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Dietary protein source and protein/carbohydrate ratio affects appetite regulation-related genes expression in gilthead seabream (Sparus aurata)

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Abstract:	This study aimed to evaluate the effect of dietary protein source (fishmeal, FM; or plant-feedstuffs, PF) and dietary protein/carbohydrate (P/CH) ratio on gilthead seabream appetite regulation and intermediary metabolism. Additionally, the effect of sampling 5h after feeding (AF) compared to 24h AF was also evaluated. Four isolipidic diets were formulated having as major protein sources FM or PF (20% FM and 80% PF), and P/CH ratios of 50/10 or 40/20, being the pregelatinized maize starch the main carbohydrate source (diets FM-P50/CH10; FM-P40/CH20; PF-P50/CH10; PF-P40/CH20). Diets were fed until satiation to 140g gilthead seabream for 41 days. The expression of appetite regulation genes was assessed at 5 and 24h AF, while other evaluated parameters were assessed only at 5h AF. Liver leptin expression was higher at 5h AF, and brain leptin receptor (lepr) expression was higher at 24h AF. Brain expression of cocaine- and amphetamine-regulated transcript (cart), leptin and ghrelin receptor (ghrr)- a and liver ghrr-b were also affected by sampling time, but the effects were dependent of the diet provided. FM-based diets promoted the expression of brain cart and leptin (at 24h AF), and liver growth hormone receptor (ghr) -ii , and increased plasma cholesterol and total lipids levels. Fish fed the PF-based diets had higher liver glycogen content, number and size of adipocytes, and expression of hepatic leptin (at 24h AF), fatty acid synthase , glucokinase , and target of rapamycin . Regarding dietary P/CH ratio, fish fed the P50/CH10 diets presented higher feed efficiency, plasma triglycerides, and expression of intestine cholecystokinin (at 5h AF), liver ghrr-b (at 24h AF), glutamate dehydrogenase and ghr-ii . The protein efficiency ratio, hepatosomatic and visceral indices, plasmatic glucose level, and brain lepr expression (at 5h AF) were higher in fish fed the P40/CH20 diets. The majority of appetite regulation related-genes were not affected by the use of PF-based diets, while the higher dietary CH seemed to lead to a shorter satiety sensation. PF-based diets promoted liver lipid deposition, hypocholesterolemia, and the activation of glycogenesis pathway, while higher CH content induced an increase in plasma glucose that appeared to be stored as lipids. In conclusion, PF-based diets with up to 20% of CH can be used in gilthead seabream without compromising growth performance and FI, and only slightly modifying appetite and metabolic parameters.
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Subject: **Submission of a manuscript for evaluation**

Dear Editors,

I am enclosing herewith a manuscript entitled "**Dietary protein source and protein/carbohydrate ratio affects appetite regulation-related genes expression in gilthead seabream (Sparus aurata)"** for publication in Aquaculture for possible evaluation. Submitted manuscript is a Research paper.
With the submission of this manuscript I would like to undertake that the above mentioned manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institute's (University of Porto, CIIMAR, University of Barcelona) representatives are fully aware of this submission. All the authors read and approved the findings of this study. None of the authors had a conflict of interest.

I sincerely hope the submitted manuscript fulfills the journal objectives.

With my best regards,

Catarina Basto Silva
Catarina Basto-Silva

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Dear editor,

Thank you for considering this manuscript for publication in Aquaculture. As reviewer #2 suggested, we checked the information contained in the Tables and made the correction suggested. Willing that this revised version of the manuscript meets with your approval, I remain.

Sincerely yours,

Catarina Basto Silva

Catarina Basto-Silva

Reviewer comment to Author

Reviewer #2

Only thing I notice is maybe some superscripts missing from some of the Tables?

Answer: We want to thank the reviewer for the comment, since we did not have noticed that mistake after changing the tables.

Highlights for

Dietary protein source and protein/carbohydrate ratio affects appetite regulation-related genes expression in gilthead seabream (Sparus aurata), by Basto-Silva et al.

- ***ghrr-a, leptin*, and *lepr* expression were affected by sampling time.**
- **Response to dietary protein sources took more time to be induced.**
- **High CH-diets promoted a shorter satiety sensation.**
- **PF diets with 20% CH did not affect FI but slightly affected appetite genes expression.**

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Dietary protein source and protein/carbohydrate ratio affects appetite regulation-related genes expression in gilthead seabream (*Sparus aurata*)

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Abstract

This study aimed to evaluate the effect of dietary protein source (fishmeal, FM; or plant-feedstuffs, PF) and dietary protein/carbohydrate (P/CH) ratio on gilthead seabream appetite regulation and intermediary metabolism. Additionally, the effect of sampling 5h after feeding (AF) compared to 24h AF was also evaluated. Four isolipidic diets were formulated having as major protein sources FM or PF (20% FM and 80% PF), and P/CH ratios of 50/10 or 40/20, being the pregelatinized maize starch the main carbohydrate source (diets FM-P50/CH10; FM-P40/CH20; PF-P50/CH10; PF-P40/CH20). Diets were fed until satiation to 140g gilthead seabream for 41 days. The expression of appetite regulation genes was assessed at 5 and 24h AF, while other evaluated parameters were assessed only at 5h AF. Liver *leptin* expression was higher at 5h AF, and brain *leptin receptor* (*lepr*) expression was higher at 24h AF. Brain expression of *cocaine- and amphetamine-regulated transcript* (*cart*), *leptin* and *ghrelin receptor* (*ghrr*)-*a* and liver *ghrr-b* were also affected by sampling time, but the effects were dependent of the diet provided. FM-based diets promoted the expression of brain *cart* and *leptin* (at 24h AF), and liver *growth hormone receptor* (*ghr*)-*ii*, and increased plasma cholesterol and total lipids levels. Fish fed the PF-based diets had higher liver glycogen content, number and size of adipocytes, and expression of hepatic *leptin* (at 24h AF), *fatty acid synthase*, *glucokinase*, and *target of rapamycin*. Regarding dietary P/CH ratio, fish fed the P50/CH10 diets presented higher feed efficiency, plasma triglycerides, and expression of intestine *cholecystokinin* (at 5h AF), liver *ghrr-b* (at 24h AF), *glutamate dehydrogenase* and *ghr-ii*. The protein efficiency ratio, hepatosomatic and visceral indices, plasmatic glucose level, and brain *lepr* expression (at 5h AF) were higher in fish fed the P40/CH20 diets. The majority of appetite regulation related-genes were not affected by the use of PF-based diets, while the higher dietary CH seemed to lead to a shorter satiety sensation. PF-based diets promoted liver lipid deposition, hypocholesterolemia, and the activation of glycogenesis pathway, while higher CH content induced an increase in plasma glucose that appeared to be stored as lipids. In conclusion, PF-based diets with up to 20% of CH can be used in gilthead seabream without compromising growth performance and FI, and only slightly modifying appetite and metabolic parameters.

Keywords

Anorexigenic/orexigenic hormones; Fishmeal; Plant-feedstuffs; Short-term effects

1. Introduction

Aquaculture is the industry with the highest growth rate among animal production sectors, with a global average annual increase of 3.2% between 1961 and 2016, compared with a 2.8% increase for livestock production (FAO, 2018). Feed represents around 60% of aquaculture production costs (Daniel, 2018). Moreover, the increase of cultured species together with the increase of aquaculture production leads to a high pressure on feeding and aquafeeds optimization.

Fishmeal (FM) is an excellent source of nutrients, namely amino acids, fatty acids, and minerals, has high digestibility and good palatability (Rust et al., 2011; Olsen and Hasan, 2012), and is the main protein source for carnivorous species (Tacon and Metian, 2008). However, FM inclusion in aquafeeds needs to decrease, due to the reduction of fisheries stocks and thus market price increase, and the need to use environmentally sustainable feedstuffs (Tacon and Metian, 2008; Olsen and Hasan, 2012). Plant-feedstuffs (PF) have high market availability, a relatively constant nutritional composition, and therefore are the most used alternative to FM (Oliva-Teles et al. 2015). Although fish do not have dietary carbohydrate (CH) requirements, the provision of an appropriate amount of digestible CH in aquafeeds is needed to spare the use of protein as an energy source (NRC, 2011). Thus, another strategy to reduce dietary FM inclusion is the optimization of the protein to CH (P/CH) ratio. However, both PF and CH were reported to affect feed intake (FI) in fish. For instance, PF-based diets decreased FI in cobia, *Rachycentron canadum* (Nguyen et al., 2013) and Atlantic salmon, *Salmo salar* (Torstensen et al., 2008), and high CH-diet decreased FI of gilthead seabream, *Sparus aurata* (Couto et al., 2008), and rainbow trout, *Oncorhynchus mykiss* (Figueiredo-Silva et al., 2012), while it increased FI of Senegalese sole, *Solea senegalensis* (Guerreiro et al., 2014). Thus, for sustainable growth of aquaculture, it is of utmost importance to have a deeper knowledge of the physiological consequences both of the dietary feedstuffs used and of the dietary nutrient composition on the regulation of FI in fish.

