Feeding frequency and dietary protein/carbohydrate ratio affect feed intake and appetite regulation-related genes expression in gilthead seabream (*Sparus aurata*)

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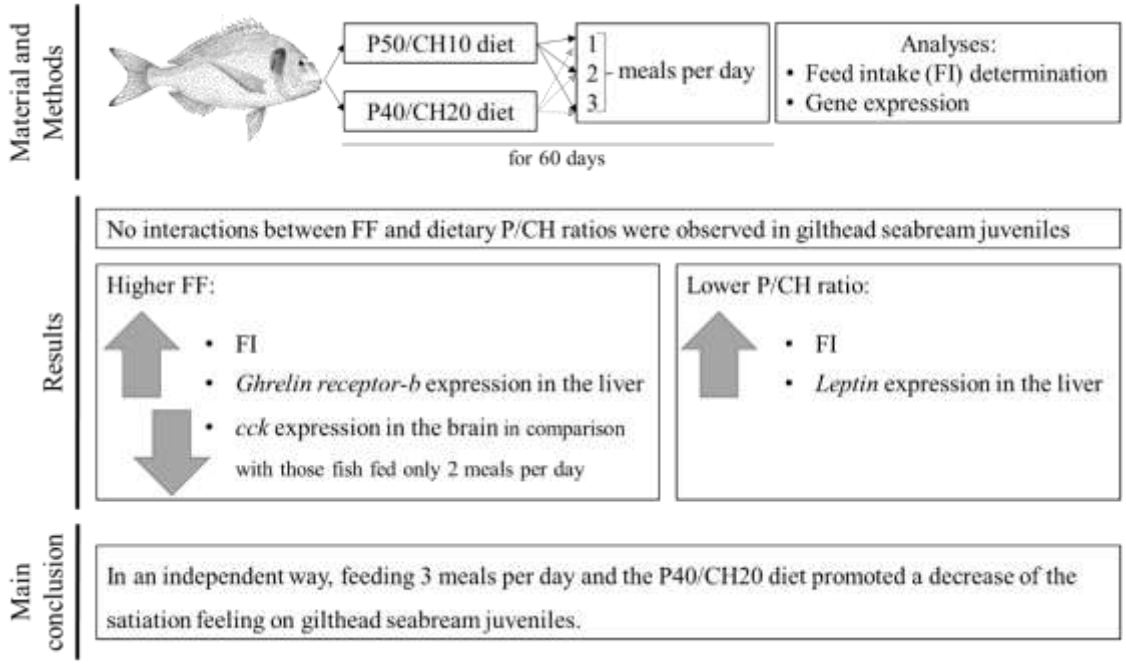
Abstract

To evaluate the effects of feeding frequency (FF) and dietary protein/carbohydrate (P/CH) ratios on appetite regulation of gilthead seabream, two practical diets were formulated to include high protein and low carbohydrate (P50/CH10 diet) or low protein and high carbohydrate (P40/CH20 diet) content and each diet was fed to triplicate groups of fish until visual satiation each meal at a FF of 1, 2, or 3 meals per day. Feed intake and feed conversion ratio were higher in fish fed 2 or 3 meals than 1 meal per day and in fish fed the P40/CH20 than the P50/CH10 diet. The specific growth rate was only affected by FF, being higher in fish fed 2 or 3 meals per day than 1 meal per day. Expression of the *cocaine-amphetamine-related transcript, corticotropin-releasing hormone, ghrelin receptor-a* (*ghsr-a*), *leptin*, and *neuropeptide y* in the brain, *cholecystokinin* (*cck*) in the intestine, and *leptin* and *ghrelin* in the stomach was not affected by FF or dietary P/CH ratio. This is the first time that ghrelin cells were immune-located in the stomach of gilthead seabream. Fish fed 3 meals per day presented lower *cck* expression in the brain than those fed twice per day and higher hepatic *ghsr-b* expression than those fed once per day. Fish fed P40/CH20 diet presented higher hepatic *leptin* expression than those fed P50/CH10 diet. In conclusion, present results indicate that feeding a P40/CH20 diet at 3 meals a day seems to decrease the satiation feeling of gilthead seabream compared to fish fed higher P/CH ratio diets or fed 1 or 2 meals a day.

Graphical Abstract



Keywords

Anorexigenic/orexigenic hormones; Ghrelin; Immunohistochemistry; Diet protein/carbohydrate ratio; Stomach.

1. Introduction

Animals survival and growth depend on the amount of energy intake and energy expenditure. Under normal conditions, when energy intake exceeds energy requirements, anorexigenic responses are produced, inhibiting fish appetite; and when energy expenditure exceeds energy requirements, fish appetite is stimulated through orexigenic responses (Volkoff, 2011). A complex regulatory network is involved in the maintenance of this energy homeostasis, including several hormones and the hypothalamus feeding center that receives or sends orexigenic or anorexigenic signals from/to peripheral organs (Delgado et al., 2017; Rønnestad et al., 2017; Soengas et al., 2018; Volkoff, 2019).

