FACULDADE DE CIÊNCIAS E TECNOLOGIA

Dietary biodiesel-derived crude glycerol in gilthead seabream juveniles (*Sparus aurata*): effects on growth performance and metabolism

Rita Isabel Pontes Barbosa Colen

Masters in Aquaculture and Fisheries

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Declaração de autoria de trabalho

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

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Rita Isabel Pontes Barbosa Colen

Dedicatory

Este trabalho é dedicado à minha irmã, a minha estrelinha brilhante que apesar de já não estar comigo estará para sempre no meu coração.
Sempre foste uma fonte de inspiração apesar de nem sempre o demonstrar.
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INDEX

List of Tables

List of Figures

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Resumo

Na União Europeia, tem-se verificado uma procura crescente de novas fontes de energia renováveis, o que tem conduzido a um aumento da produção de biodiesel a partir de óleo de colza, originando o glicerol (também conhecido como glicerina) como um valioso subproduto. Por cada litro de biodiesel produzido, são geradas aproximadamente 79 g de glicerol bruto. À medida que a produção de biodiesel aumenta geram-se quantidades consideráveis de glicerol tornando-se urgente encontrar soluções adequadas à utilização deste excedente. Uma alternativa possível é a utilização de glicerol bruto na formulação de rações para animais. O glicerol tem sido estudado como fonte de energia nas dietas de diferentes animais, incluindo peixes. O presente trabalho descreve um estudo efectuado para avaliar o efeito da incorporação de glicerol bruto derivado da produção de biodiesel (a partir de óleo de colza) em dietas, com baixo teor em farinha de peixe, para juvenis de dourada (*Sparus aurata*). Os principais parâmetros avaliados foram o desempenho global de crescimento, a capacidade digestiva e a utilização metabólica de nutrientes. Duas dietas práticas foram formuladas para serem isoproteicas (proteína bruta, 45,4% de MS), isolipídicas (18,5% de MS) e isoenergéticas (energia bruta, 21,3 kJ / g MS). A dieta de controlo (CTRL) foi produzida com níveis intermédios de derivados de proteínas marinhas (19%). A partir da mesma formulação base, 5% de glicerol foi incorporado em detrimento do trigo obtendo-se a dieta glicerol (GLY). Os tratamentos foram testados em triplicado durante 63 dias, em grupos de 20 douradas com um peso médio inicial (PI) de 27,9 ± 0,12 g. No final do ensaio, os peixes alimentados com a dieta CTRL atingiram um peso final (PF) de 84,3 ± 2,2 g, triplicando o seu peso inicial. Os peixes alimentados com a dieta GLY mostraram um crescimento significativamente superior ($P < 0,05$) em termos de peso final e taxa de crescimento específico. A ingestão de alimento foi similar entre os dois tratamentos, mas quer a eficiência alimentar quer o coeficiente de eficácia proteica melhoraram significativamente ($P < 0,05$) nos peixes alimentados com a dieta GLY. A incorporação de glicerol na dieta não teve efeito ($P > 0,05$) sobre a digestibilidade aparente da proteína. Em comparação com o tratamento de controlo, o glicerol melhorou significativamente ($P < 0,05$) a retenção de proteína e de gordura. A atividade dos enzimas digestivos foi significativamente alterada pelos diferentes tratamentos. Peixes alimentados com a dieta GLY mostraram um aumento da atividade da fosfatase alcalina (ALP) e da pepsina, enquanto a atividade da lipase e da leucina-alanina peptidase (LAP) foram pouco influenciadas pelo glicerol na dieta. A dourada demonstrou uma boa capacidade de utilizar glicerol bruto como uma fonte energética fornecida pela dieta.

Palavras-chave: Biodiesel, glicerol, dourada, *Sparus aurata*, nutrição.

Abstract

In the European Union the turn towards renewable energy sources has increased the production of biodiesel from rapeseed oil, leaving glycerol (also known as glycerin) as a valuable by-product. For every litre of biodiesel produced, approximately 79 g of crude glycerol are generated. As the biodiesel production grows, the quantity of crude glycerol generated will be considerable and its utilization will become an urgent topic. One possibility is the use of crude glycerol on animal feeds. Glycerol has been evaluated as a dietary energy source for several farm animals, including fish. A study was undertaken to assess the effect of dietary biodiesel-derived glycerol (from rapeseed oil) on the overall growth performance, digestive capacity and metabolic nutrient utilization in juvenile gilthead seabream fed a low fishmeal level diet. Two practical diets were formulated to be isonitrogenous (crude protein, 45.4% DM), isolipidic (18.5% DM) and isoenergetic (gross energy, 21.3 kJ/g DM). The control diet (CTRL) was formulated with intermediate levels of marine-derived proteins (19%). In the same basal formulation, 5% glycerol (GLY) was incorporated at the expenses of wheat. Each dietary treatment was tested in triplicate tanks over 63 days, with 20 gilthead seabream (*Sparus aurata*), with a mean initial body weight (IBW) of 27.9 ± 0.12 g. At the end of the trial, fish fed the CTRL diet reached a final body weight of 84.3 ± 2.2 g (more than 3-fold increase of initial body weight). Fish fed the GLY diet showed a significantly higher ($P<0.05$) growth, expressed in terms of final body weight and specific growth rate. Voluntary feed intake was similar between the two treatments, but both feed efficiency and protein efficiency ratio were significantly improved ($P<0.05$) in fish fed the GLY diet. Dietary glycerol had no effect ($P>0.05$) on the apparent digestibility of protein. In comparison to the control treatment, dietary glycerol significantly improved ($P<0.05$) protein and fat retention. Activities of digestive enzymes were significantly affected by the various dietary treatments. Fish fed the GLY diet showed an enhanced activity of alkaline phosphatase (ALP) and pepsin, while activities of lipase and leucine-alanine peptidase (LAP) were little affected by dietary glycerol. Fish show the ability to use crude glycerol as a dietary energy substrate.

Keywords: Biodisel, glycerol, seabream, *Sparus aurata*, nutrition.

1. Introduction

1.1. Gilthead seabream is the major farmed species in the Mediterranean

Gilthead seabream is the most important species cultured in the Mediterranean region and its production is still in rapid expansion (Del Coco *et al.,* 2009). According to the latest statistics by the Federation of European of Aquaculture Producers (FEAP, 2011), in 2010 the seabream production in the EU countries and Turkey reached a volume 158.000 tonnes (Figure 1 and Table 1) and represented a total value of 477 M€. The majority of seabream consumers are still found in Mediterranean countries but sales have risen in more Northern markets such as the UK, Germany and Russia. Even exports to the USA have increased with Greek shipments of fresh seabream reaching 692 tonnes during the first six months of 2011, up from 388 tonnes in 2010 during the same period (FAO, 2012).

