Requeni et al. (2004) verified that fish fed with 100% of fish meal replacement showed a 30% lower weight gain, with the results also indicating

a detrimental effect over time. Kissil & Lupatsch (2004) achieved a successful replacement of fish meal by plant proteins (namely soy protein concentrate, wheat gluten and corn gluten meal) in fish with an IBW of 40g.

In the current experiment, a mixture of plant glutens, concentrates and by-products were used as alternatives to fish meal. Plant ingredients accounted for almost 70% of the PLANT diet formulation. At the end of the growth trial, fish fed with PLANT and CTRL diets showed a similar body weight and weight gain. The growth results obtained with the PLANT diet were relatively lower than the ones obtained in previous experiments (Gómez-Requeni et al., 2004; Martínez-Llorens et al., 2007, 2012) for the same period in fish with similar sizes and fed with diets with different levels of fish meal. However, a positive result was obtained for FCR, since fish fed with PLANT diet ended the experiment with a significantly lower FCR than fish fed with CTRL, and lower than the ones reported in previous experiments using diets with high levels of plant protein inclusion (Martínez-Llorens et al., 2007, 2012). Plant based diets have been reported to have a low palatability (Boonyaratpalin et al., 1998; Tusche et al., 2012), which can have repercussions leading to decreased feed intake. At the end of the experiment, fish fed with the PLANT and CTRL diets did not show any significant differences in the VFI, suggesting that a good palatability was ensured by the incorporation of 5% of fish meal by-products in the PLANT feed. Fish fed with the PLANT diet, at the end of the experiment had a significantly higher PER than fish fed with CTRL. These results were better than the ones found by Martínez-Llorens et al. (2012) that in an experiment with gilthead seabream obtained lower values for PER with the increase in the incorporation of plant proteins (comparing with the control), which by the end of the experiment had a detrimental effect on fish growth. Therefore, the PLANT diet formulation used in this study, including low levels of fish meal and no soy products, resulted in positive results regarding FCR and PER.

Regarding PAP, since the utilization of these protein sources was limited within the EU countries until recent years, there is not much information about the use of these ingredients in gilthead seabream feeds (Moutinho et al., 2017;

Nengas et al., 1999; Nogueira et al., 2012). Previous studies in other species showed that a PAP incorporation of 12-24% is possible in rainbow trout (Bureau et al., 2000), 12.5% in European seabass (Campos et al., 2017), 20% in gibel carp (Yu et al., 2015), 35% in spotted rose snapper (Hernández et al., 2016) and a replacement of 54.3% of fish meal in the diets has been achieved in yellow croaker (Ai et al., 2006). To the best of my knowledge, the only fish species in which a complete replacement of fish meal by PAP has been successfully achieved was in Nile tilapia (El-Sayed, 1998).

In the current experiment, PAP diet was formulated to include feather meal (4%), porcine blood meal (3.7%) and poultry meal (39%) as alternatives to fish meal. These high levels of dietary PAP inclusion did not affect negatively seabream growth. Fish fed PAP and CTRL diets ended the experiment with a similar mean body weight and weight gain. These results agree with the ones obtained by Nogueira et al. (2012), also in an experiment with gilthead seabream fed diets with an incorporation of feather meal and blood meal, although in lower levels of incorporation. The FCR value obtained in fish fed with the PAP diet was significantly lower than the value obtained for CTRL. To the best of my knowledge, this is the first time that such a low FCR is reported in studies with gilthead seabream with this size range and with such a high fish meal replacement by PAP. In the present experiment, the VFI was not significantly different between fish fed with PAP and CTRL diets. These results are contrary the ones found in a recent study made by Moutinho et al. (2017), in which a higher incorporation of PAP lead to an increase in feed intake. In the present experiment, fish fed with the PAP diet ended the experiment with a significantly higher PER than fish fed with the CTRL diet. These results indicate positive effects of the PAP diet formulation used in the current experiment, since results obtained by Moutinho et al. (2017), Nengas et al. (1999) and Nogueira et al. (2012) showed a decrease in the PER in gilthead seabream fed with increased amounts of PAP.