Between the most important hormones of this network are cocaine-amphetamine-related transcript (cart), mainly expressed in the brain, and cholecystokinin (cck), mainly expressed in the brain and digestive tract of the fish, being both generally recognized as potent satiety factors (Volkoff and Peter, 2000; 2001; Volkoff et al., 2003; Kobayashi et al., 2008; Murashita et al., 2009; Ji et al., 2015; White et al., 2016; Pitts and Volkoff, 2017). Leptin has been also pointed as an anorexigenic hormone, since intraperitoneal and intracerebroventricular injections of this peptide promoted a reduction of feed intake (FI) in fish (Volkoff et al., 2003; Murashita et al., 2008; Li et al., 2010; Won et al., 2012). However, this anorexigenic function does not seem so clear when evaluating the fasting effects on *leptin* expression across different fish species and tissues. For instance, in gilthead seabream (*Sparus aurata*), 23 days of fasting did not affect *leptin* expression in the adipose tissue (Babaei et al., 2017), but in orange-spotted grouper (*Epinephelus coioides*), 7 days of fasting promoted an increase of *leptin* expression in the brain (Zhang et al., 2013), and in the red-bellied piranha (*Pygocentrus nattereri*), intestine *leptin* expression decreased after 7 days of fasting (Volkoff, 2015). In contrast, neuropeptide y (npy) is pointed as an orexigenic hormone mainly expressed in the brain (Volkoff et al. 2003; Wei et al. 2014; Ji et al. 2015; Li et al., 2017). The function of corticotropin-releasing hormone (crh)-related peptide is still poorly explored in fish appetite regulation, and the results seem to be controversial. Some studies described this peptide with an anorexigenic function, for instance, in goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) (Bernier and Peter, 2001; Matsuda et al., 2008;

Ortega et al. 2013). However, in *Schizothorax prenanti*, *crh* expression was not affected either by fasting for 1 or 3 h nor by fasting by up to 5 days, being necessary at least 7 days of fasting to promote a decrease in brain *crh* expression (Wang et al., 2014). While, in gilthead seabream, fasting of 21 days did not affect brain *crh* expression (Martos-Sitcha et al., 2014). Ghrelin (ghrl), a hunger hormone already identified in several fish species including gilthead seabream, is mainly expressed in the stomach but it is also expressed in other peripheral tissues, like the intestine, liver, and spleen (Unniappan et al., 2002; Murashita et al., 2009; Xu and Volkoff, 2009; Feng et al., 2013; Volkoff, 2015; Song et al., 2017; Perelló-Amorós et al., 2018). This hormone seems to participate in several physiologic mechanisms in vertebrates, such as drink behavior, reproduction, and immunological regulation (Kaiya et al., 2008), but it is in energy balance control that ghrl has one of the most relevant roles, affecting FI (Unniappan et al., 2004; Jönsson et al., 2010; Tinoco et al., 2014a; Schroeter et al., 2015; Yuan et al., 2015). In fish, ghrl role in FI regulation seems to be species-dependent. For instance, after peripheral ghrl administration, FI increased in goldfish, brown trout (*Salmo trutta*), and grass carp (*Ctenopharyngodon idellus*) (Unniappan et al., 2004; Tinoco et al., 2014a; Yuan et al., 2015) but decreased in channel catfish (*Ictalurus punctatus*) and rainbow trout (Jönsson et al., 2010; Schroeter et al., 2015). To a better ghrl characterization, some studies have used imaging techniques, namely immunohistochemistry, besides gene expression analysis (Sakata et al., 2004; Kaiya et al., 2006; Arcamone et al., 2009; Breves et al., 2009; Sánchez-Bretaño et al., 2015; Cascio et al., 2018; Opazo et al., 2019; Barrios et al., 2020). Nevertheless, ghrl-immunopositive (ip) cells in gilthead seabream tissues have not been detected to date.

However, the network between appetite-related hormones may be influenced by several factors, including feeding frequency (FF) and dietary composition. For instance, recently, Pham et al. (2021) studied the FI process in clown anemonefish (*Amphiprion ocellaris*) fed to satiety 1 or 3 meals per day, and observed that some neuropeptides already known as appetite regulators in the brain (namely agouti-related protein, AgRP, and pro-opiomelanocortin, POMC) also seem to have a role in appetite regulation associated to FF. Differently, a fixed daily ration distributed by

different meals (1, 3, or 5 meals per day, or continuous feeding) did not affect gastric *ghrelin* (*ghrl*) or intestinal *cck* gene expression in gilthead seabream (Gilannejad et al., 2021).

Regarding dietary composition effects on FI and appetite regulation mechanisms, it is important to consider dietary nutrient levels and available energy, since when provided a nutrient-balanced diet fish eat to meet energy requirements (Bureau et al., 2002). For instance, recently we evaluated the effect of different dietary P/CH ratios on appetite regulation in gilthead seabream (Basto-Silva et al., 2021) and observed a decrease in *cck* expression in fish fed a diet with a low P/CH ratio compared to a high P/CH ratio. This suggests a less satiety feeling with the former diet and agrees with previous observations in gilthead seabream, where FI was higher in fish fed diets with low P/CH ratios (Couto et al., 2008). However, different results were reported for rainbow trout, when changing the dietary P/CH ratio from 50/6 to 25/39 led to a decrease of FI but did not change the *npy* and *cartpt* expression (Figueiredo-Silva et al., 2012). This suggests that the exact mechanisms by which energy status is informed to the central or peripheral targets (i.e., cart, ghrl, leptin, npy, etc.) of appetite regulation are not yet clearly understood in fish and can vary depending on the fish species. Further, in gibel carp (*Carassius auratus gibelio*) it was reported that FI was consistently higher in fish fed simultaneously more meals per day and diets with a high P/CH ratio (Zhao et al., 2016), suggesting that FF optimization and dietary P/CH ratio can modulate fish appetite control.

Therefore, as diet composition, namely P/CH ratio, and FF affect FI in gilthead seabream, changes in the appetite-regulatory mechanisms are also expected (Couto et al., 2008; Moreira et al., 2008; García-Meilán et al., 2013; Babaei et al., 2017; Busti et al., 2020; García-Meilán et al., 2020; Basto-Silva et al., 2021; Gilannejad et al., 2021). However, the simultaneous effects of both factors in gilthead seabream appetite regulation are yet to be explored.