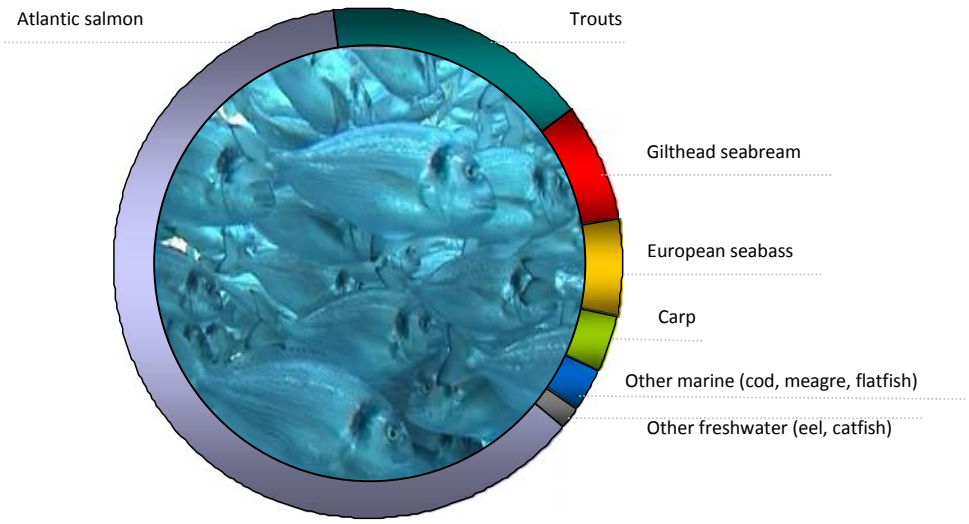


Figure 1. Major farmed species in EU. (Adapted from FEAP, 2011)

Table 1. Seabream production by EU country, in tons during the years 2003-2010 (FEAP, 2011)

COUNTRY	2003	2004	2005	2006	2007	2008	2009	2010
CROATIA	1000	1000	1200	1500	1500	1500	2200	2200
CYPRUS	1181	1356	1465	1879	1404	1600	2572	2799
FRANCE	1100	1600	1900	2200	1392	1636	1648	1377
GREECE	55000	48000	50000	66000	79000	94000	90000	74000
ITALY	9000	9050	9500	9500	11650	9600	9600	9800
MALTA	827	784	645	894	1097	1574	1984	2000
PORTUGAL	1449	1685	1519	1623	1600	1611	1600	1600
SPAIN	12442	13034	15577	20220	22320	23930	23690	20360
TURKEY	16735	20435	27634	28463	33500	34000	25000	22000
TOTAL	**98734**	**96944**	**109440**	**132279**	**153463**	**169451**	**158294**	**136136**

The seabream industry could be considered as a sector already entering its mature phase, but still needs more efficient production systems and new technologies. As in more traditional forms of animal production, nutrition plays a critical role in intensive aquaculture because it influences not only production costs (about 50% of operational costs) but also fish growth, health and waste production (NRC, 2011). To develop nutritious, cost-effective diets we must know the nutritional requirements of a given species and meet those requirements with will balanced diet formulations and appropriate feeding practices.

1.2. Current challenges on the nutrition of gilthead seabream

Being intensive aquaculture a feed based production system, a major endeavor over the past decades has been to understand and to improve digestible and metabolic utilization of dietary nutrients by fish. Fish diets are generally protein-rich, but a comparison between terrestrial monogastric animals and fish shows that the protein needs expressed per unit weight gain are only slightly different (Bowen, 1987). For more than thirty years now, two approaches have been used to reduce the protein cost involved in fish production: a) optimization of dietary protein utilization for growth; and b) reduction of dietary inclusion levels of expensive protein sources through the use of adequate substitutes.

Optimization of protein utilization by an adequate digestible energy supply

Proteins, carbohydrates and lipids are distinct nutrient groups that the body metabolizes to produce the energy it needs for numerous physiological processes and physical activities. There is considerable variation in the ability of fish species to use the energy-yielding nutrients. This variation is associated with their natural feeding habits, which are classified as herbivorous, omnivorous or carnivorous. Thus, there is a relationship between natural feeding habits and dietary protein requirements. Herbivorous and omnivorous species (e.g. carps. tilapias, catfish) require less dietary protein than some carnivorous species (NRC, 2011). Carnivorous species, such as Atlantic salmon, gilthead seabream or turbot are very efficient at using dietary protein and lipid for energy but less efficient at using dietary carbohydrates (NRC, 2011).

The value of a diet depends upon the levels and availability of all macro and micronutrients which have been shown necessary for fish (NRC, 2011). But, the energy content of the diet is of most importance, as fish like other animals adjust their feed intake to satisfy their energy requirements (Cho & Kaushik, 1990). Thereby, the actual intake of all the other nutrients is regulated by the available energy level of the diet. An improvement of dietary protein utilization, through a reduction of protein oxidation, implies an increase in the non-protein energy supply (fats or digestible carbohydrates). In rainbow trout, Médale et al. (1991) reported that under post-prandial conditions, the overall protein oxidation could be significantly decreased by the dietary use of digestible carbohydrates (gelatinized starch), which increased the contribution of carbohydrates to the overall energy supply. It is now clear for most cultivated species and particularly salmonids, that a significant dietary protein-sparring, can be achieved by increasing the dietary digestible energy (DE) levels, through the incorporation of fats and digestible carbohydrates (Cho & Kaushik, 1990).

Most studies performed with gilthead seabream seem to indicate that an increase in dietary fats can contribute to protein-sparing, while dietary crude starch is a poor non-protein energy source (Santinha *et al.,* 1996; Vergara *et al.,* 1996; Velázquez *et al.,* 2006). Research on the use of alternative dietary carbohydrate sources in fish or tools to promote its better utilization are needed.

Replacement of traditional marine-derived ingredients

Production of fishmeal and oil has remained relatively steady although the introduction of precautionary quotas and increased use for direct human consumption has resulted in reduced volumes of whole fish going for fishmeal and fish oil (IFFO, 2011). On the other hand, with the continuous global growth of aquaculture volumes (Tacon & Metian, 2008) and the concomitant increase of aquafeed production, in the past years, the use of fishmeal and fish oil as raw materials for fish feeds has become a critical element for development (Bouraoui *et al.,* 2010). A shift towards a lower usage of finite marine-harvested resources is a clearly identified as an environmental and economical sustainability challenge faced by the aquaculture industry.

It is now consensual that plant-based proteins (crude protein content ranging from 36 to 50 %) and vegetable oil sources are valid ingredients in fish feeds and nutritional alternatives to fishmeal and fish oil (Tacon & Metian, 2008; Fountoulaki *et al.,* 2009). Regarding fishmeal replacement, available knowledge shows that sensible blending of different vegetable protein sources is necessary to balance the indispensable amino acid (IAA) profile. Furthermore, under high fishmeal replacement certain crystalline IAAs and inorganic phosphorus may have to be supplemented to fulfill the requirements of a given species. There is also evidence that when including high levels of certain plant protein sources, the carbohydrate fraction and/or the presence of heat stable anti-nutritional factors may condition its nutritional value by altering digestion and nutrient utilization (Dias *et al.,* 2009).

Previous studies with gilthead seabream showed the feasibility of replacing high levels (up to 50-75%) of fishmeal by plant protein sources, provided that diets are duly supplemented with essential amino acids (i.e. lysine and methionine) and inorganic phosphorus sources (Gomez-Requeni *et al.,* 2004; De Francesco *et al.,* 2007). Substitution of up to 60% fish oil (FO) by vegetable oils does not seem to affect seabream growth (Izquierdo *et al.,* 2005; Fountoulaki *et al.,* 2009; Wassef *et al.,* 2009).

Efforts have now been directed towards the assessment of the concomitant replacement of fishmeal and fish oil by vegetable ingredients in seabream feeds. It has been reported that the partial replacement (33 and 66%) of fish oil by a blend of vegetable oils

(rapeseed, linseed and palm oils) associated to a plant-protein rich formulation had no detrimental effects on growth performance of juvenile seabream (Benedito-Palos *et al.,* 2007; Benedito-Palos *et al.,* 2008; Dias *et al.,* 2009; Matos *et al.,* 2012). However, high dietary inclusion levels of plant proteins may affect growth, feed efficiency, digestive enzyme activities and in some cases the overall immune status and hormonal control of fish (Sitjà-Bobadilla *et al.,* 2005; Bouraoui *et al.,* 2010; Silva *et al.,* 2010; Santigosa *et al.,* 2011). Despite being the only viable option at present, some recent studies assessing a broader range of the sustainability criteria (e.g. life cycle analysis, land use, carbon footprint, food mileage) tend to show that the association of vegetable-based diets and higher sustainability is not a clear-cut scenario as anticipated (Boissy *et al.,* 2011). Therefore, the search for sustainable alternative ingredients for fish feeds is still needed.