In the past years, new ingredients such as algae (micro and macro), insects and one cell organisms (namely yeasts) have been considered as a potential

replacement for fish meal (Abdel-Tawwab et al., 2008; Barroso et al., 2014; Brown, 2002; Guedes & Malcata, 2012; Henry et al., 2015; Li & Gatlin, 2003; Makkar et al., 2014). Fish fed diets with a small amount of algae (5%) have shown improved growth, feed efficiency and protein deposition (Mustafa et al., 1995). A study made by Palmegiano et al. (2005) showed that a 50% inclusion of *Spirulina* promotes a better growth rate, feed conversion ratio and protein efficiency in sturgeon. In a trial made with Nile tilapia, Silva et al. (2015) reached the conclusion that the species *Porphyra dioica* and *Ulva spp.* can be considered as potential ingredients to be included up to 10% in the diets of juveniles of this species. Vizcaíno et al. (2016) in a trial made with juvenile gilthead seabream identified a limit for the incorporation of *Gracilaria cornea* and *Ulva rigida* of 15 and 25%, respectively. Regarding the incorporation of insect meals into fish feeds, most of the published data has not been very conclusive concerning the amount in which this ingredient can be used. Only recently, in an experiment made with European seabass, Magalhães et al. (2017) concluded that 19.5% of black soldier fly pre-pupae meal can replace 45% of fish meal in diets for juveniles, without negatively affect growth, feed utilization, apparent digestibility coefficients or digestive enzyme activity. Concerning one cell organisms, namely the yeast *Saccharomyces cerevisiae*, studies have shown that the incorporation of these organisms in the diets of Nile tilapia and hybrid striped bass result in an increased growth and a better immunological response (Abdel-Tawwab et al., 2008; Lara-Flores et al., 2003; Li & Gatlin, 2003). Oliva-Teles & Gonçalves (2001), in an experiment with partial replacement of fish meal by these organisms, reached the conclusion that *Saccharomyces cerevisiae* can replace 50% of fish meal, with no negative effects in the performance of juvenile European seabass.

For the current experiment a formulation was made to include three main protein sources: insect meal (15%), micro/macroalgae meal (39%) and the yeast *Saccharomyces cerevisiae* (5%) as alternatives to fish meal. To the best of my knowledge, this is the first study in which these three protein sources were used in one single formulation, as most of the available studies use only one of these sources. Fish fed with this diet (EMERG diet) ended the experiment with a mean

body weight, weight gain, VFI and PER significantly lower than fish fed with the other experimental diets, and with a higher FCR. Although the results obtained for growth performance with the EMERG diet were not encouraging, there are previous works that prove that, when used in the right proportions, these ingredients can be functional in a fish diet. As mentioned previously, Vizcaíno et al. (2016) verified that the incorporation of *Gracilaria cornea* and *Ulva rigida* up to 15 and 25%, respectively, resulted in no differences regarding FBW and FCR when compared with a standard feed used in gilthead seabream culture. Studies regarding fish meal replacement by insect meals in gilthead seabream are very scarce. However, de Haro et al. (2016) showed that the incorporation of common green bottle fly larvae meal between 90 and 150 g kg^{-1} is not detrimental for gilthead seabream growth performance. The formulation used in this experiment for the EMERG diet did not seem to favor growth performance, but looking at the results from other studies mentioned above, it is possible to improve this diet to attain a better response regarding growth performance.