Appetite in fish, as in other vertebrates, is regulated both by orexigenic and anorexigenic responses acting as a complex network of hormones produced in the brain but also in peripheral organs, like the liver, adipose tissue, and gastrointestinal tract (Volkoff et al., 2009; Volkoff, 2016; Rønnestad et al., 2017). Further, the brain integrates metabolic information related to

nutrients availability, satiety and hunger signals, and produces responses to peripheral tissues that modulate metabolic functions (Bertucci et al., 2019).

The cocaine-and amphetamine-regulated transcript (cart) and cholecystokinin (cck), are mainly expressed by the brain and gastrointestinal tract, respectively (Rønnestad et al., 2017), and were previously described as having an anorexigenic role in several species, such as Atlantic salmon, channel catfish, *Ictalurus punctatus*, and dourado, *Salminus brasiliensis* (Valen et al., 2011; Peterson et al., 2012; Volkoff et al., 2016).

Little is known about the corticotropin-releasing hormone (crh) or corticotropin-releasing factor (crf)-related peptides responses on fish appetite regulation. However, a few studies pointed out crh as a potent anorexic peptide in goldfish, *Carassius auratus*, and rainbow trout (Bernier and Peter, 2001; Matsuda et al., 2008). In *Schizothorax prenanti*, the *crh* expression was not affected by the post-prandial period, but long-term fasting also suggests a satiety role for this peptide (Wang et al., 2014).

There is yet some contradictory data regarding the effects of hormones controlling appetite regulation. For instance, ghrelin, which is mainly expressed in the stomach, but also the gastrointestinal tract and hypothalamus, is generally considered to have an orexigenic role (Jönsson, 2013; Bertucci et al., 2019). In fish, this orexigenic role of ghrelin was confirmed in brown trout, *Salmo trutta* (Tinoco et al., 2014a), or Senegalese sole (Navarro-Guillén et al., 2017). However, in other species, such as the Atlantic cod, *Gadus morhua* (Xu and Volkoff, 2009), and rainbow trout (Jönsson et al. 2010), ghrelin was shown to have an anorexigenic role.

While in mammals the adipose tissue is the major producer of leptin (Harris, 2014), in fish leptin is mainly produced in the liver, although it is also produced in the adipose tissue, stomach, and intestine (Zhang et al. 2013; Salmerón et al., 2015; Volkoff, 2015; Volkoff et al., 2017). Like ghrelin, leptin function in appetite regulation also seems to be species-specific (Volkoff, 2016; Bertucci et al., 2019). Despite being primary described as having an anorexigenic role, as in rainbow trout, goldfish, and striped bass, *Morone chrysops* (Volkoff et al., 2003; Murashita et al., 2008; Won et al., 2012), an orexigenic role was reported in other species, such as in zebrafish,

Danio rerio, and orange-spotted grouper, *Epinephelus coioides* (Zhang et al. 2013; Tian et al., 2015).

On the other hand, neuropeptide y (npy) is one of the most studied appetite-regulating hormones in fish and appears to have an orexigenic function and a short-term response to FI (Silverstein et al., 1999; MacDonald and Volkoff, 2009; Peddu et al., 2009). This peptide has been found mainly in the brain, but also the pituitary, intestinal tract, spleen, and kidney (Bertucci et al., 2019).

Gilthead seabream represents about 7% of all marine fish produced in the world in 2017 and is one of the main species produced in the Mediterranean (FIGIS, 2019). However, despite its relevance for marine aquaculture, little is known about appetite regulation in this species, and this may be of high relevance in the new context of novel diets for carnivorous fish. Recently, Perelló-Amorós et al. (2018) studied ghrelin responses to fasting and refeeding in gilthead seabream. The authors identified the stomach as the main producer of ghrelin and the pituitary, brain, and liver as the main organs where ghrelin receptors are expressed. Moreover, it was observed that plasma ghrelin decreased significantly at 5h after feeding (AF). Regarding diet composition, Babaei et al. (2017) observed that high protein and low CH diets decreased *ghrelin* expression in the brain and increased *cck* and *ghrelin* expression in the intestine, while expression of *leptin* in the liver and adipose tissue, and *npy* in the brain, were not affected by diet composition.

Therefore, this study aimed to further evaluate the effects of diet manipulation, namely dietary protein source (FM or PF-based diets) and dietary P/CH ratio on appetite regulation and intermediary metabolism-related gene expression in gilthead seabream juveniles. Feed utilization, whole-body and liver proximate composition, plasma biochemistry, and adipose tissue and liver histomorphology were also evaluated. Additionally, the effects of short-time fasting (5h compared to 24h AF) on appetite regulation-related hormones were also studied.

2. Materials and Methods

2.1. Diets composition

Four isolipidic (18% crude lipid) diets were formulated to have different protein sources and P/CH ratios. Two diets with FM as the only protein source and with P/CH ratios of 50/10 or 40/20 (diets

FM-P50/CH10 and FM-P40/CH20, respectively), and the other two with PF as the main protein source (20% FM and 80% PF) and the same P/CH ratios (PF-P50/CH10 and PF-P40/CH20, respectively). All dietary ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA), through a 2mm diameter. Pellets were dried in an oven for 48h and then stored in plastic containers at 4ºC until use. The ingredients and proximate composition of the diets are presented in Table 1.

2.2. Fish and experimental conditions

The experiment was performed at the Marine Zoology Station, Porto University, Portugal, with gilthead seabream, *Sparus aurata,* from Atlantik Fish, Castro Marim, Algarve, Portugal, and was conducted by accredited scientists (following FELASA category C recommendations) and approved by the Portuguese Authority for Food and Animal Health (Certification number ORBEA-CIIMAR 30-2019), according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

The recirculating water system consisted of 12 cylindrical fiberglass tanks of 300 L water capacity, thermo-regulated to 22 ± 0.7ºC, and supplied with a continuous flow (6.0 L min^{-1}) of filtered seawater with 36.0 ± 1.0g L^{-1} of salinity, and a dissolved oxygen level near saturation (6.0 ± 0.5mg L^{-1}).

Fish were submitted to a quarantine period of 1 month and fed with a commercial diet (43% protein and 17% lipids; Aquasoja, Ovar, Portugal). Thereafter, 12 groups of 15 fish with an initial body weight of 140.0 ± 0.1g were randomly distributed to each tank and the experimental diets were randomly assigned to triplicate groups of these fish. The experiment lasted 41 days and during that period fish were fed by hand until apparent visual satiation, twice daily. Utmost care was taken to avoid feed losses. The FI was measured using the following equation:

$$\text{FI}(\text{g kg average body weight}^{-1}\ \text{day}^{-1}) = \frac{\left(1000 * \text{dry matter intake} \,/\, \text{fish average body weight}\right)}{\text{duration of the trial}}$$

2.3. Sampling

Fish in each tank were bulk weighed at the end of the trial, after one day of feed deprivation. For that purpose, fish were slightly anesthetized with 0.3ml L^{-1} ethylene glycol monophenyl ether. Three (n=3) fish per tank at the end of the trial were euthanized with a sharp blow to the head and pooled for whole-body composition analysis (n=3). Whole-fish, viscera, and liver weight of these fish were recorded for the determination of hepatosomatic (HSI) and visceral somatic (VSI) indices. The remaining fish continued to be fed for two more days to minimize manipulation stress. The day before sampling fish were fed at 09:00 and 16:00, and then, the following day, 6 fish from each tank were sampled 5h after the morning meal (provided at 09:00). Blood from 3 of these fish was collected from the caudal vein with heparinized syringes and immediately centrifuged at 3 000 × g for 10 min. Plasma aliquots were frozen at -80°C until performing metabolite analyses. After blood collection, fish were euthanized with a sharp blow to the head and dissected on chilled trays for collection of adipose tissue, whole-brain, anterior intestine, liver, and stomach for gene expression analysis. Three other fish were euthanized and sampled to collect adipose tissue for histology analysis, and liver for histology and proximate analyses. At 24h AF, 3 more fish from each tank were euthanized as above for the collection of adipose tissue, brain, anterior intestine, liver, and stomach for gene expression analysis. Samples for gene expression were stored in RNA later, left at 4°C overnight and subsequently stored at -80ºC until analysis. Histology samples were immediately fixed in phosphate-buffered formalin (4%, pH 7.4) for 24h and subsequently transferred to ethanol (70%) until further processing.