1.3. Emerging ingredients for aquaculture feeds

Current formulations for gilthead seabream during the grow-out stage (fish size > 20 gram) contain 35 to 42% marine protein sources (fishmeal of various qualities and smaller amounts of soluble fish protein concentrates). Regarding fish oil, current estimations point to a 12-14% feed inclusion level.

To be a viable alternative feedstuff to fish meal in aquafeeds, a candidate ingredient must possess certain characteristics, including wide availability, competitive price, plus ease of handling, shipping, storage and use in feed production. Furthermore, it must possess certain nutritional characteristics, such as low levels of fibre, starch, especially non soluble carbohydrates and anti-nutrients, plus have a relatively high protein content, favorable amino acid profile, high nutrient digestibility and reasonable palatability (Gatlin *et al.,* 2007).

For novel ingredients, the issue is largely dependent on economics and large-scale production capacity to be cost-competitive in a global market. Nowadays, emerging ingredients are:

- Marine by-products: discards, by-catches, wastes from industrial fisheries and target exploitation of zooplanckton stocks (copepods, Antarctic krill);

- Processed animal proteins (PAPs) and oils derived from terrestrial animal by-products (e.g. blood meals, feathermeal hydrolisates, gut mucosa hydrolisates, poultry fat, porcine lard);
- Single-cell proteins (e.g. yeasts, bacteria and microalgae) grown on agrofood or other industrial wastes;
- Insect meals grown on agrofood-industrial wastes (substrates such as dairy products, by-products of the alcoholic beverage, cereal husks) which otherwise would have to be eliminated through landfills at rather high costs;
- A series of by-products associated to the biodiesel industry (e.g. corn and wheat distillates, glycerol, microalgae biomass).

Discards from fisheries, estimated to be some 20 MT/year (Hall & Mainprize 2005), and by-catches and wastes from processing, estimated to be 25 to 30 MT/year (Naylor et al. 2009), are both potential sources for fish feed. None of these resources are easily available, but improved logistics in fish processing and an efficient international regime for managing marine catches can make better use of the resources available (Naylor et al. 2009). Nevertheless, none of these resources can alone support a major expansion in marine aquaculture.

The world's oceans have large stocks of herbivore copepods and krill that are abundant and potentially exploitable. The standing stock biomass of Antarctic krill is estimated to be 500 MT and the annual production must at least be well above 100 MT/year (Olsen, 2011). In recent years, Antarctic krill has been industrially harvested, but landings are less than 1 MT/year, well below the allowed quota of 6 MT/year (Naylor et al. 2009). A similar scenario can be found for the industrial exploitation of copepod populations of *Calanus* species. These issues are controversial, but science should nevertheless thoroughly explore the possibilities, constraints and consequences of an alternative strategy for harvesting large zooplankton stocks in the oceans.

Draft legislation published by the European Commission confirms the safety, feed value and environmental importance of processed animal protein, according to EFPRA, the body which represents European animal by-product processors. A wider use of porcine and avian processed animal proteins (PAP) in fish feeds is expected to be legally authorized by mid 2013. These PAPs are not only a sustainable source of protein, of

high biological value (adequate amino acid profile, highly digestible, low phosphorus) and large quantities are available in abattoirs throughout the world (Udo *et al.,* 2012).

Single cell proteins, including yeast, microalgae and bacteria, have been viewed as promising substitutes for fishmeal and fish oil in fish diets (Li & Gatlin III, 2003). Interest in unicellular proteins has increased as a result of continuously growing fermentation industries which produce microorganism biomass as a by-product. Yeast biomass is not only a source of proteins but also an excellent source of B-complex vitamins, nucleic acids, vitamins and minerals (Ferreira *et al.,* 2010). A dietary inclusion of up to 30% brewers yeast improved feed efficiency and protein utilization of European seabass (Oliva-Teles & Gonçalves, 2001). A bacterial protein meal produced based on methane from natural gas is a new resource, and feeding experiments with rainbow trout using a feed containing up to 27% bacterial protein meal has produced positive results (Aas *et al.,* 2006). Microalgae have recently attracted considerable attention as possible sources of bioenergy (Patil et al. 2008, Xu et al. 2009), and major research efforts made to explore these possibilities will probably also be useful for aquaculture. What makes microalgae attractive as an ingredient for aquaculture feed is that some species are not only protein rich, but have high contents of HUFA, in particular of DHA (Olsen, 2011).

World energy demand continues to rise. The most feasible way to meet this growing demand is by using alternative fuels (Demirbas, 2008). Biodiesel and ethanol have been developed as bio-fuel to substitute petroleum derived fuels (Borugadda & Goud, 2012). Biofuels are expected to meet increasing share of the demand growth in liquid fuel (Figure 2).

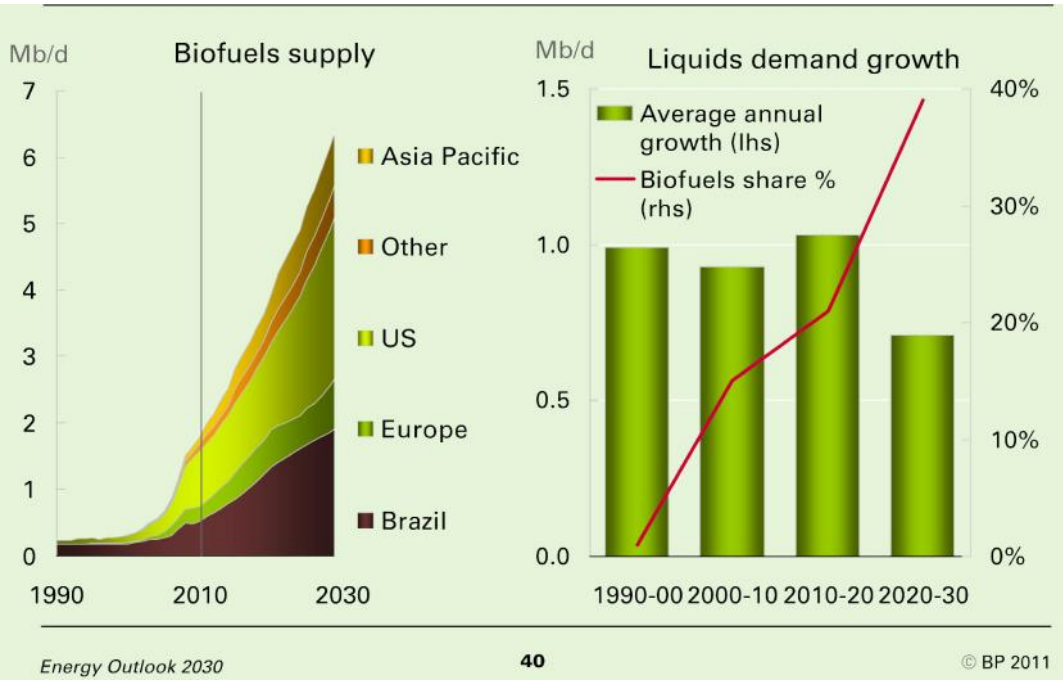
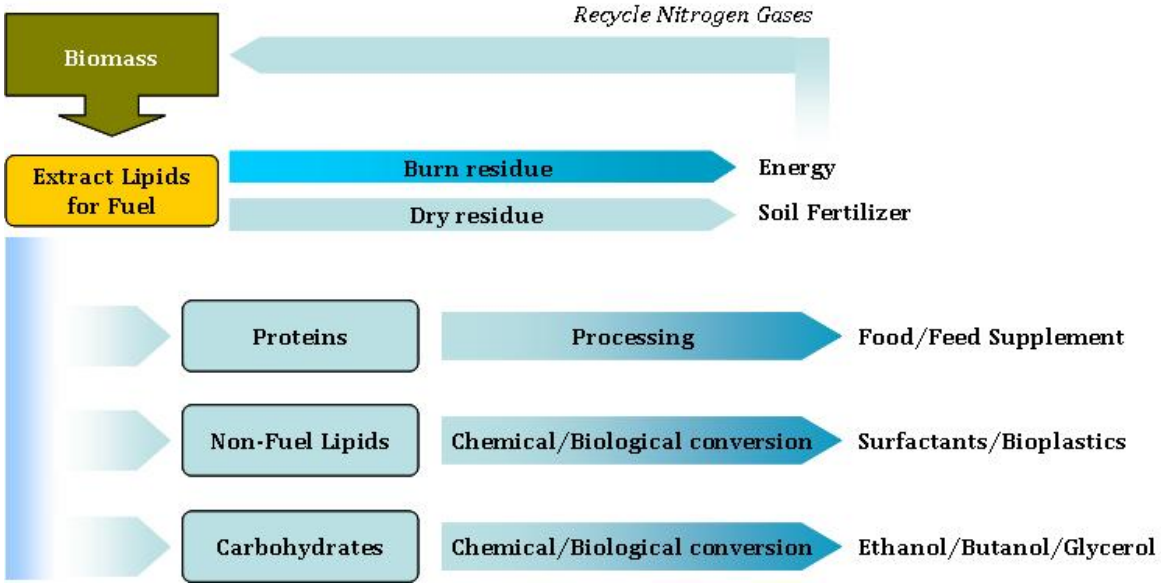