Viscera, liver and skin have been reported as an important fat storage tissues in fish (Cabral et al., 2013). Although no statistical differences were verified between HSI and VSI in fish fed with PLANT and CTRL diets, the values for liver lipid content were significantly higher in fish fed with the PLANT diet. In a similar experiment, Sitjà-Bobadilla et al. (2005) obtained results that showed the same trend found in this experiment. Results obtained for body composition of fish fed with the PAP diet showed an increase in the lipid body content along the experiment. Previous studies in which fish meal was replaced by PAP ingredients in feeds for gilthead seabream (Nogueira et al., 2012), spotted rose snapper (Hernández et al., 2016), cuneate drum (Wang et al., 2006) and gibel carp (Zhang et al., 2006) also showed the trend verified in this experiment. Fish fed with the EMERG diet ended the experiment with a considerable lower fat content than fish from the beginning of the experiment. These results are in agreement with the ones obtained with Nile tilapia (Takeuchi et al., 2002) and gilthead seabream (Vizcaíno et al., 2016) that tested high incorporation of algae meals. A possible explanation for this fact was made by Vizcaíno et al. (2016) that referred that

gilthead seabream fed on macroalgae utilize more efficiently the dietary lipids, resulting in decreased body lipid accumulation. Aside the differences referred above, the formulations used in this study did not seem to affect more aspects of the body composition in seabream with this size.

Concerning the effect of these novel diets on nutrient retention by the seabream juveniles, the results obtained in this experiment with the PLANT diet showed an opposite trend to the results obtained in previous works (Cabral et al., 2011; Regost et al., 1999), in which an increase in the incorporation of plant proteins led to a decrease in nutrient retention. This positive result for nutrient retention in fish fed the PLANT diet are in agreement with the good growth performance obtained in the growth trial. In a recent study, Campos et al. (2017) studied the dietary incorporation of hydrolyzed feather meal up to 12.5% in European seabass. In that experiment, an improvement in the retention of nitrogen, lipids and energy was seen, fact that was also observed in fish fed with the PAP diet used in the present experiment, when compared with fish fed with the CTRL feed. Information regarding nutrient retention with the ingredients (or type of ingredients) used in the formulation of the EMERG diet is very scarce. Anyhow, in an experiment with European seabass, Oliva-Teles & Gonçalves (2001) tested the partial replacement of fish meal by the yeast *Saccharomyces cerevisiae* with an improvement in protein and energy retention. The same result was not verified in the present experiment with the EMERG diet, but the lower nutrient retention is in agreement with the decreased growth performance observed in this treatment. Therefore, the formulations used in the PAP and PLANT diets seem to promote a better nutrient retention than a standard formulation for gilthead seabream.

The presence of ANFs, the chemical and physical composition of the ingredients in the feeds can have an effect in their digestibility (Martínez-Llorens et al., 2012). Results obtained for protein digestibility in fish fed with PLANT diet were very similar to the ones obtained for fish fed with the CTRL diet, while energy digestibility was lower in fish fed with the PLANT diet. Martínez-Llorens et al. (2012) obtained similar results when the dietary incorporation levels of plant

proteins increased. The cause for the differences in energy digestibility between plants and fish meal were explained by Kissil & Lupatsch (2004), and these can be related to the higher relative carbohydrate content in plant ingredients, as carbohydrates are less available as an energy source in carnivorous fish, such as gilthead seabream. Phosphorus digestibility in PLANT diets was significantly higher than in CTRL. Plant ingredients usually have low phosphorus content and might be rich in phytates, which cannot be broken down by non-ruminants, and their presence in feeds can reduce the availability of phosphorus (Dias et al., 2009; Francis et al., 2001). These results show that the availability of phosphorus was not compromised in the PLANT diet used in the present study.

At the end of the experiment, nutrients (proteins, lipids and phosphorus) and energy digestibility of the PAP diet was not different from the CTRL diet, showing the similarity of both diets. Protein digestibility coefficients between fish fed with the CTRL and EMERG diets were not significantly different on transformed data. Nevertheless, EMERG protein digestibility was considerably lower than CTRL (62.79 and 92.83%, respectively), which may have affected growth performance in fish fed with the EMERG diet. Lipid and energy digestibility was significantly lower than the ones observed either in CTRL and in the other experimental diets. These results have also been reported in turbot fed with grasshopper meal (Kroeckel et al., 2012). This issue can be related to the levels of chitin present in the diet, which were not assessed in this experiment. Insects are usually a poor source of carbohydrates, but they contain chitin, which is a primary component of their exoskeleton (Henry et al., 2015). Although gastrointestinal bacteria and chitinase activity can degrade this component, a reduced chitinase activity may be the result of a long-term adaptation to chitin-free diets (Kroeckel et al., 2012), which was probably the case in this experiment as fish were previously fed with a standard diet for gilthead seabream.