2.4. Proximate analysis

Fish collected for whole-body composition were pooled by tank, thus n=3 per treatment, dried at 100°C until constant weight, and moisture content calculated. Analyses of dry matter, protein, lipid, and ash of whole-body, diets, and dietary ingredients were done following the Association of Official Analytical Chemists methods (AOAC, 2000). Energy content was determined by direct combustion in an adiabatic bomb calorimeter (PARR model 1261; PARR Instruments, Moline, IL, USA) and starch according to Beutler (1984). Liver glycogen and lipid content were

determined as described by Plummer (1987) and Folch et al. (1957), respectively, with an n=9 for each treatment.

2.5. Plasma metabolites

Plasma metabolites, with an n=9 by treatment, were determined using enzymatic colorimetric kits from Spinreact, Girona, Spain (glucose kit, code 1001191; cholesterol kit, code 1001091; triglycerides kit, code 1001312; total protein kit, code 1001291, and total lipids kit, code 1001270).

2.6. Histological processing and morphological evaluation

Adipose tissue and liver were processed and sectioned using standard histological techniques and stained with hematoxylin and eosin. Adipose tissue was analyzed regarding adipocytes size and relative frequency, as described by Bou et al. (2014). Liver samples were evaluated giving attention to lipid droplets as described by Papadakis et al. (2013) with slight modifications. Briefly, the images were converted to greyscale, all structures that could be confused by the software as lipid vacuoles (such as blood capillaries and adipose tissue) were manually removed, and then, a threshold filter and dark background condition were applied. To evaluate lipid vacuoles, the dark pixels were selected, corresponding to the empty cytoplasm space after images processing. Digital images were acquired with Zen software (Blue edition; Zeiss, Jena, Germany), and analyzed using Image J, version 1.46 (National Institutes of Health, Maryland, USA). One image for each sample was obtained with a 10x magnification, thus an n=9 was determined for each treatment.

2.7. RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR)

Samples for RNA extraction were processed as described by Vélez et al. (2016). Total RNA samples (1100 ng) were processed for cDNA synthesis using DNase I enzyme (Life Technologies, Alcobendas, Spain), and Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) according to the manufacturer's recommendations, and cDNA

samples were stored at -20ºC until used. Quantitative real-time PCR (qPCR) was performed as described in Riera-Heredia et al. (2019), with minor variations. All samples were analyzed in duplicate, using 2.5µL of iTaq Universal SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250nM of forward and reverse primers (presented in Table 2), 1µL of each cDNA sample and autoclaved water until a final volume of 5µL. The qPCR reactions followed Salmerón et al. (2013) procedure. Relative expression of each transcript individual sample was normalized using the corresponding geometric mean expression of the translation elongation factor 1*a* (*ef1a*) and ribosomal protein S18 (*rps18*) as reference genes, which were constitutively expressed and not affected by the experimental treatments. Since some of the expressed genes did not have efficiency curves within the optimum range (i.e. 95-105%), although all genes were specifically amplified (i.e. only one melting peak was observed), the Pfaffl method (Pfaffl, 2001) was used to determine the relative expression (n=9 for each treatment).

2.8. Statistical analysis

All data are presented as the mean and standard error of the mean (SEM), except in histomorphological evaluation where the standard error is used. Statistical analyses were done by two-way ANOVA and in the case of interaction between factors, one-way ANOVA was performed for the P/CH ratio within each protein source, and protein source within each P/CH ratio. Time effect on appetite regulation-related genes within each diet was analyzed by one-way ANOVA, followed by Tukey´s test. A statistical significance of $p<0.05$ was set to all the statistical tests performed. Data were tested for normality by the Shapiro-Wilk test and homogeneity of variances by the Levene's test. When normality was not verified, data were transformed before ANOVA. All statistical analyses were done using the SPSS 25 software package for Windows (IBM® SPSS® Statistics, New York, USA).

3. Results

Fish promptly accepted the experimental diets and no mortality was recorded during the trial. Dietary protein source did not affect fish growth but, within the FM-based diets, fish fed diet FM-

P40/CH20 presented lower growth than fish fed diet FM-P50/CH10 (Table 3). While, there were no differences in FI between groups. Feed efficiency (FE) and protein efficiency ratio (PER) were only affected by P/CH ratio, with FE being higher and PER lower in fish fed P50/CH10 diets.

The fish whole-body composition was not affected by dietary composition, while HSI and VSI were higher in fish fed the P40/CH20 than the P50/CH10 diets (Table 4). Fish fed the FM-based diets had lower liver glycogen content than fish fed PF-based diets. Within the PF-based diets, liver lipid content was lower in fish fed the P50/CH10 than those fed the P40/CH20 diets, while within the P40/CH20 groups, liver lipid was higher in fish fed the PF-based diets than the FM-based diets.

Independently of the dietary protein source, plasma glucose was higher in fish fed the P40/CH20 than in the P50/CH10 diets and, within the P40/CH20 it was higher in fish fed the FM- than the PF-based diets (Table 5). Plasma cholesterol and total lipids levels were higher in fish fed the FM- than the PF-based diets, while plasma triglycerides were lower in fish fed the P40/CH20 than the P50/CH10 diets. Plasma total protein content was not affected by dietary composition.

Regarding adipocyte cell size, only the two smaller adipocyte classes were affected by dietary protein sources (Figure 1). Thus, fish fed the FM-based diets had a higher number of smaller adipocytes cells (30-314μm^2), while fish fed the PF-based diets had a higher amount of medium-size adipocytes (315-2827μm^2). The liver area covered by lipid vacuoles was not affected by dietary composition (Figure 2).

Concerning appetite regulation-related genes, under the current experimental conditions undetectable levels of expression were observed for *leptin* in the adipose tissue, intestine, and stomach; for *ghrelin* and *ghrelin receptor-a* (*ghrr-a*) in the intestine and liver; and for *ghrelin receptor-b* (*ghrr-b*) in the brain. The *crh* and *npy* in the brain, and *ghrelin* in the stomach were not affected by sampling time or diet composition (Table 6). Hepatic *leptin* expression was higher at 5h than at 24h AF in all dietary treatments, while the opposite was true for brain *leptin receptor* (*lepr*). Brain *leptin* expression was higher at 24h AF than at 5h in all treatments, except for fish fed diet PF-P50/CH10, where no time effect was observed. Brain *ghrr-a* and hepatic *ghrr-b* expression were higher 24h AF in fish fed the P50/CH10 diets and PF-P50/CH10 diet,

respectively. The *cart* expression in the brain was higher at 24h than at 5h AF, only in fish fed the FM-P50/CH10 diet.

At 24h AF, but not at 5h, liver *leptin* expression was higher in fish fed the PF- than the FM-based diets, while the opposite was observed in the brain *leptin* expression. Moreover, at 5h AF, but not at 24h, brain *lepr* expression was higher in fish fed the P40/CH20 than the P50/CH10 diets. The *cart* gene expression in the brain was not affected by diet composition at 5h AF, while at 24h AF the expression was higher in fish fed the FM- than the PF-based diets. Brain *ghrr-a* expression was not affected by diet composition, while in the liver *ghrr-b* expression was higher at 24h AF, but not at 5h, in fish fed the P50/CH10 diets. In the intestine, the *cck* expression, at 5h AF, was higher in fish fed the P50/CH10 than the P40/CH20 diets. At 24h AF, *cck* expression was also higher with the P50/CH10 diets, but only in fish fed the FM-based diets, while the opposite was observed in the PF-based diets.

Liver *fatty acid synthase* (*fas*), *glucokinase* (*gk*), and *target of rapamycin* (*mtor*) gene expression were higher, while expression of *growth hormone receptor-ii* (*ghr-ii*) was lower, in fish fed the PF- than the FM-based diets (Table 7). The *ghr-ii* and *glutamate dehydrogenase* (*gdh*) expression were lower in fish fed the P40/CH20 than the P50/CH10 diets. The *growth hormone receptor-i* (*ghr-i*) gene expression was lower in fish fed the FM-P40/CH20 diet than the other diets. In the FM-based diets, but not in the PF-based diets, *insulin-like growth factor-1* (*igf-1*) expression was higher in fish fed the P50/CH10 diets. The expression of *3-hydroxyacyl-CoA dehydrogenase* (*hoad*) and *fas* in the adipose tissue, and of *hoad* and *glucose-6-phosphatase* (*g6pase*) in the liver were not affected by the dietary treatments.