Figure 2. Biofuels meet an increasing share of the demand growth in liquid fuel
(Data source: BP Statistical Review- Energy Outlook 2030. January 2011 in http://www.jatrogreentech.com/energy/bio/)

In a European context, biodiesel can be produced from oilseeds (rapeseed, sunflower), cereals (corn, barley) and animal fats. There are a multitude of fast-evolving options for recovering economic value from the lipid-extracted biomass. Despite in some cases, still under assessment at a lab scale, most biorefinery concepts rely on the following processes:



Given the large amounts of lipid-extracted biomass residues that will likely be generated in future biofuels industry, the valorization of every component of remaining biomass raw material and co-products must be considered as a means to enhance the economics of the process.

Among others valuable compounds, glycerol, also known as glycerine, is a by-product of the biodiesel generation process. Crude glycerol obtained as a co-product of biodiesel from vegetable oils contains minimum 80 % of glycerine, maximum 0.5 % of methanol and about 1.2 % of non-glycerine organic material (EFSA, 2010). Glycerol can be produced either by microbial fermentation or by chemical synthesis from petrochemical feedstocks or can be recovered as a by-product of soap manufacture from fats (Wang *et al.,* 2001). For which liter of biodiesel produced, approximately 79 g of crude glycerol are generated and although glycerol is extensively used in food, cosmetic, and pharmaceutical industries, the purification of biodiesel-derived crude glycerol is not economically viable for the biodiesel plants. As more and more crude glycerol is continuously generated, the development of high-value co-products from biodiesel-derived crude glycerol is critically important for the long-term sustainability of biodiesel industries (Thompson & He, 2006, Nitayavardhana & Khanal, 2011). Currently glycerine is classified as a feed material according to Commission Regulation (EU) 892/2010 (EFSA, 2010). Despite being dependent on the type and conditions of biodiesel processing, the typical composition of glycerol is reported in Table 2 (Nitayavardhana & Khanal, 2011).

Glycerol is a polyhydroxyl alcohol (polyol), or a sugar alcohol. However, from a nutritional standpoint, glycerol is considered as a carbohydrate source. The experts seem to disagree as to whether glycerol should be considered a carbohydrate or a simple energy source (Dasari, 2007). Metabolically, glycerol can serve as a precursor for synthesis of triacylglycerols and phospholipids in the liver and adipose tissue. But also, during periods of nutrient/energy deprivation glycerol can be released by lipolysis of the adipose tissue. When released into the bloodstream, glycerol is taken up by the liver for the synthesis of glucose (gluconeogenic precursor) and may serve as energy substrate. Because the liver also synthesizes a significant amount of triacylglycerol during fasting, it would be logical to assume that glycerol could also participate on the glyceride-glycerol step for triacylglycerol synthesis (Kalhan *et al.,* 2001).

Table 2. Characteristics of crude glycerol.

Parameters	Values
pH	4.00–4.50
Glycerol (g/L)	887.00 ± 59.59
Methanol (g/L)	N/D
Lactic acid (g/L)	N/D
Acetic acid (g/L)	N/D
Total solids (TS) (%)	6.72 ± 0.52
Volatile solids (VS) (%)	3.58 ± 0.42
Total suspended solids (TSS) (%)	0.27 ± 0.03
Volatile suspended solids (VSS) (%)	0.16 ± 0.02
Total chemical oxygen demand (COD) (g/L)	1259.44 ± 26.01
Soluble chemical oxygen demand (SCOD) (g/L)	1227.08 ± 12.36
Total nitrogen (mg/L)	4.04 ± 0.46
Potassium (K) (mg/L)	23,833.33 ± 288.68
Phosphorus (P) (mg/L)	25.57 ± 0.15
Calcium (Ca) (mg/L)	1.72 ± 0.01
Magnesium (Mg) (mg/L)	9.47 ± 0.22
Sodium (Na) (mg/L)	596.50 ± 12.49
Sulfur (S) (mg/L)	N/D
Iron (Fe) (mg/L)	2.55 ± 0.06
Manganese (Mn) (mg/L)	0.13 ± 0.00
Zinc (Zn) (mg/L)	0.32 ± 0.01
Copper (Cu) (mg/L)	0.26 ± 0.00
Boron (B) (mg/L)	0.25 ± 0.02
Molybdenum (Mo) (mg/L)	N/D
Aluminum (Al) (mg/L)	N/D
Silicon (Si) (mg/L)	N/D

Note: N/D, non-detectable.
* All analyses are based on n (sample size) = 6.

In land-based animals the inclusion of crude glycerol in the diets has been investigated, and several studies show that the addition of glycerol in levels between 5 and 10% increased growth rates and feed intake, improve meat yield in poultry (Cerrate *et al.,* 2006, Swiatkiewicz & Koreleski, 2009, Min *et al.,* 2010, Kroupa *et al.,* 2011), pigs (Della Casa *et al.,* 2009, Schieck *et al.,* 2010a, Schieck *et al.,* 2010b, Shields *et al.,* 2011) and cows (Chung *et al.,* 2007, Donkin *et al.,* 2009, Carvalho *et al.,* 2011).

Knowledge on the use of glycerol in fish diets is extremely scarce and somehow contradictory. Menton et al. (1986) suggested that rainbow trout had lower feeding activity and could not efficiently utilize dietary glycerol as an energy source. On the other hand, more recent studies with channel catfish and Nile tilapia showed that glycerol may be successfully used as a dietary carbohydrate or energy source (Li *et al.,* 2010; Meurer *et al.,* 2012).

1.4. Objectives

The general objective of this thesis was to evaluate the potential of using biodiesel-derived crude glycerol (from rapeseed oil) as a feed ingredient in low fishmeal diets for gilthead seabream juveniles.

Studies undertaken during the thesis comprised the assessment of:

- Overall growth performance of fish
- Apparent digestibility and digestive capacity
- Metabolic nutrient utilization

2. Material and methods

2.1. Experimental diets

Two practical diets were formulated with practical ingredients to contain 45.4% crude protein, 18.5% crude fat, 21.3 kJ/g gross energy and fulfilled the known nutritional requirements (essential amino acids and phosphorus) of juvenile seabream. In the Control diet (CTRL) the total marine-derived ingredients accounting as major protein sources represented 19% of the formula, while fish oil was the main fat source. The Glycerol diet (GLY) has the same basal formulation, but 5% glycerol was incorporated at the expenses of wheat (Table 3).

