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Feed composition and feeding frequency effects on gilthead seabream (*Sparus aurata*): focus on fish appetite regulation, metabolism, intestine functionality and health

Catarina Raquel Basto Correia da Silva

Tese de Doutoramento apresentada à
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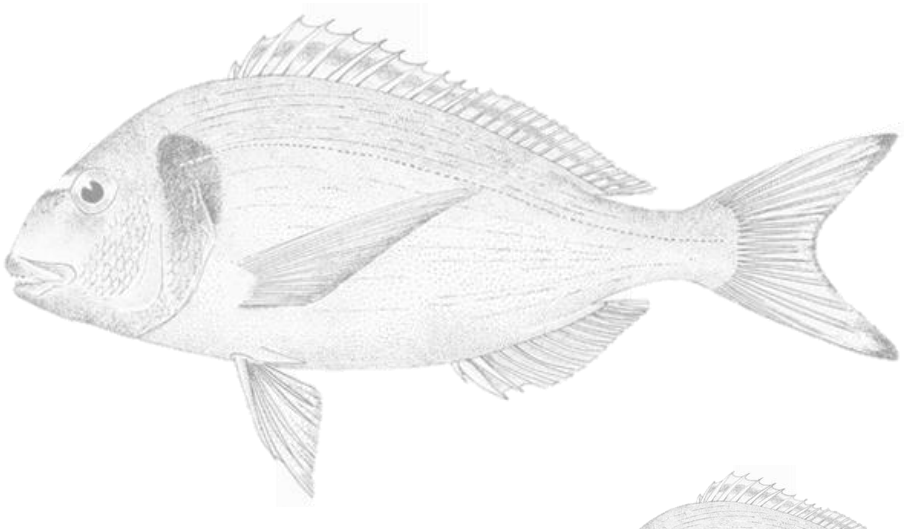

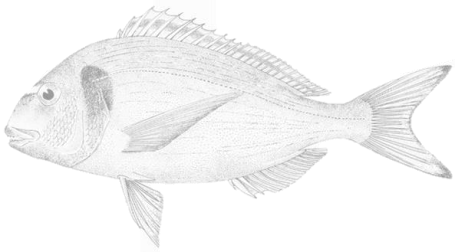

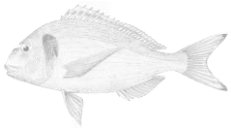

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Doutoramento em Biologia

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NOTA PRÉVIA

Dissertação apresentada à Faculdade de Ciências da Universidade do Porto para a obtenção do grau de Doutor em Biologia, no âmbito do programa doutoral em Biologia.

Orientador: Prof. Doutor Aires Oliva-Teles
Co-orientador: Doutora Inês Guerreiro
Co-orientador: Prof. Doutora Encarnación Capilla

Esta tese é composta por um conjunto coerente de trabalhos de investigação, já publicados ou submetidos a revistas de mérito internacional. Serve para clarificar que apesar de os artigos terem sido escritos em colaboração com outros autores, a candidata participou ativamente no desenho e trabalho experimental, obtenção, análise e discussão dos dados e por fim na preparação e publicação dos artigos.

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Em todas as publicações decorrentes deste trabalho é devidamente referido que as instituições de origem da doutoranda Catarina Raquel Basto Correia da Silva são:

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- Basto-Silva, C., Enes, P., Oliva-Teles, A., Balbuena-Pecino, S., Navarro, I., Capilla, E., Guerreiro, I., 2021. Dietary protein source and protein/carbohydrate ratio affects appetite regulation-related genes expression in gilthead seabream (*Sparus aurata*). Aquaculture 533, 736142. doi: 10.1016/j.aquaculture.2020.736142.
- Basto-Silva, C., Couto, A., Rodrigues, J., Oliva-Teles, A., Navarro, I., Kaiya, H., Capilla, E., Guerreiro, I., 2022. Feeding frequency and dietary protein/carbohydrate ratio affect feed intake and appetite regulation-related genes expression in gilthead seabream (*Sparus aurata*). Comp Biochem Physiol A Mol Integr Physiol 267,111168. doi: 10.1016/j.cbpa.2022.111168.
- Basto-Silva, C., Serra, C. R., Castro, C., Nóvoa, G. S., Oliva-Teles, A., Capilla, E., Guerreiro, I., 2022. Effects of feeding frequency and dietary protein/carbohydrate ratios on gilthead seabream (*Sparus aurata*) intestinal functionality and health. Aquaculture Nutrition, 8435786. doi: 10.1155/2022/8435786.
- Basto-Silva, C., García-Meilán, I., Couto, A., Serra, C., Enes, P., Oliva-Teles, A., Capilla, E., Guerreiro, I., 2022. Effect of dietary plant-feedstuffs and protein/carbohydrate ratio on gilthead seabream (*Sparus aurata*) gut health and functionality. Fishes 7, 59. doi: 10.3390/fishes7020059.
- Basto-Silva, C., Enes, P., Oliva-Teles, A., Capilla, E., Guerreiro, I., 2022. Dietary protein/carbohydrate ratio and feeding frequency affect feed utilization, intermediary metabolism, and economic efficiency of gilthead seabream (*Sparus*