The present study aimed to evaluate the effects of different FF (1, 2, or 3 meals per day) and dietary P/CH ratios (P50/CH10 or P40/CH20) on appetite regulation-related genes expression and FI of gilthead seabream, one of the most important species in European aquaculture. The present study also aimed to locate, for the first time, ghrl cells in gilthead seabream stomach and intestine for a better characterization of this hormone.

2. Materials and methods

2.1. Diets composition

Two isolipidic (17% crude lipids) and isoenergetic (20 kJ g^{-1}) practical diets were formulated to include 50% protein and 10% carbohydrates, or 40% protein and 20% carbohydrates (diets P50/CH10 or P40/CH20, respectively). All dietary ingredients were carefully mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA), using a 2 mm die. Pellets were dried in an oven for 48 h at 50 ºC and then stored in plastic containers at 4 ºC until use. The experimental diet composition and proximate analysis are presented in Table 1. Dry matter, protein, lipid, and ash analyses of the diets were done following the Association of Official Analytical Chemists methods (AOAC, 2000), and dietary starch was determined as described by Beutler (1984).

2.2. Experimental conditions and sampling

The experiment was performed at the Marine Zoology Station, University of Porto, Portugal, with gilthead seabream (*Sparus aurata*) obtained from Sonríonansa, Pesués, Cantabria, Spain. Upon arrival at the experimental facilities, fish were submitted to a quarantine period of 19 days and fed a commercial diet (43% protein, 21% nitrogen free extract, 15% lipids, and 15% lipids, 1% fiber, and 9% ash; Aquasoja, Ovar, Portugal).

The trial was performed in a recirculating water system equipped with 18 fiberglass tanks (100 L water capacity), thermo-regulated to 24 ± 1 ºC, and each tank was supplied with a continuous flow of filtered seawater (6.0 L min^{-1}). During the trial, salinity was 36.0 ± 1.0 g L^{-1}, dissolved oxygen was kept near saturation (6.0 ± 0.5 mg L^{-1}), and fish were under a 12 h light/12 h dark photoperiod. Eighteen groups of 20 fish with an individual body weight of 9.1 ± 0.01 g (mean ± standard deviation) were established into each tank, and the diets and FF conditions were randomly assigned to triplicate groups of fish. Fish were fed by hand for 60 days, 6 days a week, until visual satiation, 1 meal per day (9:00 h), 2 meals per day (9:00 and 17:00 h), or 3 meals per

day (9:00, 13:00, and 17:00 h). The amount of feed provided by meal was recorded for FI determination.

At the end of the trial, 5 h after the morning meal (14:00 h), three fish from each tank (nine fish per experimental treatment) were euthanized by decapitation and dissected on chilled trays for collection of the stomach and anterior intestine for immunohistochemistry (IHC), and whole-brain (including hypophysis), stomach, anterior intestine, and liver for gene expression analyses. The samples for IHC were rinsed in phosphate-buffered saline (PBS), blotted dry with a paper towel, immediately fixed in Bouin (#57211, Thermo Scientific - Richard-Allan Scientific, USA) for 24 h, and subsequently transferred to 70% ethanol until further processing. The samples for gene expression were immediately stored in RNA later, left at 4 °C overnight, and subsequently stored at -80 ºC until analyses. The sampling time was selected since it was shown to provide the best results concerning appetite regulation in a previous study (Basto-Silva et al., 2021).

The experiment was performed by accredited scientists (following FELASA category C recommendations) and was conducted according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

2.3. Immunohistochemistry processing

Tissues were processed and sectioned using standard histological techniques. Transversal sections with 4 µm thickness were collected in Poly-L-Lysine slides (#J2800AMNT, Fisher Scientific, UK), dewaxed with xylene, and rehydrated in descending concentrations of alcohol. The IHC procedure was performed as described in (Kaiya et al., 2006) with slight modifications. Thus, all sections were delimited with a Dako pen (#5200230-2, LusoPalex Lda, Portugal), incubated in proteinase K (20 µg ml^{-1} in Tris-EDTA buffer) for 20 min, at room temperature (RT), washed in deionized running-water for 5 min, and in PBS for 5 min more. Then, the sections were incubated in 3% H_2O_2 (#31642, Merck KGaA, Germany) in methanol for 40 min at RT, rinsed in PBS for 10 min, incubated for 30 min with the Ultra V Block reagent from UltraVision Detection System Anti-Polyvalent, HRP kit #TP-060-HL (Thermo Fisher Scientific, USA), and quickly dipped 2-3 times in PBS. Then, the sections were incubated overnight on a humidity chamber, at 4 ºC, in

anti-octanoylated rat ghrelin [1-11] rabbit serum diluted 1/50,000 in a solution of 1% bovine serum albumin/tris-buffered saline (BSA/TBS). After the incubation, slides were rinsed in PBS for 10 min, and the sections were incubated with the secondary antibody (Biotinylated Goat Anti-Polyvalent Secondary from kit #TP-060-HL) for 30 min at RT. A new wash in PBS for 10 min was performed before incubation with Streptavidin Peroxidase reagent (from kit #TP-060-HL) for 30 min at RT and washed again with PBS. The sections were reacted with 3,3' diaminobenzidine, DAB Quanto kit #TA-060-QHDX (Thermo Fisher Scientific, USA) according to the manufacturers' instructions, and rinsed in deionized running water for 10 min. Finally, the sections were dehydrated through a crescent series solution of alcohol, cleared in xylene, and mounted in DPX mounting media (#4112; Thermo Scientific, USA). To verify the specificity of the immunohistochemical staining reaction, two negative control sections were performed for each sample: one without anti-rat ghrelin serum and another without secondary antibody. The anti-rat ghrelin serum was kindly offered by Professor Hiroyuki Kaiya, from National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan.