All diets were manufactured by extrusion (pellet size 2.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105-110ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, United Kingdom) for 2 hours at 60ºC. Following drying, pellets were allowed to cool at room temperature, and subsequently the oil fraction (blend of fish and rapeseed oils) and glycerol was added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands). During the trial, all experimental diets were stored at room temperature, but in a cool and aerated emplacement. Additionally, to measure apparent digestibility by the indirect method, 5 kg of each complete diet was re-grinded, chromic oxide was incorporated at 1% and the mixtures were dry-pelleted (screen diameter: 4.5 mm), using a steamless pelleting machine (CPM-300, San Francisco, USA).

Table 3. Formulation and proximate composition of experimental diets.

Ingredients, %	CTRL	GLY
Fishmeal 70 LT[1]	7.0	8.0
Fishmeal FAQ[2]	7.0	7.0
CPSP 90[3]	5.0	5.0
Pea protein concentrate[4]	5.3	5.3
Wheat gluten[5]	9.0	9.0
Corn gluten[6]	14.0	14.0
Soybean meal 48[7]	8.0	8.0
Wheat DDGS	21.5	15.5
Wheat meal	5.0	5.0
Fish oil	10.0	10.0
Rapeseed oil	3.2	3.2
Vitamin & Mineral Premix[8]	1.0	1.0
Crude glycerol[9]	0.0	5.0
Di-calcium phosphate	2.0	2.0
L-Lysine	1.5	1.5
DL-Methionine	0.5	0.5
Dry matter (DM) (%)	94.2 ± 0.1	93.4 ± 0.1
Crude protein (%DM)	45.4 ± 0.2	45.2 ± 0.2
Lipid (%DM)	18.2 ± 0.2	18.2 ± 0.2
Ash (%DM)	6.7 ± 0.0	6.8 ± 0.0
Phosphorus (%DM)	1.3 ± 0.1	1. 2 ± 0.1
Gross energy (kJ/g DM)	21.4 ± 0.2	21.3 ± 0.1

[1]Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2]Fair Average Quality (FAQ) fishmeal: 60% CP, 11%CF, COFACO, Portugal.
[3]Fish solubles protein concentrate: 84% CP, 12% CF, Sopropêche, France.
[4]Peas protein concentrate: 78% CP, 8% CF, ROQUETTE, France.
[5]VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
[6]GLUTALYS: 61% CP, 8% CF, ROQUETTE, France.
[7]Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL, Portugal.
[8]Premix for marine fish, PREMIX Lda, Portugal.
[9]Biodiesel derived crude glycerol: 82% glycerol, <0.03% methanol, IBEROL, Portugal

2.2. Growth trial

The fish were originated from Estação Piloto de Piscicultura de Olhão (IPIMAR, Portugal) and transported to the Experimental Research Station of CCMAR (Faro, Portugal). Fish were adapted to these new conditions fro several weeks, during which they were fed a commercial seabream feed. Six homogenous groups of 20 seabream each, with a mean initial body weight of 27.9 ± 0.12 g were stocked in 100 L circular plastic tanks supplied with flow-through seawater (temperature: 24±2°C; salinity: 33-

34g·L^{-1}, dissolved oxygen above 5mg·L^{-1}) and subjected to 12/12 fluorescent light/dark cycle. Each dietary treatment was tested in triplicate tanks over 63 days. Fish were fed to apparent satiety, by hand, three times a day (09.30, 14.30 and 16.30h) and feed intake was recorded and utmost care was taken to avoid feed losses.

2.3. Sampling

At the beginning of the growth trial, the fish were individually weight and six fish from the initial stock were sampled for subsequent analysis of whole-body composition. To follow growth and feed utilization, the fish from each tank were bulk weighed every 21 days. At the end of the trial, the fish were individually weight and six fish from each triplicate tank were sampled for analysis of whole-body composition. At the same occasion, samples of the proximal intestine from three fish per tank (9 per dietary treatment) were collected, internally rinsed, frozen in liquid nitrogen and stored at -80°C prior to analysis of digestive enzyme activities. All samplings were done within the 18 hours following the last meal.

2.4. Apparent digestibility measurements

At the end of the growth trial and following all associated samplings, the remaining fish were used to determine, the apparent digestibility coefficients (ADC) of the dietary components, by the indirect method with diets containing 1% chromic oxide as inert tracer. Duplicate groups of fish were stocked in cylindro-conical tanks (volume: 100 L) in which the outlet water passes through a faeces settling decantation system. After an adaptation period of one week, fish were fed daily (10.00h) with the two experimental diets. After feeding, tanks and decantation system were thoroughly clean to eliminate any feed residues. Faecal samples were collected daily over 2 weeks. After daily collection, faeces were frozen at -20°C. Pooled faeces from each group of fish were freeze-dried prior to analysis. ADC of the dietary nutrients and energy were calculated as follows:

$$\text{ADC } (\%) = 100 \times \left[1 - \frac{\text{dietary } Cr_2O_3 \text{ level}}{\text{faecal } Cr_2O_3 \text{ level}} \times \frac{\text{faecal nutrient or energy level}}{\text{dietary nutrient or energy level}} \right]$$

2.5. Analytical methods

Proximate composition analysis of the diets, whole fish and faeces was made by the following procedures: dry matter by drying at 105°C for 24 h; ash by combustion at 550°C for 12 h; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428); fat after petroleum ether extraction by the Soxhlet method and gross energy in an adiabatic bomb calorimeter (IKA). Chromic oxide in the diets and faeces was determined according to Bolin et al. (1952), after perchloric acid digestion and total phosphorus according to the ISO/DIS 6491 method using the vanado-molybdate reagent.

In the proximal intestine samples, alkaline phosphatase activity (ALP) was measured using p-nitrophenylphosphate as a substrate (Bessey *et al.,* 1946). Pepsin activity (PEP) was determined using bovine heamoglobin as a substrate (Anson, 1938). Lipase (LIP) activity was measured using p-nitrophenyl myristate as a substrate (Lijima *et al.,* 1998). Leucine-alanine peptidase (LAP) activity was determined using leucine-alanine as a substrate (Nicholson & Kim, 1975). Enzyme activity was expressed as specific (U/mg protein). Protein was determined by means of the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

2.6. Statistical analysis

Data are expressed as means ± standard deviation. Data were previously checked for normal distribution and homogeneity of variances and when necessary arcsin transformed. Following ANOVA and if appropriate, means were compared by the Student-Newman-Keuls multiple range test. Statistical significance was tested at a 0.05 probability level. All statistical tests were performed using the STATISTICA version 8.0 software (StatSoft Inc., 2007).

3. Results

Data on growth performance, feed conversion and protein efficiency of seabream juveniles fed the two experimental diets is reported in Figures 3-4 and Table 4. Fish mortality throughout the trial was negligible and not associated to dietary treatments. Feed intake values (4%BW/day) for both treatments were high and confirm that the 5% crude glycerol addition to the diets had no significant effect (P>0.05) on feed palatability. After 63 days of experimental feeding, final body weigh (FBW) of fish fed the GLY diet was significant higher (P<0.05) than that found in fish fed the CTRL diet.