aurata) juveniles. Aquaculture 554, 738182. doi: 10.1016/j.aquaculture.2022.738182.

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À minha família

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The text below is for my close friends and my family so I will be writing in Portuguese.

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ABSTRACT

Aquaculture is one of the industries with the highest growth rate among the animal production sectors. Within fed animal aquaculture, feed represents the major production costs. To reduce dietary costs, optimization of dietary composition and feeding frequency (FF) assume an important position ensuring aquaculture growth and sustainability. Fishmeal (FM) is considered the most adequate protein source for carnivorous fish. However, dietary FM inclusion needs to be reduced and replaced by more sustainable, available, and economic alternatives. Plant-feedstuffs (PF) have high market availability, a relative constant nutritional composition, and acceptable costs, and therefore are the most used alternative to FM. Although fish do not have dietary carbohydrate (CH) requirements, the provision of an appropriate amount of digestible CH in aquafeeds can spare the use of protein as an energy source. The use of diets including PF as an alternative to FM, and diets with different protein (P)/CH ratios have been already extensively explored in gilthead seabream (*Sparus aurata*), which is one of the most important marine fish species produced in Europe, but the integrated effects of these strategies are poorly explored. Another strategy to improve feed utilization and to ensure aquaculture sustainability is through FF optimization, which can improve fish growth, health, and welfare, as well as industrial economic profits. However, more knowledge is needed on the effects of FF manipulation in gilthead seabream and the possible interactive effects between FF and dietary composition.

The present thesis used a holistic approach to explore the above-mentioned strategies for improving feed utilization, including the evaluation of fish growth performance, feed intake (FI) and utilization, whole-body composition, histomorphology and immunohistochemistry (IHC) techniques, intestine microbiota characterization, digestive and oxidative stress-related enzymes activity, plasmatic metabolites, and expression of selected genes involved in some metabolic pathways, namely appetite regulation, intermediary metabolism, immunology, and oxidative stress.

Chapters 2 and 5 investigated the integrated effects of dietary protein sources (FM or PF) and dietary P/CH (P50/CH10 and P40/CH20) ratios on gilthead seabream (140 g) appetite regulation, intermediary metabolism, and intestinal functionality and health. The appetite regulation related-response focused on different fish tissues, namely adipose tissue, brain, intestine, liver, and stomach, while intermediary metabolism response was focused on the liver and adipose tissue. Additionally, short-time fasting effects on some

appetite regulation genes were also assessed by the comparison of the expression at 5 h and 24 h after feeding (AF).

Interactions between dietary protein source and dietary P/CH ratios were only observed in final body weight (FBW), hepatic lipid content, plasmatic glucose, proteolytic activity in the pyloric caeca (PC), and expression of *cholecystokinin* (*cck*) in the intestine (24 h AF), *growth hormone receptor* (*ghr*)-*i*, and *insulin-like growth factor-1* (*igf-1*). The remaining observed effects were due to protein source or dietary P/CH ratio independently of each other.

FM-based diets led to an increase of plasma cholesterol and total lipid level, α-amylase activity in the PC and intestine, expression of *cocaine- and amphetamine-regulated transcript* (*cart*) and *leptin* (24 h AF) in the brain, *ghr-ii* in the liver, and *glutathione reductase* (*gr*) and *glutathione peroxidase* in the intestine. While PF-based diets led to higher hepatic glycogen content, number and size of adipocytes, histomorphological alterations in the intestine, number of operational taxonomic units (OTUs), microbial richness and diversity indices in intestine mucosa, and expression of hepatic *leptin* (24 h AF), *fatty acid synthase* (*fas*), *glucokinase* (*gk*), and *target of rapamycin* (*mtor*).