4. Discussion

4.1. Appetite regulation-related genes expression

Sampling time effect

The knowledge of appetite regulation mechanisms is still limited in several fish species, including gilthead seabream (Babaei et al., 2017; Perelló-Amorós et al., 2018). In this section, we discuss

the effects of two short-time fasting periods (5h compared to 24h AF) on appetite regulation hormones, to get a preliminary understanding of these hormones functions.

cart and *cck* were previously described as having an anorexigenic role in several species, such as Atlantic salmon, channel catfish, and dourado (Valen et al., 2011; Peterson et al., 2012; Volkoff et al., 2016). However, in the present study, these hormones did not respond to the short-fasting periods, except fish fed FM-P50/CH10 which presented higher *cart* gene expression at 24h AF. A lack of response of these hormones in fish under different fasting periods was also observed in winter skate, *Raja ocellata*, hypothalamus and in cobia brain (MacDonald and Volkoff, 2009; Nguyen et al., 2013). Moreover, fasting may induce a translational and/or post-translational response of *cart*, affecting protein levels, but without influencing the mRNA levels (MacDonald and Volkoff, 2009). Since in the present study protein levels were not assessed, such a response can not be disregarded. It is also possible that another *cart* or *cck* isoform more sensitive to fasting could exist for the studied fish species (MacDonald and Volkoff, 2009). In fact, diverse cart and cck isoforms were reported for a few fish species (Volkoff and Peter, 2001; Murashita et al.,2009; Peterson et al., 2012). Another possibility might be that these hormones could need more time to induce expression changes (Nguyen et al., 2013).

In the present study, no changes in brain *crh* expression were detected with short-time fasting time. Similarly, in *Schizothorax prenanti* no changes in hypothalamus *crh* gene expression were observed at 3h AF (Wang et al., 2014). However, after 7 days of fasting, *crh* gene expression decreased compared to the fed group, suggesting that it may have an anorexigenic function. Thus, in gilthead seabream, 24h may be a short time to induce a *crh* response, and this subject needs to be further evaluated.

In the present study, *ghrelin* expression was detected in the stomach but not in the intestine and liver. However, no variation in the stomach *ghrelin* expression with short-time fasting was detected. In some fish species, ghrelin has been described as an orexigenic hormone (Tinoco et al., 2014a; Volkoff, 2015; Blanco et al., 2016; Navarro-Guillén et al., 2017), while in other species it was reported as an anorexigenic hormone (Peddu et al., 2009; Xu and Volkoff, 2009; Jönsson et al., 2010; Schroeter et al., 2015). Previously, in gilthead seabream, Perelló-Amorós et al. (2018)

described an anorexigenic role of stomach *ghrelin* expression at 24h AF, while plasma ghrelin concentration followed an orexigenic role, decreasing significantly its concentration 5h AF. As in the present study, a lack of variation in stomach *ghrelin* expression at 24h AF, or even during a period of 4 or 8-days of fasting, was also reported in Mozambique tilapia, *Oreochromis mossambicus*, and in channel catfish (Fox et al., 2009; Peterson et al., 2012).

In the present study, *ghrr* expression was dependent on diets and tissues. *Ghrr-a* was expressed in the brain, while *ghrr-b* was expressed in the liver. Further, brain *ghrr-a* expression was higher at 24h AF but only in fish fed the higher CH-diets, pointing to an orexigenic function under these feeding conditions. In the liver, *ghrr-b* expression followed a similar trend, but only in fish fed the PF-P50/CH10 diet (further discussed in section 4.2). Also in gilthead seabream, the *ghrr-a* expression was previously described in the pituitary as having an orexigenic role, decreasing at 5h AF, while such a decrease was not observed for pituitary *ghrr-b*, where no significant short-term fasting effects were reported (Perelló-Amorós et al., 2018). Differently, in Mozambique tilapia, brain *ghrr-a* expression was not affected by short-term fasting, but *ghrr-b* expression significantly decreased at 3h AF (Peddu et al., 2009).

Though the role of leptin on fish appetite regulation is well known, its mechanisms of action are still unclear. Overall, intraperitoneal (IP) and intracerebroventricular (ICV) injections of leptin decreased feed ingestion in several fish species, suggesting an anorexigenic behavior (Volkoff et al., 2003; Murashita et al., 2008; Won et al., 2012). However, leptin seems to have a tissue and species-specific behavior. For example, in goldfish and orange-spotted grouper, brain *leptin* expression was not affected by a short-term fasting period, while hepatic *leptin* gene expression increased 9h after fasting, suggesting an orexigenic function (Zhang et al., 2013; Tinoco et al., 2014b). On the other hand, in red-bellied piranha, *Pygocentrus nattereri*, brain *leptin* expression was not affected by 7 days fasting, but intestine *leptin* gene expression was decreased, which suggests that intestine leptin has an anorexigenic behavior (Volkoff, 2015). In the present study, while the brain leptin appeared to have an orexigenic function, reflected by its higher gene expression observed at 24h than at 5h AF, liver *leptin* expression was higher at 5h than at 24h AF, suggesting an anorexigenic function. However, since these are the first results on the effects of

short-term fasting on gilthead seabream *leptin* expression, further studies, with different short-fasting timings, are needed to support the present findings.

In this study, brain *lepr* expression increased at 24h AF, suggesting an orexigenic role. However, such an increase was not observed in orange-spotted grouper and goldfish, where brain *lepr* was not affected at 3 or 7-days of fasting, and 24h of fasting, respectively (Zhang et al., 2013; Tinoco et al., 2014b).

An orexigenic function of npy has been reported in several fish species (Silverstein et al., 1999; MacDonald and Volkoff, 2009; Peddu et al., 2009). In the present study, as also previously observed in this species (Babaei et al. 2017), brain *npy* expression was not significantly affected by sampling time, although a trend for higher expression at 24h was noticed.

Overall, the short-term periods of fasting evaluated in the present study may have been too short to detect sensible expression changes in appetite regulation hormones, thus difficulting a clear definition of their orexigenic or anorexigenic functions.

Diet composition effect

Differences in appetite regulation gene expression related to dietary protein sources were only noticed at 24h AF, none being detected at 5h AF, which could suggest that fish response to dietary protein sources takes a relatively longer time to be induced.

Although appetite regulation mechanisms are still poorly understood in fish, several authors reported a decrease of FI in fish fed PF-based diets (Hevrøy et al., 2008; Nguyen et al., 2013; Tuziak et al., 2014). Despite dietary protein source did not significantly affect FI in the present study, the PF-based diets seemed to promote longer satiety feeling than the FM-based diets, inhibiting brain *leptin* expression, and increasing hepatic *leptin* expression, which seems to have an orexigenic and anorexigenic behavior, respectively. In several fish species, *cart* and *npy* brain expression were not affected by PF-based diets (Hevrøy et al., 2008; Nguyen et al., 2013; Volkoff et al., 2017). However, in the present study, *cart* gene expression decreased in fish fed PF-based diets, suggesting that in gilthead seabream this hormone could be affected by dietary protein source.

In pacu, *Piaractus mesopotamicus*, a decrease in intestine *cck* expression was observed 30min AF in fish fed diets with 25 and 50% of soy protein as FM replacement, compared with fish fed diets without soy protein (Volkoff et al., 2017). Despite the differences on sampling time, in the present study, intestine *cck* expression was lower at 24h AF in fish fed the diet PF-P50/CH10, which had 25% of soybean dietary incorporation, when compared to fish fed the FM-P50/CH10 diet with no soybean. However, it should not be discarded that the changes in intestine *cck* expression could be related to changes in digestive physiology, and not to appetite regulation, since cck is also a regulator of digestive processes in fish (Volkoff et al., 2017). Indeed, PF-based diets did not affect *cck* brain gene expression in Atlantic salmon and cobia, leading the authors to conclude that under the tested conditions *cck* mRNA levels could not be defined as an appetite/satiety signal (Hevrøy et al., 2008; Nguyen et al., 2013).

Concerning the P/CH ratio, higher CH diets promoted brain *lepr* gene expression and inhibited the intestine *cck* gene expression at 5h AF. These results suggest that high dietary CH content leads to a less satiety sensation, considering that lepr and cck have orexigenic and anorexigenic functions, respectively. A decrease in *cck* gene expression with the increase of dietary CH inclusion was previously observed in gilthead seabream, which led the authors to conclude that dietary condition modulates the expression of appetite regulation genes (Babaei et al., 2017).

4.2. Diet composition effect on nutritional and metabolic parameters

In the present study, neither protein source or P/CH ratio significantly affected FI. Nonetheless, it is important to mention that a trend for higher FI was observed in fish fed diet PF-P40/CH20. The energy content of this diet was the lowest between the tested diets, moreover PF proteins are generaly less digestible than FM protein (Glencross et al. 2007). This together with the fact that fish as other animals, within limits, eat to meet energy needs (Bureau et al. 2002), might explain this observed trend for higher FI.