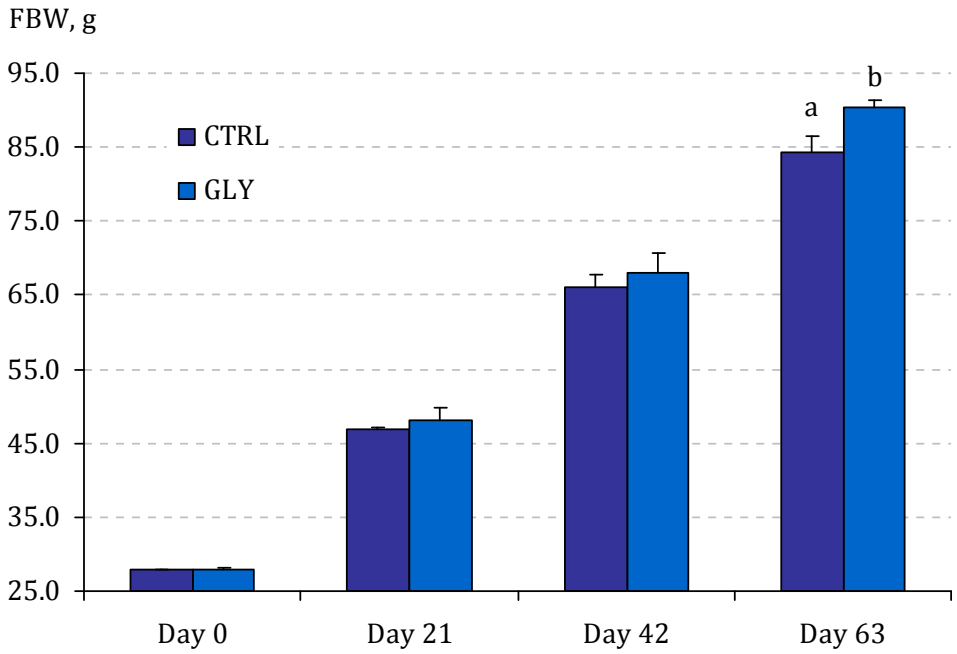


Figure 3. Final body weight (FBW) in seabream fed the dietary treatments.
Bars are means ± sd (n=3). Bars with different letters differ significantly (P<0.05).

At the end of the trial, data shows that voluntary feed intake was similar between treatments. In comparison to CTRL fish, those fed the GLY diet showed a significant enhancement (P<0.05) of the specific growth rate, feed efficiency and protein efficiency ratio. Moreover, the apparent digestibility of dry matter and protein was not affected (P>0.05) by the dietary inclusion of 5% crude glycerol.

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Table 4. Growth performance at day 63 (IBW: 27.9 ± 2.1 g).

	CTRL	GLY
FBW (g)	84.31 ± 2.26[a]	90.43 ± 0.84[b]
Weight gain (%IBW/day)	3.15 ± 0.19	3.52 ± 0.08
SGR (%/day)	1.76 ± 0.04[a]	1.86 ± 0.01[b]
VFI (%BW/day)	4.04 ± 0.07	3.98 ± 0.10
FE	0.78 ± 0.04[a]	0.88± 0.02[b]
PER	1.83 ± 0.10[a]	2.09 ± 0.04[b]
ADC Dry matter (%)	83.61 ± 0.41	83.57 ± 0.22
ADC Protein (%)	90.72 ± 0.12	91.24 ± 0.17

Values are means ± sd (n=3). Row means with different letters differ significantly (P<0.05).
IBW (g): Initial mean body weight.
FBW (g): Final mean body weight.
Specific growth rate, SGR (%/day): (Ln FBW – Ln IBW) x 100/days.
Voluntary feed intake, VFI (%BW/day): (crude feed intake/IBW/days) x 100.
Feed efficiency, FE: wet weight gain/crude feed intake
Protein efficiency ratio, PER: wet weight gain/crude protein intake.
Apparent digestibility coefficient, ADC (%): 100–[(% Cr_2O_3 feed/Cr_2O_3 faeces) x (% nutrient faeces/ % nutrient feed)].

Feed efficiency for seabream juveniles fed the GLY diet were significantly improved (P<0.05) than those fed the CTRL diet as show in Figure 4.

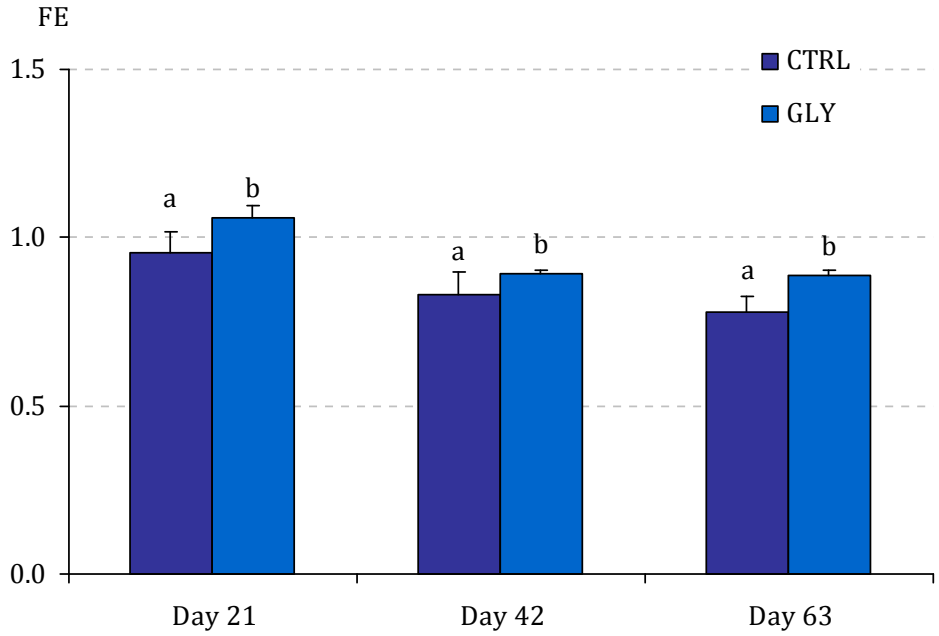

Figure 4. Feed efficiency (FE) in seabream fed the dietary treatments.
Bars are means ± sd (n=20). Bars with different letters differ significantly (P<0.05).

Data on the whole-body composition of seabream after 63 days of experimental feeding are presented in Table 5 and show that dietary treatments had no significant effect (P>0.05) on the nutrient and energy contents of whole-fish.

Table 5. Whole-body composition of seabream fed the experimental diets.

	Initial fish	CTRL	GLY
Dry matter (DM) (%)	30.94	34.84 ± 0.65	35.30 ± 1.08
Protein (%DM)	50.82	49.99 ± 2.23	47.52 ± 0.22
Lipid (%DM)	23.23	36.57 ± 0.80	36.08 ± 0.47
Ash (%DM)	13.87	11.09 ± 1.03	11.43 ± 1.35
Phosphorus (%DM)	2.36	1.97 ± 0.17	2.09 ± 0.24
Gross energy (kJ/g DM)	21.55	23.74 ± 0.17	23.32 ± 0.13

Values are means ± sd (n=3).

Based on weight gain, feed intake and whole-body composition of fish, values for protein and fat retention, and nitrogen budget are reported in Figures 5 and 6. In comparison to the CTRL treatment, fish fed the GLY diet showed a significant increase (P<0.05) of protein and fat retention (as % of nutrient intake).

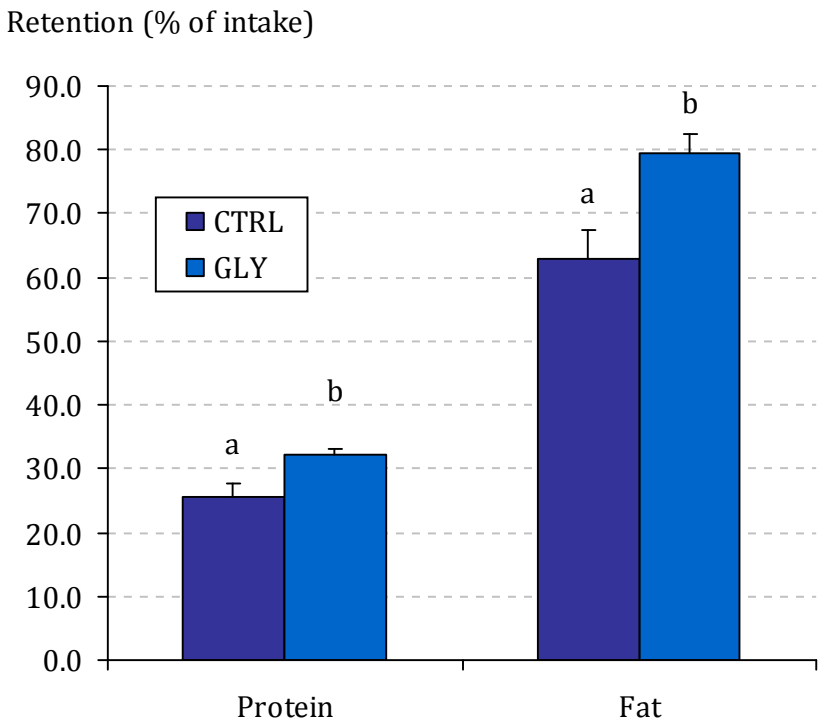

Figure 5. Protein and fat retention in seabream fed the dietary treatments.
Bars are means ± sd (n=3). Bars with different letters differ significantly (P<0.05).
Retention (%): 100 x (FBW x final body nutrient content - IBW x initial body nutrient content) / nutrient intake.