Amino acid digestibility in PLANT diets was high, with taurine having the lowest value. Taurine is present in high concentrations in fish meal but it is almost non-existent in plant ingredients, and even when all indispensable amino acid

requirements are met in plant-based diets for carnivorous fish, growth is often reduced due to the lack of this amino acid (Lunger et al., 2007). This information disagrees with the results obtained in the present experiment in which fish fed with PLANT diet presented the highest growth. However, Pinto et al. (2013) have shown that gilthead seabream is probably able to biosynthesize taurine from its precursors (methionine/cysteine), which may explain why growth was not affected in the current study. Amino acid digestibility amongst fish fed with the EMERG diet was significantly lower than in the other three diets, except for cystine. As diets had similar levels of protein (≈50%), the lower amino acid digestibility may have led to a lower amino acid availability, which might explain the lower growth observed in fish fed with this diet.

The environmental impact of fish farming is closely associated to excessive feed wastage and sub-optimal nutrient utilization. Therefore, the development of eco-friendly diets should target the reduction of nitrogen and phosphorus outputs (Cabral et al., 2013; Dias et al., 2009). In this experiment, the EMERG diet presented significantly higher results for the faecal N losses when compared with the other diets used, fact that can be explained with the relatively low digestibility coefficients obtained with this feed. It is also important to note that the results obtained for faecal N losses with PAP and PLANT diets are very similar to the ones obtained to the diet with the commercial formulation (CTRL), meaning that the high fish meal replacement did not affect faecal N losses. These results are in agreement to the ones obtained by Cabral et al. (2013) in an experiment with Senegalese sole, in which fish meal was replaced by plant proteins. Regarding P budget, the only significant difference among treatments was verified in the fecal losses, where the diets rich in either processed animal proteins or plant proteins led to lower excretions of P. This trend was also verified in previous works regarding fish meal replacement by plant proteins (Cabral et al., 2011, 2013; Dias et al., 2009) and processed animal proteins (Campos et al., 2017).

In summary, the results obtained in this work indicate that fish meal can be almost completely replaced by high levels of PAP and plant proteins, without impairing growth, feed intake and nutrient utilization. Moreover, the present results also suggest that fecal and metabolic N and P losses in fish fed with the PAP and PLANT diets result in lower waste outputs to the environment, making these feeds more environmentally friendly than the ones that are commonly used in gilthead seabream culture.

Conclusions

The results obtained in the present study show that the growth performance of juvenile gilthead seabream can be sustained and even improved by a practical dietary formulation containing as few as 5% of fish meal and a high incorporation of processed animal proteins or plant proteins based on concentrates, glutens and by-products. The diets PAP and PLANT showed a high nutrient and amino acid digestibility. No differences were observed on nitrogenous fecal losses among fish fed CTRL, PAP and PLANT diets, and regarding phosphorous fecal losses, these were reduced in fish fed with PAP and PLANT diets compared with CTRL. A slight improvement was observed regarding nitrogenous metabolic losses in the PAP and PLANT diets, resulting in less quantities of this nutrient being released into the environment when fish are fed with these diets.

Fish fed with the EMERG diet clearly had the lowest growth performance and the lowest nutrient digestibility, releasing also higher levels of nitrogenous and phosphorous compounds into the environment. Although this diet resulted in reduced growth performances, it is important to note that the utilization of these ingredients is relatively new in aquaculture feeds. Therefore, more research is clearly needed on this subject to find a suitable combination of ingredients that will allow to attain the desired response in terms of fish growth and nutrient utilization.

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