2.3.1. Morphometric evaluation

The morphological evaluation was only performed on the stomach sections since the IHC technique was not well-succeed in the intestine samples. Digital images were acquired using a light microscope (Axio Imager.A2; Zeiss, Germany) equipped with the Zen software (Blue edition; Zeiss, Germany) and analyzed individually. Ghrelin cell density was calculated as the number of ghrl-ip cells per unit area (cells mm^{-2}). A double-blinded evaluation (i.e. two different person without previous knowledge of the treatments) was repeated for three times in each fish stomach section. The mean of the three counts from the same section was considered for ghrl cell density determination in this specific section. The ghrl-ip cells were only considered after verification of the negative control sections. The area of each section was measured using Image J, version 1.46 (National Institutes of Health, USA). For each experimental condition, nine fish were used (n = 9).

2.4. Gene expression

Whole-brain (including hypophysis), stomach, intestine, and liver samples for RNA extraction were processed as described by Basto-Silva et al. (2021). RNA samples were used for cDNA synthesis using a DNase I (Life Technologies, Alcobendas, Spain) to remove genomic DNA contamination, followed by the Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) according to the manufacturer's recommendations, from a starting amount of 3300 ng of total RNA. Samples were stored at -20 ºC until used. Quantitative real-time PCR (qPCR) was performed as described in Basto-Silva et al. (2021) and the forward and reverse primers used were designed based on the deposited nucleotide sequences in the GenBank database (https://www.ncbi.nlm.nih.gov/) and are presented in Table 2. Translation elongation factor alpha (*ef1a*) and ribosomal protein s18 (*rps18*) genes were selected as reference genes since they were constitutively expressed and were not affected by the experimental treatments. Since some of the expressed genes did not have optimum efficiency curves (between 95-105%) thus, to normalize gene expression, the Pfaffl method (Pfaffl, 2001) was used. For each experimental condition, nine fish (n=9) were used.

2.5. Statistical analysis

All data are presented as the mean and standard deviation. Statistical analyses were done by two-way ANOVA, with FF and dietary P/CH ratio as factors, using SPSS 27 software package for Windows (IBM® SPSS® Statistics, USA). Data were tested for normality by the Shapiro-Wilk test and homogeneity of variances by Levene's test. When normality was not verified, data were transformed before ANOVA. For the leptin receptor (*lepr*) gene expression in the brain, where interaction between factors was observed, a one-way ANOVA was performed for the P/CH ratio within each FF, and for FF within each P/CH ratio. Significant differences among FF groups were determined by the Tukey multiple range test. A statistical significance of $p \leq 0.05$ was set for all the statistical tests performed.

3. Results

Fish promptly accepted the experimental diets, and during the trial, neither FF nor diet composition affected mortality, which was very low (1.67-3.33%). Specific growth rate (SGR) was only affected by FF, being higher in fish fed 2 or 3 meals per day than in those fed only 1 meal per day. FI and feed conversion ratio (FCR) were also higher in fish fed 2 and 3 meals than 1 meal per day and, independently of the FF protocol, in fish fed the P40/CH20 diet than the P50/CH10 diet (Table 3).

Gene expression levels were undetectable for *leptin* in the anterior intestine; *ghrl* in the brain, anterior intestine, and liver; *ghrelin receptor-a* (*ghsr-a*) in the anterior intestine; and *ghsr-b* in the brain. The expression of *npy*, *cartpt*, *crh*, *leptin*, and *ghsr-a* in the brain, *cck* in the intestine, and *leptin* and *ghrl* in the stomach was not affected by FF nor dietary P/CH ratio (Fig. 1). Fish fed 3 meals per day presented lower *cck* expression in the brain than those fed twice per day, and higher hepatic *ghsr-b* expression than fish fed 1 meal per day. Fish fed the P40/CH20 diet presented higher hepatic *leptin* expression than those fed the P50/CH10 diet. In fish fed twice per day, the expression of *lepr* in the brain was higher with the P40/CH20 diet than with diet P50/CH10. The expression of this receptor was also higher in fish fed P40/CH20 diet 2 times per day than in fish fed 1 meal per day the same diet.

In the stomach, ghrl-ip cells presented a small and round shape and were mainly encountered at the base of the gastric folds in the mucosal layer. No effect of FF or diet composition was observed on the density of ghrl-ip cells in the stomach (Fig. 2).

4. Discussion

A cumulative effect between FF and dietary P/CH ratio was previously reported in gibel carp since FI was consistently higher in fish fed simultaneously more meals per day and diets with higher P/CH ratios (Zhao et al., 2016). Moreover, interactions between FF and dietary P/CH ratio might also be expected, since starch digestibility can be compromised by an increase in FF (Yamamoto et al., 2007). Carnivorous fish not only have limited capacity to use dietary CH (Enes et al., 2011; Kamalam et al., 2017) but also nutrients digestion and absorption might be decreased by the increase in gut transit when fed at a higher FF (Liu and Liao, 1999; Thongprajukaew et al.,

2017). Thus, under those conditions, fish may possibly present a higher FI to fulfill their nutritional requirements and energy needs. In the present study, however, despite independent effects are being reported, no major interactions between FF and dietary P/CH ratios were observed.

Contrary to what we have observed, other studies on gilthead seabream did not report any significant effects of FF on FI (Yilmaz and Eroldogan, 2011; Busti et al., 2020) or in associated appetite regulation mechanisms (Gilannejad et al., 2021). In the study by Gilannejad et al. (2021) fish were fed a fixed daily amount of feed, while in the present study gilthead seabream were fed until apparent satiation, and this can contribute to explaining the apparently contradictory results between the two studies.