After 63 days of feeding, the dietary glycerol incorporation had no effect (P>0.05) on daily nitrogen gain. However, in comparison to CTRL fish, those fed with the GLY diet showed a significant reduction (P<0.05) of total nitrogenous losses, which was clearly associated to lower metabolic and faecal losses (Figure 6).

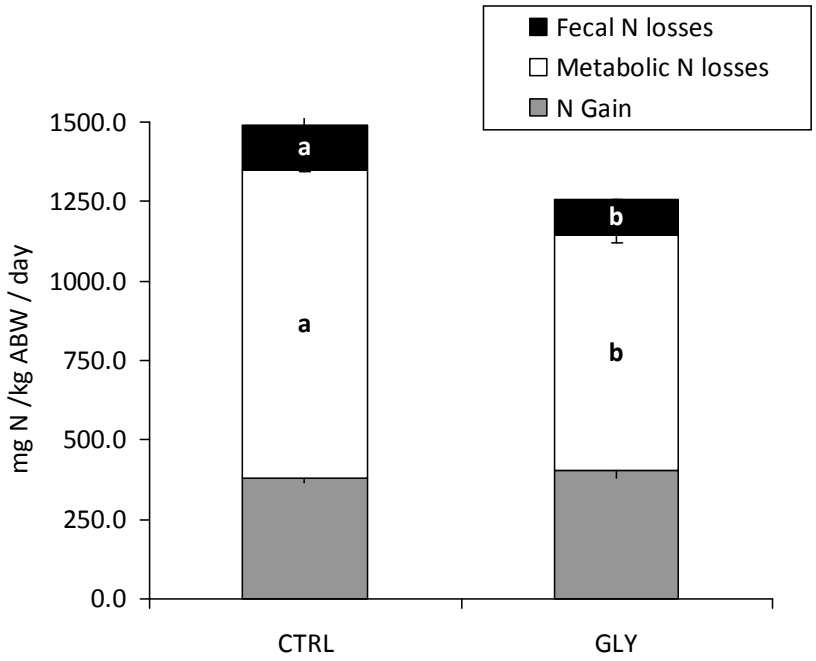

Figure 6. Nitrogen (N) budget in seabream fed the dietary treatments.
Bars are means ± sd (n=3). Bars with different letters differ significantly (P<0.05).
N gain: (final whole-body N content - initial whole-body N content)/ [(FBW+IBW)/2]/days.
Faecal N losses: [N intake – (protein intake x %ADC protein)/6.25]/ [(FBW+IBW)/2]/days.
Metabolic N losses: N intake – (N gain + faecal N losses).

Activities of selected digestive enzymes were affected by the various dietary treatments (Table 6). In relation to levels found in fish fed the CTRL diet, activities of intestinal alkaline phosphatase (ALP) and pepsin at the intestinal level were significant enhanced (P<0.05) in fish fed the GLY diet. Dietary treatments had no effect (P>0.05) on the activities of leucine-alanine peptidase (LAP) and lipase.

Table 6. Digestive enzyme activities in seabream fed the experimental diets.

	CTRL	GLY
ALP (mU/mg protein)	1.25 ± 0.19^{a}	1.84 ± 0.21^{b}
LAP (U/mg protein)	1.21 ± 0.09	1.28 ± 0.09
Pepsin (μU/mg protein)	44.1 ± 5.0^{a}	61.3 ± 5.7^{b}
Lipase (mU/mg protein)	1.91 ± 0.27	1.92 ± 0.36

Values are means ± sd (n=9). Row means with different letters differ significantly (P<0.05).

4. Discussion

The global annual biodiesel production is projected to be about 41 billion liters in 2019 (OECD-FAO, 2010). Crude glycerol is the major by-product of biodiesel production. Therefore, the development of high-value co-products from biodiesel-derived crude glycerol is critically important for the long-term sustainability of biodiesel industries. Because glycerol is expensive to purify for use in food, pharmaceutical, or cosmetics industries, biodiesel producers must seek alternative methods for its disposal. Further, the discharge of unrefined glycerol may cause a serious environmental problem. The biological conversion of crude glycerol into high-value biochemical's is one of the most promising alternatives. Crude glycerol has been successfully used as a sole carbon source for microbial and fungal cultivations (Abad & Turon, 2012; Nitayavardhana & Khanal, 2011). Crude glycerol was also tested as a carbon source for fermentation of microalgae *Schizochytrium limacinum*, which is a prolific producer of docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (Abad & Turon, 2012). However, the most promising alternative that has been attracting attention in recent years is the use of crude glycerol from biodiesel production in animal feeds. With this possibility, a large volume of glycerol is expected to be utilized by the high demand and fast growing production animal nutrition industry.

Regarding land-based animals, like cows (DeFrain *et al.,* 2004, Chung *et al.,* 2007, Schieck *et al.,* 2010a, Ogborn, 2006), pigs (Kijora & Kupsch, 1996, Mourot *et al.,* 1994, Lammers *et al.,* 2008a, Lammers *et al.,* 2008b, Della Casa *et al.,* 2009, Zijlstra *et al.,* 2009, Kerr *et al.,* 2009, Schieck *et al.,* 2010b, Shields *et al.,* 2011, Duttlinger *et al.,* 2012, Hansen *et al.,* 2009, Mader, 2011) and poultry (Dozier *et al.,* 2011, Dozier *et al.,* 2008, Kroupa *et al.,* 2011, Swiatkiewicz & Koreleski, 2009, Cerrate *et al.,* 2006, Mader, 2011, Min *et al.,* 2010), there is a great wealth of studies regarding glycerol-inclusion in feeds and, at least for inclusion levels below 10%, it was often found to increase growth rates, increase feed intake, improve meat yield, increase ultimate muscle pH, replenish muscle glycogen reserves and improve water holding capacity. On the other hand, some of these studies also shown that glycerol can have some impact on body composition, fatty acid profile and other organoleptic properties.

In fish and other marine organisms, although there are already some studies in this sense, knowledge on the dietary use of crude glycerol is still scarce and limited to a few species (Li *et al.,* 2010, Meurer *et al.,* 2011). For gilthead seabream specifically, the only relevant study we found relates to addition of pure glycerol to the rearing water of pre-larvae and larvae, for the purpose of boosting hepatic glycogen reserves, in order to improve survival during early stages of development (Maurizi *et al.,* 2000). Results of this study confirm a dose-dependent hepatic glycogen deposition due to glycerol uptake, suggesting that gilthead seabream have a generally good tolerance for glycerol as exogenous energy source, at least in early life stages, and that it contributes towards hepatic glycogen deposition. However, no information was found regarding the long-term use of glycerol as exogenous energy source in later life stages.