According to Benedito-Palos et al. (2007), in gilthead seabream, *ghr-i* mediates the expression of *growth hormone* and hepatic *igf-1*, while *ghr-ii* is a more constitutive gene that does not require intact igf-pathways to exert a growth-promoting action. Moreover, a decrease in *ghr* and *igf-1*

gene expression was also reported in gilthead seabream fed a 100% PF diet (Gómez-Requeni et al., 2004). However, in the present study, the dietary protein sources led to an unclear response in both *ghr-i* and *igf-1* gene expression, which could be justified by the tested sampling time, 5h AF, instead of overnight fasting as in the study by Gómez-Requeni et al. (2004). In the present study, *ghr-i* gene expression was lower in fish fed the FM-P40/CH20 diet than in fish fed PF-P40/CH20 diet. Although statistical significant growth differences were not observed on those fish, the ones fed PF-P40/CH20 had higher final body weight, which is in accordance with the observed higher *ghr-i* gene expression. On the other hand, *ghr-ii* gene expression was lower in fish fed the PF-based diets. Thus, further studies are required to elucidate the effect of diet composition on these hormones and receptors, and their relationship with FI and the remaining appetite regulation mechanisms or metabolic parameters.

Athough dietary protein source did not affect growth, FE nor PER, the PF-based diets may lead to an increase in lipid deposition, as suggested by Pratoomyot et al. (2010). Cruz-Garcia et al. (2011) and Riera-Heredia et al. (2019) further reported that PF-based diets promote adipocyte hypertrophy, thus leading to less functional adipose tissue. In the present study, despite changes were not observed in the area covered by liver lipid vacuoles, an increase in the size and number of adipocytes, liver lipid content, and hepatic *fas* and *mtor* gene expression, was observed in fish fed the PF-based diets. In accordance, mtor inhibition in rainbow trout led to a decrease of *fas* and *gk* gene expression, leading the authors to conclude that the activation of mtor signalling is necessary for the post-prandial regulation of hepatic lipogenesis and gk (Dai et al., 2003). In agreement, in the present study, *mtor*, *gk*, and *fas* gene expression, were all consistently higher in fish fed PF-based diets. In addition, Kim et al. (2012) also described a relationship between *mtor* and *npy* gene expression. However, in the present study, *mtor* increased in fish fed PF-based diets, but no effect of dietary protein source was observed in *npy* gene expression, supporting the evidence that *mtor* function is more evident in relation to lipid synthesis and storage (Ricoult and Manning, 2013).

PF-based diets induced hypocholesterolemia, as also previously reported in gilthead seabream (Gómez-Requeni et al. 2004). This hypocholesterolemia may be related to precipitation by plant

sterols of the marginally soluble cholesterol into a non-absorbable state, or the displacement of cholesterol from the micelles that assist its absorption into the enterocytes (Hicks and Moreau 2001). PF-based diets also seem to have promoted glycogenesis, as suggested by the increased liver *gk* gene expression and liver glycogen content. As expected, plasma glucose was higher in fish fed the high CH-diets (diets P40/CH20). However, within these diets, plasma glucose was higher in fish fed the FM-based diet. This might be related to the fact that the starch present in the FM-based diets was pregelatinized maize starch, which is more easily digested than the starch present in the plant ingredients of the PF-based diets. Similarly, an increased plasma glucose level in fish fed FM-based diets compared with fish fed PF-based diets was already reported in European seabass, *Dicentrarchus labrax* (Guerreiro et al., 2015).

Fish fed FM-P50/CH10 diet presented a higher growth than fish fed FM-P40/CH20 diet, which might be at least, partially explained by the higher FI (not statistically significant), FE, and dietary protein and energy content. This higher growth is in accordance with the observed higher expression of *ghr-i, ghr-ii* and *igf-1* in fish fed FM-P50/CH10 diet. Similary, Pérez-Sánchez et al. (1995) previously observed in gilthead seabream that the growth stagnation could be linked to a decrease in plasma igf-1 immunoreactivity and hepatic growth hormone binding sites. Nevertheless, PER was decreased in fish fed diets with higher dietary protein content, suggesting that gilthead seabream did not efficiently use the excess protein provided.

Present results showed that though a higher dietary CH content induced an increase in plasma glucose levels, liver *gk* gene expression was not affected. Similar results were previously observed in gilthead seabream fed diets with different gelatinized starch levels, where gk activity was not affected by different circulating glucose levels (Couto et al., 2008). *g6pase* gene expression was not affected by dietary CH content. The absence of dietary CH effects on gluconeogenesis was also observed in gilthead seabream fed diets with different starch levels (Enes et al., 2008). According to Enes et al. (2006), in European seabass, gluconeogenic regulation was mainly influenced by amino acid catabolic mechanisms rather than by dietary CH, and this was probably the case in the present study, as *gdh* gene expression increased in fish fed the high protein diets. Excess glucose can be stored in the liver as glycogen or as lipids (Enes et al., 2009). In this study,

liver glycogen was not affected by dietary CH level, but liver lipid content was higher in fish fed PF-based diets with higher CH content, in line with the increase of HSI and VSI in fish fed higher CH levels. However, no changes were observed in the area covered by liver lipid vacuoles.

Additionally, in Mozambique tilapia, a reduction of brain *ghrr* mRNA levels 6h after an IP glucose injection was reported (Riley et al., 2009). In the present study, a similar negative feedback was observed in fish fed higher CH-diets, since with an increase of plasma glucose levels, a decrease in the hepatic *ghrr-b* gene expression 24h AF was found.

5. Conclusion

This study indicates that in gilthead seabream, among the appetite-related genes evaluated in the present study, only *ghrr-a*, *leptin*, and *lepr* gene expression are affected by the short-term fasting periods evaluated, at 5h and 24h AF. However, these tested periods may have been too short to detect sensible expression changes in appetite regulation hormones, difficulting a clear definition of their orexigenic or anorexigenic roles.

The effects of FM and PF-based diets on appetite-related genes are only noticed at 24h AF, suggesting that fish response to dietary protein sources takes a relatively longer time to be induced. Further, PF-based diets seem to affect *cart*, *cck*, and *leptin* gene expression, and its implication in appetite-regulation should be deeply evaluated in future studies. PF-based diets promote liver lipid deposition, hypocholesterolemia, and the activation of the glycogenesis pathway.

The high dietary CH content seems to lead a shorter satiety sensation, by affecting *lepr* and *cck* gene expression. Even so, the connection between FI, dietary composition, and fish appetite-related genes expression remains unclear. Thus, more studies should be done for a complete understanding of this relationship, for instance using diets with even higher CH levels or longer sampling times AF.

High dietary CH content induced an increase in plasma glucose but did not affect *gk* and *g6pase* gene expression. Gluconeogenic regulation seems to be mainly influenced by amino acid catabolism, as confirmed by the increase of *gdh* gene expression observed in fish fed the high

protein diets. The excess of plasmatic glucose seems to be stored as lipids, since fish fed the high CH diets present higher hepatic lipid content and higher HSI and VSI. Overall, PF-based diets with up to 20% of CH-content can be used in this specie without compromising growth performance and FI, although slightly modifying appetite-related genes expression and metabolic parameters.

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Table 1. Ingredients and proximate composition of the experimental diets.

	Diets			
	FM-P50/CH10	FM-P40/CH20	PF-P50/CH10	PF-P40/CH20
Ingredients (%DM)				
Fishmeal[1]	64.8	51.9	13.0	10.4
Soybean meal[2]	-	-	25.0	19.1
Wheat gluten[3]	-	-	12.7	9.0
Corn gluten[4]	-	-	22.6	20.0
Fish oil[5]	10.4	11.9	15.2	15.7
Pregelatinized maize starch[6]	10.0	20.0	5.9	16.6
Cellulose[7]	11.3	12.7	-	2.9
Monocalcium phosphate[8]	-	-	1.5	2.1
Lysine[9]	-	-	0.6	0.5
Taurine[10]	-	-	0.2	0.2
Vitamin mix[11]	1.0	1.0	1.0	1.0
Mineral mix[12]	1.0	1.0	1.0	1.0
Binder[13]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Proximate analysis (%DM)				
Dry matter	92.1	92.9	93.8	90.3
Crude protein	51.3	39.1	50.6	38.0
Crude fat	18.7	18.6	18.7	18.4
Ash	8.6	7.5	6.4	5.6
Starch	9.0	17.2	11.4	18.2
Gross energy (kJ g $^{-1}$)	23.7	21.2	22.1	20.6

CH: Carbohydrate; CP: Crude protein; DM: Dry matter; FM: Fishmeal; GL: Gross lipid; P: Protein; PF: Plant-feedstuffs.