In the present study, we have observed that gilthead seabream fed 3 meals per day presented higher FI and gene expression of hepatic *ghsr-b* than fish fed 1 meal per day, suggesting that eating more meals per day increases fish appetite, which might partially justify the increased FI and weight gain observed in those fish. These observations might also suggest that in gilthead seabream ghsr-b has an orexigenic action. Nonetheless, the role of ghsr-b in FI regulation in fish is poorly understood. Contrary to present results, fasting did not affect *ghsr-b* expression either in gilthead seabream brain or liver (Perelló-Amorós et al., 2018). In zebrafish (*Danio rerio*), this receptor seems to mediate an orexigenic effect (Eom et al., 2014), while in Mozambique tilapia (*Oreochromis mossambicus*) it seems to have an anorexigenic role (Peddu et al., 2009). Therefore, more studies should be done to better understand the role of ghsr in fish.

We also observed lower brain *cck* expression in fish fed 3 meals per day comparing with fish fed 2 meals per day. A clear anorexigenic role for cck has been shown in several fish species (Volkoff et al., 2003; Valen et al., 2011; Feng et al., 2012; Penney and Volkoff, 2014; Yuan et al., 2014; Ji et al., 2015; Volkoff et al., 2016; White et al., 2016). However, in the present study, we did not observe any FI differences between fish fed 3 or 2 meals per day.

Gilthead seabream fed the P40/CH20 diet exhibited a similar growth to fish fed the P50/CH10 diet, but had higher FI and presented higher *leptin* expression in the liver. The *lepr* expression in the brain was also higher in fish fed the P40/CH20 diet but that was only observed when fish were

fed 2 meals per day. The interactive effect of FF and P/CH ratio on brain *lepr* expression was not expected since no interaction was observed regarding FI. However, both leptin and lepr results might suggest that diets with a lower dietary P/CH ratio promote a less satiety feeling. Nonetheless, this lower satiety feeling can only be considered if both leptin in the liver and lepr in the brain have an orexigenic role. An orexigenic function of lepr in the brain was also suggested in a previous study in gilthead seabream (Basto-Silva et al., 2021), although in that study hepatic leptin was reported to have contrarily an anorectic role. Nonetheless, hepatic leptin seemed to present an orexigenic role in other fish species, like goldfish and orange-spotted grouper, since it only increased several hours after feeding (Tinoco et al. 2012; Zhang et al. 2013; Tinoco et al. 2014b). It must be kept in mind that fish eat to meet nutrients and energy needs (Bureau et al., 2011; NRC 2011), thus the less satiation feeling and the increased FI in fish fed P40/CH20 diets can be related to the lower dietary protein content of that diet, which does not meet the requirements for gilthead seabream (Vergara and Jauncey 1993; Santinha et al., 1996; Lupatsch et al., 2003). Hence, fish needed to consume more feed to satisfy their protein requirement.

Previously, some studies also suggested that in gilthead seabream lower dietary P/CH ratios promote a smaller satiation feeling. That was the case of our previous work (Basto-Silva et al., 2021), where gilthead seabream fed P40/CH20 diets presented higher expression of *lepr* in the brain and lower expression of *cck* in the intestine than fish fed P50/CH10 diets. Or the study by Babaei et al. (2017), where fish fed P39/CH37 diets presented lower *cck* and *ghrl* expression in the gastrointestinal tract and higher *ghrl* expression in the brain than fish fed P58/CH15 diets. The activation of different physiological mechanisms reported in various studies can be also related to the distinct diets used, as some genes might be activated at different times post-feeding depending on dietary components (Bonacic et al., 2017; Murashita et al., 2019). For instance, in Senegalese sole (*Solea senegalensis*) fed 18% of fish oil, *cartpt* expression in the brain peaked at 1 h after feeding but in fish fed 8% of fish oil the peak occurred only 3 h after feeding (Bonacic et al., 2017). Similarly, in yellowtail fish (*Seriola quinqueradiata*) fed a low fishmeal diet (15%), *cck* expression was lowest at 2 h after feeding, but in fish fed a 50% fishmeal no differences were observed in *cck* expression at any of the post-feeding sampling points (Murashita et al., 2019).

However, no other significant differences were observed regarding gene expression, which might be connected with the observed high standard deviations, not allowing to make stronger conclusions. These high variation in appetite-relates genes expression was already presented in some other studies (Hernández-Cruz et al., 2015; Perelló-Amorós et al., 2018; Torrecillas et al., 2021). Moreover, due to the small fish size and as previously done in other studies on appetite regulation in gilthead seabream we analyzed the whole-brain (Babaei et al., 2017; Perelló-Amorós et al., 2018; Basto-Silva et al., 2021; Pulido-Rodriguez et al., 2021). Nonetheless, this might have masked certain modifications that could have been detected if we had analyzed specific regions as the telencephalon and hypothalamus as observed in other studies reporting different levels of activity depending on the analyzed brain section (MacDonald and Volkoff, 2009; Babichuk and Volkoff, 2013; Volkoff, 2015; Blanco et al., 2016). Thus, in future studies, the brain should be sectioned, and gene expression results might be supported through complementary methodologies, such as protein measurement and quantification.