In our work, at the end of the 63 days of experimental feeding, the overall growth performance can be considered as good and within the normal range for seabream juveniles, with SGR values varying from 1.76 to 1.86 (%/day). In all treatments, fish had more than a 3-fold increase of their initial body weight. Previous studies show the ability of juvenile seabream to cope with low dietary levels of fish-derived proteins, provided that essential amino acid requirements are met, the palatability of the feed is guaranteed and the levels of anti-nutritional factors are low (Gómez-Requeni *et al.,* 2003, Sánchez-Muros *et al.,* 2003, Gomez-Requeni *et al.,* 2004, Benedito-Palos *et al.,* 2007, Benedito-Palos *et al.,* 2008, Montero *et al.,* 2008, Benedito-Palos *et al.,* 2009, Bouraoui *et al.,* 2010). Our performance data confirms that both experimental diets were formulated in accordance with the known nutritional requirements of seabream (NRC, 2011).

Data from our study clearly demonstrates that the dietary incorporation of 5% crude glycerol, at the expenses of wheat, originates a significant improvement of growth performance criteria, including growth rate, feed efficiency and protein efficiency. Moreover, glycerol incorporation had no effect on the body composition of juvenile seabream. Finally, glycerol-rich diet significantly enhanced the retention of dietary proteins and fats and reduced total nitrogenous losses. Available literature data shows that at a dietary incorporation level of 10%, crude glycerol from biodiesel production has also been found adequate as a complement of catfish diet without adverse effects on

feed consumption, weight gain, feed efficiency and liver lipids (Li *et al.,* 2010). A recent study by Silva et al. (2012) reports that 5% inclusion of crude glycerol in a diet for gilthead seabream of commercial size (>300 gram) had no deleterious effects in terms of growth, proximate composition of fish, fatty acid profile, oxidative state and organoleptic properties (aroma, color and texture). In the same study, histological and biochemical assays confirmed that glycerol-fed fish displayed increased glycogen deposition in muscle, along with increased levels of ATP. This suggests that inclusion of crude glycerol in gilthead seabream diets (particularly in the finishing phase), seems like a viable strategy to increase glycogen deposition in muscle without negatively impacting fish welfare and quality (Silva *et al.,* 2012).

Our data shows that the apparent digestibility of protein was high in both dietary treatments (92%) and little affected by the incorporation of 5% crude glycerol. However, the slightly higher protein digestibility value observed in fish fed the glycerol diet has an effect on reducing the daily nitrogenous fecal losses. Additionally, the significant reduction of metabolic nitrogen losses observed in seabream fed the GLY diet is a clear sign that in comparison to wheat, glycerol is a better energy-yielding substrate, leading to a higher efficiency on the use of dietary protein. In Nile tilapia, crude glycerol derived from the biodiesel production was considered an energy source with good digestibility (Meurer *et al.,* 2011; Neu *et al.,* 2012). The beneficial effects of glycerol on the overall growth performance criteria can be attributed to a higher protein deposition due to the sparing effect glycerol can have on glucogenic amino acids like alanine and glutamate.

It is important to note that glycerol metabolism can be different among species and there is indeed evidence that different species of fish use glycerol in different ways (Menton *et al.,* 1986, Furne *et al.,* 2009, Savina & Wojtczak, 1977). In some cases, glycerol is not used itself as a gluconeogenic precursor, but still provides energy, sparing other gluconeogenic precursors (like lactate) and therefore indirectly contributing towards glucose production and glycogen replenishing, which seems to be the case for rainbow trout (Kam & Milligan, 2006). Metabolically, gilthead seabream is relatively flexible, having a high tolerance towards dietary carbohydrates, compared to other carnivores like rainbow trout (Moon, 2001, Enes *et al.,* 2009, Blin *et al.,* 1999). Specifically, studies have shown that gilthead seabream does not display the same type of

hyperglycemia that rainbow trout does when fed carbohydrates, but only a postprandial transient peak (at least in part due to repression of hepatic gluconeogenesis) (Panserat *et al.,* 2002, Panserat *et al.,* 2001). On the other hand, during fasting or feed-restriction periods, gilthead seabream liver displays both higher gluconeogenic rates and lower glucose catabolic capacity (due to decreased expression of glucokinase) (Caseras *et al.,* 2000). Finally, there are indications that, at least for gilthead seabream, 3-carbon compounds may have a higher importance than glucose in providing energy and carbon for short-term hepatic glycogen replenishment during feeding-fasting cycles (Metón *et al.,* 2003), as well as during stressful situations associated with feed deprivation (Alves *et al.,* 2010), possibly because triacylglycerols are an important secondary store of energy in gilthead seabream and their mobilization entails higher glycerol availability.

The mechanisms whereby dietary components change the expression of specific enzymes activity occur at the gene level (transcription), the mRNA level (processing, extranuclear transport, cytoplasmic stability or translation efficiency), and the protein stability level. The possible mediators of nutritional adaptation of digestive enzymes are manifold: gut regulatory peptides released in response to the presence in the lumen of nutrients or hydrolysis products from dietary substrates and subsequent metabolites, or even absorbed circulating plasma metabolites (Le Huerou-Luron *et al.,* 1993). Our data on the effect of dietary glycerol on digestive enzymes, suggests a positive relationship with pepsin and alkaline phosphatase activities. On the other hand, dietary glycerol had no effect on the activities of lipase and leucine-alanine peptidase. Available information regarding the effect of dietary glycerol levels on the activity of digestive enzymes in animals is almost inexistent and makes difficult a non-speculative discussion of our data. Pepsin is an enzyme whose function is the digestion of proteins, while alkaline phosphatase is mainly associated to the dephosphorylation and subsequent proteolytic degradation of peptides into amino acids (Cara *et al.,* 2003). Despite not clearly explicable, their increase in glycerol fed fish is in accordance with the zootechnical data regarding a slightly higher digestibility of proteins. Previous results with bulls suggest that feeding with glycerol had a stimulatory effect on rumen microorganisms and digestive enzymes (Wang *et al.,* 2009). It is also important to highlight that glycerol is commonly used as an effective enzyme-stabilizer agent (Simon *et al.,* 2002). The lack of effect of lipase activity is somehow expectable, since both diets had an identical lipid supply.

A major potential concern regarding the use of biodiesel-derived crude glycerol in animal feeds is the fact that it often contains significant amounts of salts and some methanol (or, less commonly, ethanol) residues, which could potentially induce oxidative stress. For example, residual levels of potassium may result in wet litter or imbalances in dietary electrolyte balance in broilers (Cerrate *et al.,* 2006). However, a proteomic analysis showed a low impact of glycerol-supplementation on muscle metabolism, with most changes probably reflecting increased stress coping capacity in glycerol-fed fish (Silva *et al.,* 2012).

5. Conclusions

Effective utilization of crude glycerol is crucial to the commercialization and further development of biodiesel production. In the long term, utilization of biomass-derived glycerol will not only contribute to reducing society's dependence on nonrenewable resources but also will promote the development of integrated biorefineries. Within this context, the use of crude glycerol as an animal feed ingredient holds great potential. Our study generated new knowledge on the incorporation of crude glycerol in practical diets for gilthead seabream, the major farmed fish species in the Mediterranean region. Results obtained in this study, demonstrate that the dietary incorporation of 5% crude glycerol, at the expenses of wheat, originates a significant improvement of overall growth performance criteria and suggests a better metabolic utilization of dietary proteins.

Further studies on this topic are required to validate the observed effects over a longer feeding period and over specific phases of the production cycle (e.g. winter feeding). It is also imperative to gain a better knowledge on the underlying mechanisms regulating the metabolic fate of glycerol in fish. Additionally, besides its direct nutritional role, glycerol shows also interesting hygroscopic properties, being often used in the context of food and feed technology as a non-toxic humectant. It allows the manufacture of products with higher moisture content, but with low water activity levels, reducing the risk of microbial growth and contributing towards prolonged shelf-life.

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