[1]Sorgal. S.A. Ovar. Portugal (CP: 77.1% DM; GL: 11.8% DM).

[2]Sorgal. S.A. Ovar. Portugal (CP: 52.0% DM; GL: 1.9% DM).

[3]Sorgal. S.A. Ovar. Portugal (CP: 83.1% DM; GL: 1.4% DM).

[4]Sorgal. S.A. Ovar. Portugal (CP: 70.1% DM; GL: 2.8% DM).

[5]Sorgal. S.A. Ovar. Portugal.

[6]C-Gel instant 12018. Cerestar. Mechelen. Belgium.

[7]α- Cellulose (C-8002). Sigma-Aldrich. Sintra. Portugal.

[8]Sorgal. S.A. Ovar. Portugal.

[9]Feed-grade lysine. Sorgal. S.A. Ovar. Portugal.

[10]Feed-grade taurine. Sorgal. S.A. Ovar. Portugal.

[11]Vitamins (mg kg^{-1} diet): retinol acetate. 18000 (IU kg^{-1} diet); cholecalciferol. 2000 (IU kg^{-1} diet); alpha tocopherol acetate. 35; sodium menadione bisulphate. 10; thiamin-HCl. 15; riboflavin. 25; calcium pantothenate. 50; nicotinic acid. 200; pyridoxine HCl. 5; folic acid 10; cyanocobalamin. 0.02; biotin. 1.5; ascorbic acid. 50; inositol. 400. Premix. Lda. Viana do Castelo. Portugal.

[12]Minerals (mg kg^{-1} diet): copper (II) sulphate. 5; ferrous carbonate. 40; fluorine. 1; potassium iodide. 0.6; magnesium oxide. 500; manganese oxide. 20; sodium selenite. 0.3; zinc oxide. 30; Minerals content (%): Calcium. 17; Phosphorus. 13; Potassium. 6; Cloride. 7; Sodium chloride. 4. Premix. Lda. Viana do Castelo. Portugal.

[13]Liptosa. Madrid. Spain.

Table 2. Genes and primers used for qPCR.

Gene	ID primer	Sequence (5´- 3´)	Accession nº	Tm (ºC)	Efficiency (%)
translation elongation factor 1a	*ef1a*	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	AF184170	60	76.5
ribosomal Protein S18	*rps18*	F: GGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACCA	AM490061.1	60	79.6
3-hydroxyacyl-CoA dehydrogenase	*hoad*	F: GAACCTCAGCAACAAGCCAAGAG R: CTAAGAGGCGGTTGACAATGAATCC	JQ308829	60	81.8
cholecystokinin	*cck*	F: CTGTGTACGAGCTGTTTGGGG R: AGCCGGAGGGAGAGCTTT	KP822925	60	84.6
cocaine- and amphetamine-regulated transcript	*cart*	F: CTGAGGAGCAAAGAGATGCCCTTAGAGAAA R: GCGTCACACGAAGGCAGCCA	MG570186	60	95.5
corticotropin-releasing hormone	*crh*	F: ATGGAGAGGGGAAGGAGGT R: ATCTTTGGCGGACTGGAAA	KC195964	60	82.6
fatty acid synthase	*fas*	F: TGGCAGCATACACACAGACC R: CACACAGGGCTTCAGTTTCA	AM952430	60	93.6
ghrelin	*ghrelin*	F: CCCGTCACAAAAACCTCAGAAC R: TTCAAAGGGGGCGCTTATTG	MG570187	60	90.3
ghrelin receptor-a	*ghrr-a*	F: GTCGGCGGCTGTGGCAAAGA R: GGCCAACACCACCACCACCAAC	MG570188	60	90.0
ghrelin receptor-b	*ghrr-b*	F: CGCACACGCATAACTTTGTC R: GAGGAGGATGAGCAGGTGAA	MG570189	60	122.0
glucokinase	*gk*	F: GACGCTATCAAGAGACGA*GGGAC R: CCACGGTCCTCATCTCCTCCAT	AF053330	60	79.9
glucose-6-phosphatase	*g6pase*	F: CTGCTGTGGACGATGGAGAAAG R: TGTTGAGGGGCGAGTGAAGAC	AF151718	60	88.3
glutamate dehydrogenase	*gdh*	F: GGTATCCACGGTCGTATCTCAGCC R: GAGACCCACATTACCAAAGCCCTG	JX073708	60	92.1
growth hormone receptor-i	*ghr-i*	F: ACCTGTCAGCCACCACATGA R: TCGTGCAGATCTGGGTCGTA	AF438176	60	88.0
growth hormone receptor-ii	*ghr-ii*	F: GAGTGAACCCGGCCTGACAG R: GCGGTGGTATCTGATTCATGGT	AY573601	60	90.9
insulin-like growth factor-1	*igf-1*	F: ACAGAATGTAGGGACGGAGCGAATGGAC R: TTCGGACCATTGTTAGCCTCCTCTCTG	EF688016	60	86.6
leptin	*leptin*	F: TCTCTTCGCTGTCTGGATTCCTGGAT R: CTCCTTCTTGCTCTGTAGCTCTT	KP822924	60	95.1
leptin receptor	*lepr*	F: GGCGGAACTGATTCTACTCTG R: AGTATCGGACCTCGTATCTCA	MG570178	60	108.2

Gene	ID primer	Sequence (5´- 3´)	Accession nº	Tm (ºC)	Efficiency (%)
neuropeptide Y	*npy*	F: AAACCGGAGAACCCCGGGGAGG R: CTGGACCTTTTTCCATACCTCTG	KP822926	60	73.2
target of rapamycin	*mtor*	F: CAGACTGACGAGGATGCTGA R: AGTTGAGCAGCGGGTCATAG	Vélez et al. (2016)	60	94.0

F: Forward; R: Reverse; Tm: Melting temperature.

Table 3. Growth performance and feed utilization efficiency of gilthead seabream fed the experimental diets.

Protein source	FM			PF			*Two-way ANOVA*		
P/CH ratio	P50/CH10	P40/CH20	SEM	P50/CH10	P40/CH20	SEM	PS	P/CH	I
Final body weight (g)	217.4[b]	195.9[a]	4.59	205.0	206.9	3.52	ns	ns	*
FI (g kg ABW^{-1} day^{-1})	13.68	12.19	0.44	12.97	14.13	0.62	ns	ns	ns
FE[1]	0.77	0.66	0.02	0.71	0.66	0.02	ns	**	ns
PER[2]	1.51	1.70	0.04	1.40	1.75	0.07	ns	***	ns

ABW: Average body weight; CH: Carbohydrate; FE: Feed efficiency; FI: Feed intake; FM: Fishmeal; I: Interaction; P: Protein; PER: Protein efficiency ratio; PF: Plant-feedstuffs; PS: Protein source; SEM: Standard error of the mean.

Values presented as means (n=3 tanks).

Different lower-case letters denote significant differences between dietary P/CH ratios.

ns: not significant; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

ABW: (initial body weight + final body weight)/2; [1]FE: wet weight gain/dry feed intake. [2]PER: wet weight gain/crude protein intake.

Table 4. Whole-body and liver composition (wet weight basis), hepatosomatic (HSI) and visceral somatic (VSI) indices of gilthead seabream fed the experimental diets.

Protein source	FM			PF			*Two-way ANOVA*		
P/CH ratio	P50/CH10	P40/CH20	SEM	P50/CH10	P40/CH20	SEM	PS	P/CH	I
Body									
Protein (%)	16.43	15.97	0.18	16.32	15.43	0.28	ns	ns	ns
Lipid (%)	14.85	14.12	0.51	13.83	14.50	0.31	ns	ns	ns
Ash (%)	4.01	3.92	0.13	4.03	4.11	0.06	ns	ns	ns
Dry matter (%)	34.36	33.85	0.35	33.62	33.27	0.47	ns	ns	ns
Energy (kJ g^{-1})	9.02	9.15	0.23	8.77	8.83	0.15	ns	ns	ns
HSI (%)[1]	1.61	2.15	0.10	1.43	2.16	0.12	ns	***	ns
VSI (%)[2]	5.51	6.07	0.21	4.95	6.17	0.24	ns	**	ns
Liver									
Lipid (%)	8.16	7.08^{A}	0.60	8.89^{a}	13.49^{bB}	1.12	****	ns	*
Glycogen (%)	10.55	12.97	0.52	13.25	13.46	0.56	*	ns	ns

CH: Carbohydrate; FM: Fishmeal; HSI: Hepatosomatic index; I: Interaction; P: Protein; PF: Plant-feedstuffs; PS: Protein source; SEM: Standard error of the mean; VSI: Visceral somatic index.
Values presented as means, body (n=3), liver lipid and glycogen, VSI, and HSI (n=9).
Different lower-case letters denote significant differences between dietary P/CH ratios; upper-case letters denote significant differences between dietary protein sources.
ns: not significant; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
[1]Hepatosomatic index: (liver weight/body weight) × 100. [2]Visceral somatic index: (viscera weight/body weight) × 100.