In the present study, it was detected for the first-time gilthead seabream ghrl-ip cells in the stomach. As in rainbow trout, summer flounder (*Paralichthys dentatus*), European seabass (*Dicentrarchus labrax*), Japanese eel (*Anguilla japonica*), Streaked prochilodus (*Prochilodus lineatus*), and goldfish (Sakata et al., 2004; Kaiya et al., 2006; Arcamone et al., 2009; Breves et al., 2009; Sánchez-Bretaño et al., 2015; Barrios et al., 2020), ghrl-ip cells were small and round and were found mainly at the base of gastric folds in the mucosal layer of the stomach. In rainbow trout and Japanese eel two types of ghrl cells were observed (Sakata et al., 2004; Kaiya et al., 2006): opened-type cells, which seem to be in contact with the lumen and could have as a function to receive the luminal information, e.g., type and quality of the nutrients or pH; and closed-type cells, which do not have a luminal connection, and seem to be regulated by other hormones, neuronal stimulation, or mechanical distention (Sakata and Sakai, 2010). However, the distinction between those two types of cells was not possible in this study. We also tried but did not succeed in immune-locating ghrl cells on the anterior intestine of gilthead seabream. This is in agreement with gene expression data, both in this study and that of Basto-Silva et al. (2021), where *ghrl*

expression was undetectable in the anterior intestine. These results further support that in gilthead seabream ghrl is mainly expressed in the stomach (Perelló-Amorós et al., 2018).

The lack of FF and P/CH ratio effects on the density of ghrl-ip cells in the stomach is in agreement with the absence of effects observed on *ghrl* expression in this organ. In zebrafish larvae, it was suggested that ghrl might not be essential for appetite control, since neither *ghrl* expression nor peptide levels (measured through an IHC approach) were affected during fasting (Opazo et al., 2019). However, the limited and diverse data available for gilthead seabream does not allow to conclude about the importance of ghrl on appetite control in this species. Indeed, contrary to what was observed in the present study and that of Basto-Silva et al. (2021), the work of Babaei et al. (2017) appeared to indicate that a low dietary P/CH ratio promotes *ghrl* expression in the brain and lower expression in the gastrointestinal tract. Perelló-Amorós et al. (2018) further showed that ghrl seems to have an important role during fasting, exhibiting a strong down-regulation at the post-prandial stage. Thus, ghrl role in gilthead seabream appetite regulation seems to be complex and needs to be further clarified.

In conclusion, either 3 meals per day and low P/CH diets seem to decrease the satiation feeling of gilthead seabream juveniles, increasing FI and affecting the expression of some appetite-related genes. The present study also confirmed, for the first time in this species, the presence of ghrl cells in the base of gastric folds.

Declaration of Competing Interest The authors declare no competing interests.

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

	Diets	
	P50/CH10	P40/CH20
Ingredients (% DM)		
Fishmeal[1]	15.6	12.5
Fish oil[2]	14.0	14.7
Soybean meal[3]	25.0	20.0
Corn gluten[4]	20.0	15.0
Wheat gluten[5]	11.4	6.4
Wheat meal[6]	9.4	26.2
Monocalcium phosphate[7]	0.7	1.0
Lysine[8]	0.1	0.5
Taurine[9]	0.2	0.2
Vitamin mix[10]	1.0	1.0
Mineral mix[11]	1.0	1.0
Binder[12]	1.0	1.0
Choline chloride (50%)	0.5	0.5
Proximate analysis (% DM)		
Dry matter	93.6	93.0
Crude protein	51.9	42.2
Crude fat	17.5	17.4
Ash	6.0	5.4
Starch	9.8	17.4
Gross energy (kJ g^{-1})[13]	20.8	19.8

CH: Carbohydrates; CP: Crude protein; D: Diet; DM: Dry matter; GL: Gross lipid; P: Protein.

[1]Sorgal. S.A. Ovar. Portugal (CP: 73.5% DM; GL: 17.0% DM).

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

[3]Sorgal. S.A. Ovar. Portugal (CP: 54.3% DM; GL: 1.8% DM).

[4]Sorgal. S.A. Ovar. Portugal (CP: 70.0% DM; GL: 3.3% DM).

[5]Sorgal. S.A. Ovar. Portugal (CP: 84.2% DM; GL: 1.0% DM).

[6]Sorgal. S.A. Ovar. Portugal (CP: 13.8% DM; GL: 1.1% DM).

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

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

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

[10]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.

[11]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.

[12]Liptosa. Madrid. Spain.

[13]Gross energy calculated based on theoretical values (CP: 23.6 kJ g^{-1}; GL: 39.5 kJ g^{-1}; carbohydrates: 17.2 kJ g^{-1}): (23.6 × % dietary CP) + (39.5 × % dietary GL) + (17.2 × % dietary CH).

716 **Table 2**. Appetite regulation-related genes and primers used for qPCR.

Gene	ID primer	Sequence (5´- 3´)	[1]Accession nº	Tm (ºC)	Efficiency (%)
cholecystokinin	*cck*	F: CTGTGTACGAGCTGTTTGGGG R: AGCCGGAGGGAGAGCTTT	KP822925	60	90.5
cocaine- and amphetamine-regulated transcript	*cartpt*	F: CTGAGGAGCAAAGAGATGCCCTTAGAGAAA R: GCGTCACACGAAGGCAGCCA	MG570186	60	81.8
corticotropin-releasing hormone	*crh*	F: ATGGAGAGGGGAAGGAGGT R: ATCTTTGGCGGACTGGAAA	KC195964	60	85.3
ghrelin	*ghrl*	F: CCCGTCACAAAAACCTCAGAAC R: TTCAAAGGGGGCGCTTATTG	MG570187	60	98.7
ghrelin receptor-a	*ghsr-a*	F: GTCGGCGGCTGTGGCAAAGA R: GGCCAACACCACCACCACCAAC	MG570188	60	112.0
ghrelin receptor-b	*ghsr-b*	F: CGCACACGCATAACTTTGTC R: GAGGAGGATGAGCAGGTGAA	MG570189	60	114.2
leptin	*leptin*	F: TCTCTTCGCTGTCTGGATTCCTGGAT R: CTCCTTCTTGCTCTGTAGCTCTT	KP822924	60	104.3
leptin receptor	*lepr*	F: GGCGGAACTGATTCTACTCTG R: AGTATCGGACCTCGTATCTCA	MG570178	60	105.5
neuropeptide y	*npy*	F: AAACCGGAGAACCCCGGGGAGG R: CTGGACCTTTTTCCATACCTCTG	KP822926	60	78.8
Reference genes					
translation elongation factor	*ef1a*	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	AF184170	60	96.5
ribosomal protein S18	*rps18*	F: GGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACCA	AM490061.1	60	98.0

717 F: Forward; R: Reverse; Tm: Melting temperature. [1]from the GenBank database (https://www.ncbi.nlm.nih.gov/).

718 **Table 3.** Growth performance, feed intake, and feed utilization efficiency of gilthead seabream
719 fed the experimental diets at different feeding frequencies.

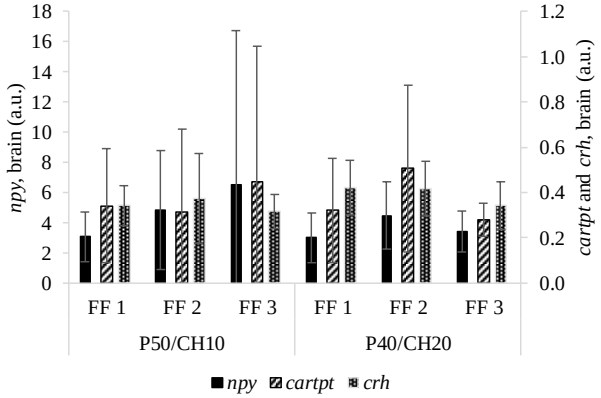
P/CH ratio	P50/CH10			P40/CH20		
FF	1	2	3	1	2	3
SGR (%)[1]	2.5 ± 0.0	2.8 ± 0.0	2.7 ± 0.1	2.4 ± 0.0	2.8 ± 0.2	2.7 ± 0.1
FI[2] (g kg ABW^{-1} day^{-1})	1.2 ± 0.0	1.5 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
FCR[3]	1.1 ± 0.0	1.2 ± 0.1	1.2 ± 0.0	1.2 ± 0.0	1.3 ± 0.0	1.3 ± 0.0

Two-way ANOVA

				Ratio P/CH		FF		
	P/CH	FF	I	P50/CH10	P40/CH20	1	2	3
SGR (%)[1]	ns	***	ns	-	-	a	b	b
FI[2] (g kg ABW^{-1} day^{-1})	**	***	ns	A	B	a	b	b
FCR[3]	***	***	ns	A	B	a	b	b

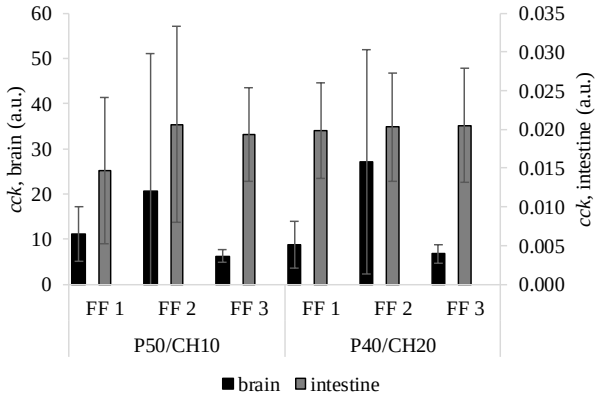
720 Values presented as means (n=3) and standard deviation. Different upper-case letters denote for significant
721 differences between dietary P/CH ratio and different lower-case letters denote for significant differences
722 between feeding frequencies.
723 ns: not significant; **P ≤ 0.01; ***P ≤ 0.001.
724 CH: Carbohydrates; FBW: Final body weight; FF: Feeding frequency; I: Interaction; P: Protein.
725 [1]Specific growth rate, SGR: [(ln (FBW) – ln (IBW))/time in days] × 100.
726 [2]Feed intake, FI (g kg ABW^{-1} day^{-1}): FI (kg $fish^{-1}$)/ABW/time in days.
727 Average body weight, ABW: (IBW + FBW)/2.
728 [3]Feed conversion ratio, FCR: dry FI/wet WG.
729

(a) *npy*, *cart* and *crh*



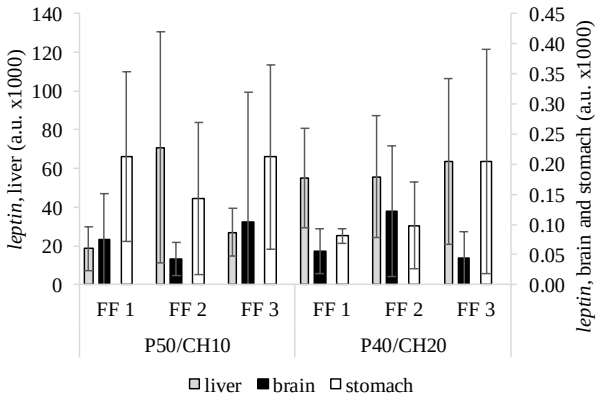
		FF	P/CH ratio	FF x P/CH
Two-way *p*-value	*npy*	0.164	0.607	0.890
	cartpt	0.718	0.329	0.172
	crh	0.340	0.117	0.794

(b) *cck*



		FF	P/CH ratio	FF x P/CH	† FF		
					1	2	3
Two-way *p*-value	*cck*, brain	≤ 0.01†	0.792	0.329	ab	b	a
	cck, intestine	0.514	0.410	0.631	-	-	-