Table 5. Plasma glucose, cholesterol, triglycerides, total protein, and total lipids of gilthead seabream fed the experimental diets, 5h after feeding.

Protein source	FM			PF			*Two-way ANOVA*		
P/CH ratio	P50/CH10	P40/CH20	SEM	P50/CH10	P40/CH20	SEM	PS	P/CH	I
Glucose (mg dL^{-1})	71.9^{a}	156.9^{Bb}	11.6	70.4^{a}	113.3^{Ab}	6.2	***	***	***
Cholesterol (mg dL^{-1})	231.5	218.3	8.2	160.9	142.2	5.5	***	ns	ns
Triglycerides (mg dL^{-1})	636.4	517.2	31.8	580.3	527.2	24.3	ns	**	ns
Total proteins (g dL^{-1})	2.93	2.96	0.05	3.02	3.04	0.06	ns	ns	ns
Total lipids (g dL^{-1})	2.34	2.13	0.07	1.95	1.95	0.05	**	ns	ns

CH: Carbohydrate; FM: Fishmeal; I: Interaction; P: Protein; PF: Plant-feedstuffs; PS: Protein source; SEM: Standard error of the mean.
Values presented as means (n=9).
Different lower-case letters denote significant differences between dietary P/CH ratios; upper-case letters denote significant differences between dietary protein sources.
ns: not significant; **P ≤ 0.01; ***P ≤ 0.001.

Table 6. Expression[1] of appetite regulation-related genes in gilthead seabream at 5h and 24h after feeding the experimental diets.

Sampling time	5h								24h							
PS	FM		PF			***Two-way ANOVA***			FM		PF			***Two-way ANOVA***		
P/CH ratio	P50/CH10	P40/CH20	P50/CH10	P40/CH20	SEM	PS	P/CH	I	P50/CH10	P40/CH20	P50/CH10	P40/CH20	SEM	PS	P/CH	I
Brain																
cart	0.09#	1.63	0.85	0.37	0.25	ns	ns	ns	0.48#	0.23	0.14	0.19	0.04	*	ns	ns
crh	6.75	10.79	6.12	10.81	1.74	ns	ns	ns	6.12	4.45	4.49	4.32	0.44	ns	ns	ns
ghrr-a	0.05#	0.07	0.06#	0.07	0.01	ns	ns	ns	0.14#	0.14	0.21#	0.08	0.03	ns	ns	ns
leptin	0.03#	0.02#	0.02	0.02#	0.00	ns	ns	ns	0.12#	1.62#	0.11	0.07#	0.24	*	ns	ns
lepr	0.08#	0.15#	0.08#	0.15#	0.02	ns	*	ns	0.35#	0.29#	0.21#	0.25#	0.03	ns	ns	ns
npy	36.81	62.85	70.98	128.59	17.18	ns	ns	ns	35.57	78.71	121.65	143.87	39.00	ns	ns	ns
Intestine																
cck	379.42	220.50	341.64	295.66	26.28	ns	*	ns	347.34^{Bb}	190.89^{Aa}	302.25^{Aa}	360.68^{Bb}	32.14	ns	ns	**
Liver																
ghrr-b	0.78	0.61	0.38#	0.52	0.08	ns	ns	ns	2.10	0.88	1.75#	1.08	0.23	ns	**	ns
leptin	0.31#	0.17#	0.18#	0.28#	0.03	ns	ns	ns	0.0008#	0.0007#	0.0033#	0.0019#	0.0003	**	ns	ns
Stomach																
ghrelin	597.19	579.30	735.59	807.70	47.41	ns	ns	ns	730.81	607.18	529.85	661.31	50.36	ns	ns	ns

[1.] All values expressed as arbitrary unit x 10^3, except for *ghrr-b* that was expressed as arbitrary unit x 10^7.

cart: cocaine- and amphetamine-regulated transcript; *cck*: *cholecystokinin*; CH: Carbohydrate; *crh*: *corticotropin-releasing hormone*; FM: Fishmeal; *ghrr-a*: *ghrelin receptor-a*; *ghrr-b*: *ghrelin receptor-b*; I: Interaction; *lepr*: *leptin receptor*; *npy*: *neuropeptide y*; P: Protein; PF: Plant-feedstuffs; PS: Protein source; SEM: Standard error of the mean.

Values presented as means (n=9).

Different lower-case letters denote significant differences between dietary P/CH ratios; upper-case letters denote significant differences between dietary protein sources. Significant differences between sampling times within each diet were indicated by #.

ns: not significant; *P ≤ 0.05; **P ≤ 0.01.

Table 7. Liver and adipose tissue normalized expression[1] of genes related to growth and intermediary metabolism of gilthead seabream fed the experimental diets.

Protein source	FM			PF			*Two-way ANOVA*		
P/CH ratio	P50/CH10	P40/CH20	SEM	P50/CH10	P40/CH20	SEM	PS	P/CH	I
Fatty acid metabolism									
Adipose tissue									
hoad	9.75	10.22	0.44	11.27	10.71	0.60	ns	ns	ns
fas	6.34	7.71	0.96	14.42	7.79	2.66	ns	ns	ns
Liver									
hoad	6.31	5.56	0.61	6.35	7.41	0.55	ns	ns	ns
fas	10.80	8.95	1.75	35.47	23.15	3.30	***	ns	ns
Liver glycolysis									
gk	313.55	261.66	16.47	391.75	392.29	38.45	*	ns	ns
Liver gluconeogenesis									
g6pase	2.55	3.03	0.41	3.91	2.13	0.61	ns	ns	ns
Liver amino acid catabolism									
gdh	15.91	9.84	1.84	18.45	13.71	1.25	ns	*	ns
Liver growth-related genes									
ghr-i	14.26^{b}	9.88^{Aa}	1.02	12.20	13.75^{B}	0.86	ns	ns	ns
ghr-ii	0.84	0.58	0.06	0.58	0.51	0.04	**	**	ns
igf-1	38.88^{Bb}	22.28^{a}	2.66	30.44^{A}	30.57	2.33	ns	**	ns
mtor	0.93	0.82	0.04	1.05	0.96	0.05	*	ns	ns

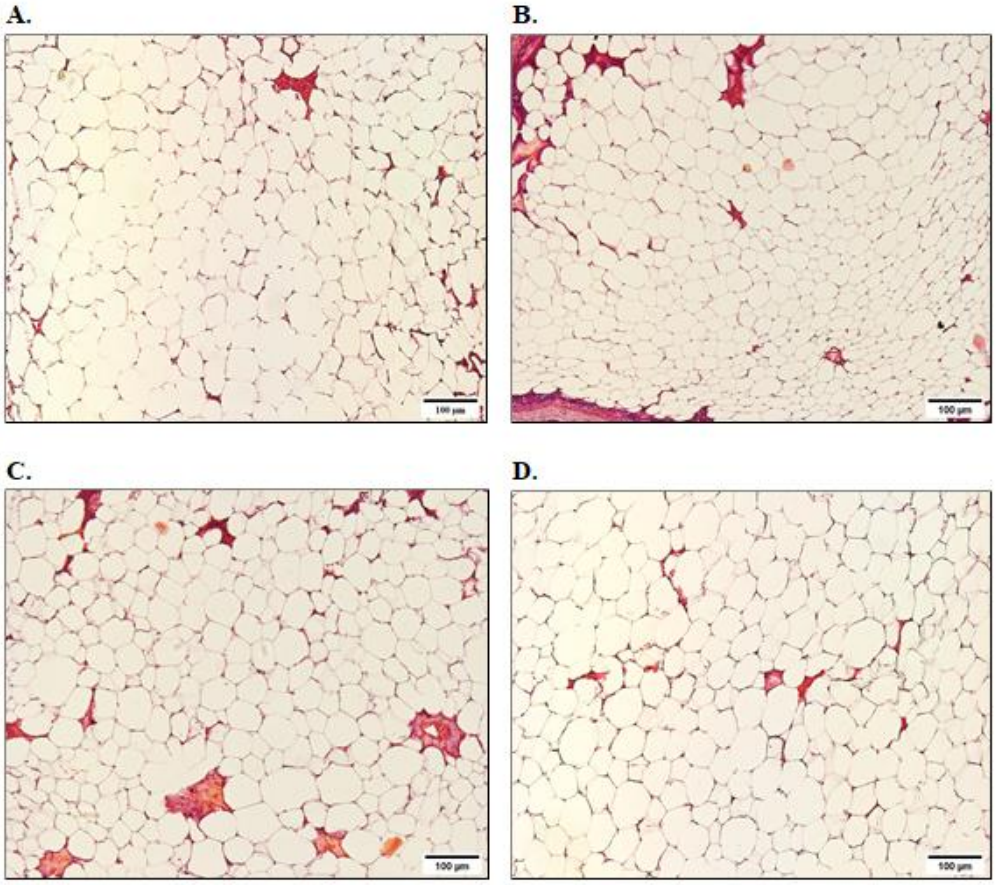
[1.] All values expressed as arbitrary unit x 10^3.