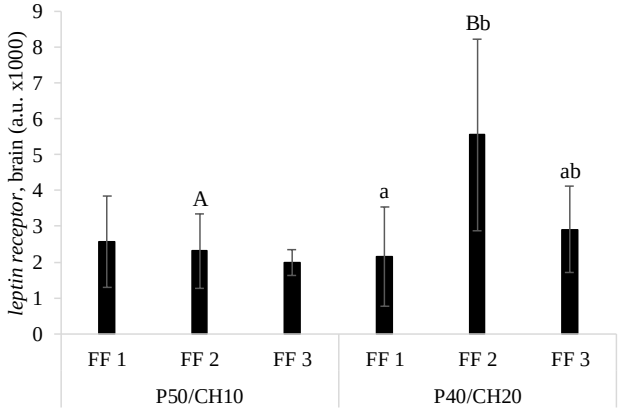
(c) *leptin*



		FF	P/CH ratio	FF x P/CH	‡ P/CH	
					50/10	40/20
Two-way *p*-value	*leptin*, liver	0.256	≤ 0.05‡	0.194	A	B
	leptin, brain	0.366	0.474	0.328	-	-
	leptin, stomach	0.347	0.181	0.764	-	-

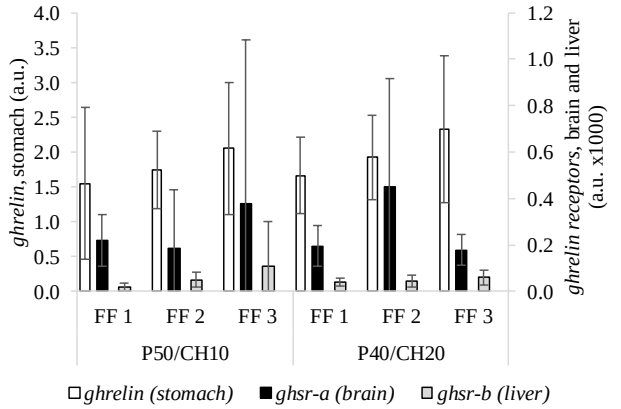
730

(d) *leptin receptor*



		FF	P/CH ratio	FF x P/CH
Two-way *p*-value	*leptin receptor*	≤ 0.05	≤ 0.05	≤ 0.05

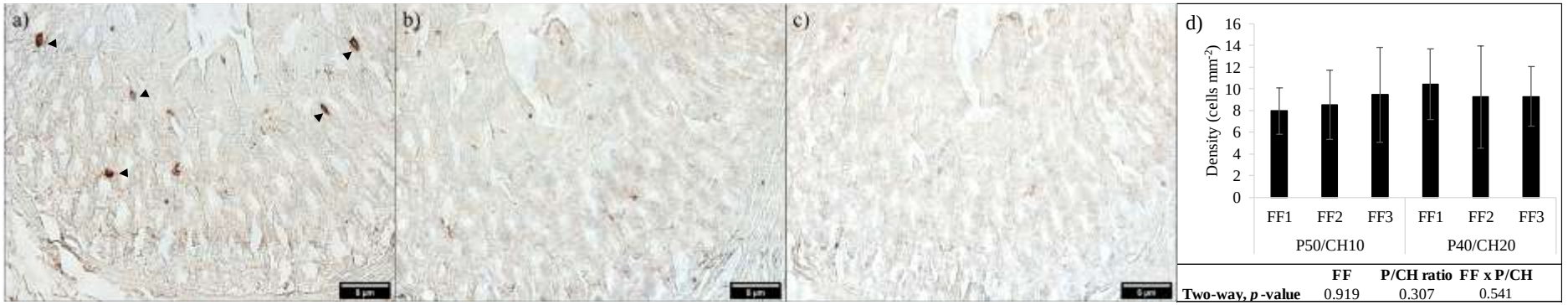
(e) *ghrelin* and their *receptors*



		FF	P/CH ratio	FF x P/CH	† FF		
					1	2	3
Two-way *p*-value	*ghrelin* , stomach	0.119	0.305	0.984	-	-	-
	ghsr-a , brain	0.934	0.283	0.124	-	-	-
	ghsr-b , liver	≤ 0.05†	0.168	0.158	a	ab	b

Fig. 1 Normalized appetite regulation-related genes expression of gilthead seabream fed the experimental diets at different feeding frequencies (FF). *cocaine- and amphetamine-regulated transcript* (*cartpt*), *corticotropin-releasing hormone* (*crh*) and *neuropeptide y* (*npy*) in the brain (a), *cholecystokinin* (*cck*) in the brain and intestine (b), *leptin* in the brain, liver, and stomach (c), *leptin receptor* in the brain (d), and *ghrelin* and their receptors (*ghsr-a* and *ghsr-b*) in the stomach, brain, and liver (e). Values presented as means (n=9) and standard deviation. † (FF) and ‡ (P/CH ratio) statistical significances are shown in the gray column in the tables. In case of interaction between FF and dietary P/CH ratio, one-way ANOVA was performed, and significant differences are indicated within the graph. Different lower-case letters denote significant differences between the FF, and upper-case letters denote significant differences between the dietary P/CH ratio, ($p \leq 0.05$). All values are expressed as arbitrary units (a.u.).

CH: carbohydrates; P: protein.



	FF	P/CH ratio	FF x P/CH
Two-way, *p*-value	0.919	0.307	0.541

Fig. 2 Representative immunopositive ghrelin cells (►) in the middle part of the stomach (a), negative control without primary antibody (b), negative control without secondary antibody (c), density of immunopositive ghrelin cells (cells mm^{-2}) in the stomach of gilthead seabream fed the experimental diets at different feeding frequencies (FF) (d). Images captured at 40× magnification from a gilthead seabream fed P50/CH10 diet, 2 meals per day. Values presented as means (n = 9) and standard deviation. No significant differences were found ($p > 0.05$) between the experimental conditions. CH: carbohydrate; P: protein.