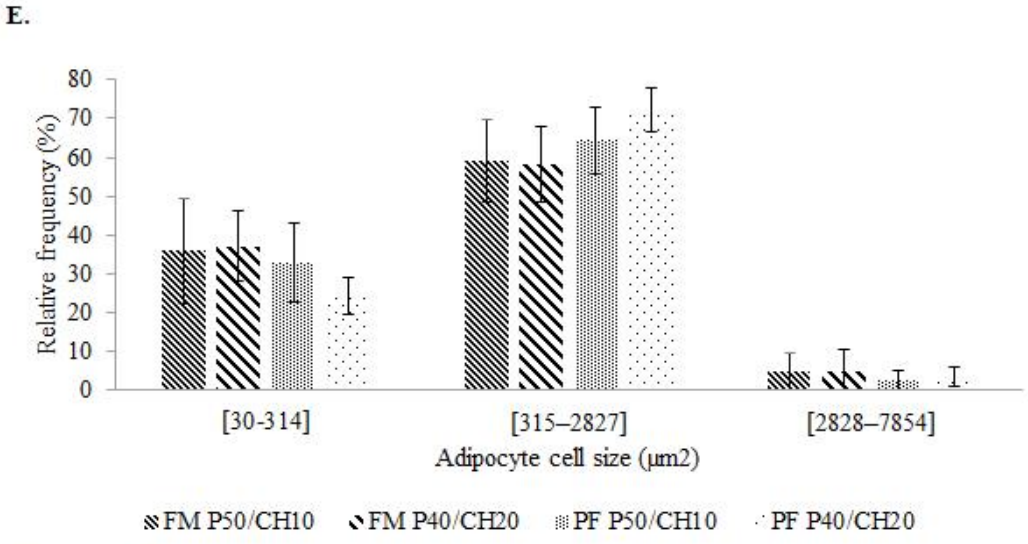
CH: Carbohydrate; *fas*: *fatty acid synthase*; FM: Fishmeal; *gk*: *glucokinase*; *g6pase*: *glucose-6-phosphatase*; *gdh*: *glutamate dehydrogenase*; *ghr-i*: *growth hormone receptor-i*; *ghr-ii*: *growth hormone receptor-ii*; *hoad*: *3-hydroxyacyl-CoA dehydrogenase*; I: Interaction; *igf-1*: *insulin-like growth factor-1*; *mtor*: *target of rapamycin*; P: Protein; PF: Plant-feedstuffs; PS: Protein source; SEM: Standard error of the mean.

Values presented as means (n=9).

Different lower-case letters denote significant differences between dietary P/CH ratios; upper-case letters denote significant differences between dietary protein sources.

ns: not significant; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.





Two-way ANOVA

				Protein source		P/CH ratio	
	Protein source	P/CH ratio	Interaction	FM	PF	P50/CH10	P40/CH20
Classes							
[30-314]	*	ns	ns	B	A	-	-
[315–2827]	**	ns	ns	A	B	-	-
[2828–7854]	ns	ns	ns	-	-	-	-

835

Figure 1. Representative hematoxylin and eosin-stained histological sections of adipose tissue from fish fed FM-P50/CH10 (A), FM-P40/CH20 (B), PF-P50/CH10 (C), and PF-P40/CH20 (D); and frequency distribution by classes (%) of adipocyte cell size from gilthead seabream fed the experimental diets (E). Images captured at 10× magnification. Values presented as means (n=9) and standard error. ns: not significant; *P ≤ 0.05; **P ≤ 0.01. CH: Carbohydrate; FM: Fishmeal; PF: Plant-feedstuffs; P: Protein.

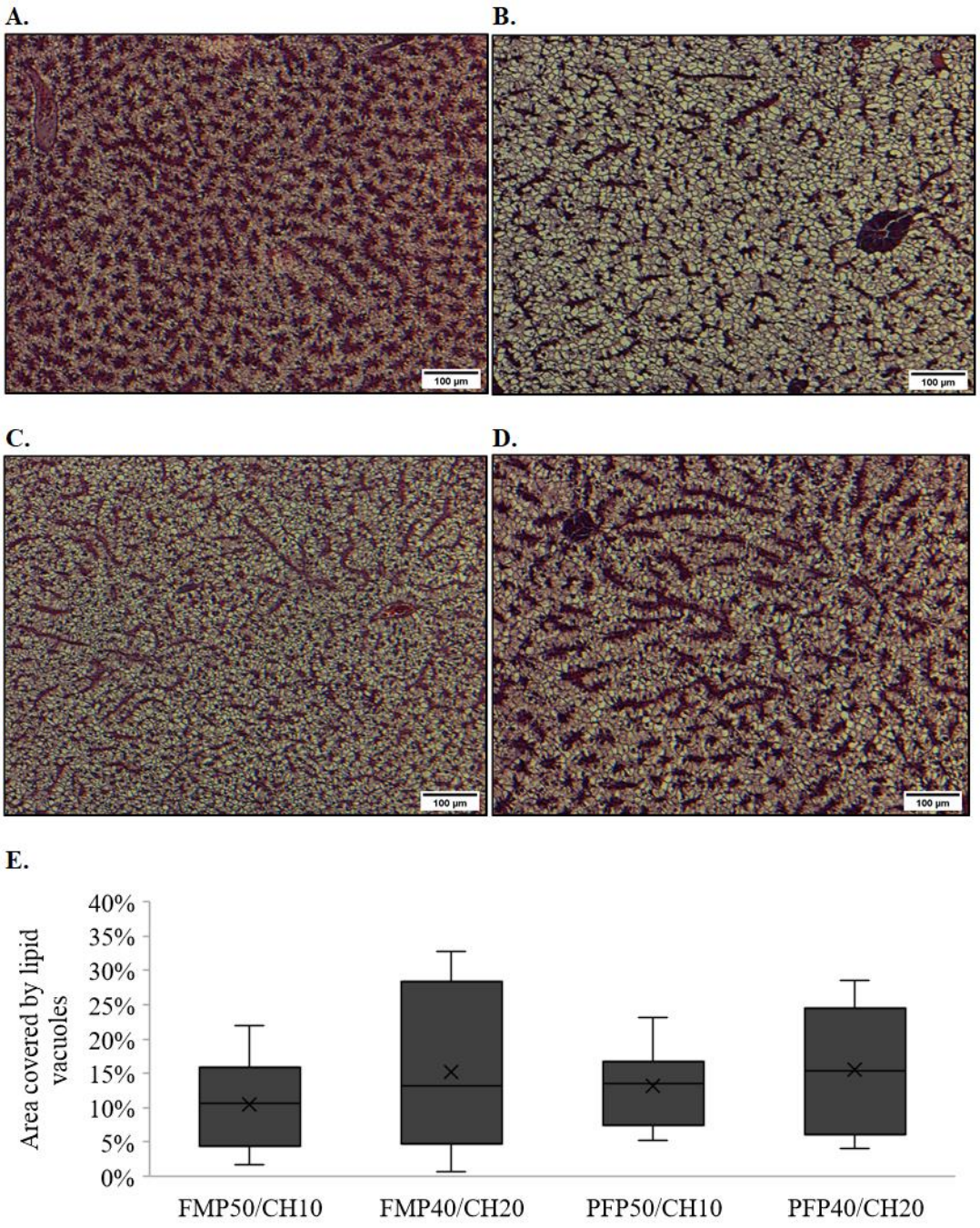


Figure 2. Representative hematoxylin and eosin-stained histological sections of liver from fish fed FM-P50/CH10 (A), FM-P40/CH20 (B), PF-P50/CH10 (C), and PF-P40/CH20 (D); and area covered by lipid vacuoles (%) in the liver of gilthead seabream fed the experimental diets (E). Images captured at 10× magnification. Values presented as means (n=9) and standard error. No significant differences were found ($P<0.05$). CH: Carbohydrate; FM: Fishmeal; PF: Plant-feedstuffs; P: Protein.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT author statement for

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All authors contributed equally to the original manuscript, namely in planning, writing, and editing the manuscript, and in data acquisition, analysis, and interpretation.