Regarding dietary P/CH ratio, fish fed the P50/CH10 diets presented higher feed efficiency (FE), plasmatic triglycerides, α-amylase activity in the PC, expression of *cck* (5 h AF), *cyclooxygenase-2* (*cox2*), and *superoxide dismutase* (*sod*) in the intestine, and *ghrelin receptor* (*ghrr*)-*b* (24 h AF), *glutamate dehydrogenase* (*gdh*) and *ghr-ii* in the liver. Fish fed the P40/CH20 diets presented higher protein efficiency ratio (PER), hepatosomatic (HSI) and visceral indices (VSI), plasmatic glucose levels, and brain *leptin receptor* (*lepr*) expression (5 h AF). Moreover, dietary P/CH ratio had no relevant effects on intestine histomorphology nor in microbiota composition.

From the above results it seems that it can be concluded that in gilthead seabream, PF-based diets promoted a longer satiation feeling, enhanced lipogenesis, glycogenesis, and hypocholesterolemia, and affected intestine histomorphology and microbiota composition. On the other hand, lower dietary P/CH ratios seemed to promote a lower satiety feeling, inhibition of the amino acid (AA) catabolism, and an enhancement of lipogenesis.

The integrated effects of dietary P/CH ratios (P50/CH10 and P40/CH20) and FF (1, 2, or 3 meals per day) on gilthead seabream (9.1 g) juveniles appetite regulation, intermediary metabolism, and intestine functionality and health were evaluated in Chapters 3, 4, and

6. Interactions between dietary P/CH ratio and FF were only observed in plasmatic glucose, cholesterol, and total lipids levels, GR activity in the intestine, expression of *lepr* in the brain, and of *ghr-ii* and *igf-1* in the liver. The remaining effects observed due to dietary P/CH ratio or FF were independent of each other.

The P50/CH10 diets led to an increase of FE, α-amylase activity, and hepatic *gdh* expression, while P40/CH20 diets led to higher FI, PER, hepatic lipid and glycogen content, hepatocyte area covered by lipid vacuoles, number of OTUs, microbial richness and diversity indices in intestine mucosa, and expression of hepatic *leptin* and *gk* 5 h AF.

Regarding FF, fish fed more meals per day presented higher FI, FBW, and expression of *ghrr-b* in the liver, while fish fed only 1 meal per day presented higher FE, PER, plasmatic triglycerides and total protein levels, α-amylase activity, and expression of *ghr-i*, *gk*, and *fas* in the liver. Glucose-6-phosphate dehydrogenase and catalase activity in the intestine was lower in fish fed 2 meals per day in comparison with those fed 1 or 3 meals per day, respectively, and *cck* expression in the brain was higher in fish fed 2 meals than 3 meals per day.

From the above results, it seems that it can be concluded that lower dietary P/CH ratios promoted a lower satiation feeling and an enhancement of glycogenesis and glycolysis while reducing AA catabolism in gilthead seabream juveniles. A higher FF also promoted a lower satiation feeling, increased growth, and reduced glycolysis and lipogenesis pathways.

Overall, no consistent interactions were observed between the use of FM- or PF-based diets and dietary P/CH ratios, neither between P/CH ratios and the tested FF protocols on gilthead seabream appetite regulation, metabolism, and intestinal functionality and health. Thus, it seems that no potential beneficial interactive effects can be achieved by applying the two diet formulation strategies tested in this thesis. However, it might be concluded that diets with a lower P/CH ratio (P40/CH20 vs. P50/CH10) distributed in 2 meals per day seem to be a good strategy for this species since it did not compromise growth performance and only slightly affected appetite and metabolic parameters. Furthermore, PF-based diets should be used with caution to avoid abnormalities in the absorptive and digestive functions.

The present thesis also aimed to further improve the knowledge on appetite regulation mechanisms in gilthead seabream, particularly focusing on ghrelin and leptin functions. For the first time, immunopositive ghrelin cells were detected in the stomach of gilthead

seabream through an IHC technique (Chapter 3). The immunopositive ghrelin cells were small and round and were found mainly at the base of the gastric folds in the mucosal layer of the stomach.

The present thesis also aimed to further explore the effects of leptin and ghrelin in the adipogenic process using an *in vitro* approach (Chapter 7). Ghrelin was shown to decrease the expression of *peroxisome proliferator-activated receptor-γ* (*pparγ*) in the early differentiating phase of adipocytes but did not reduce intracellular lipid content. Leptin was shown to inhibit lipid accumulation and reduce the *pparγ* and *cluster of differentiation-36* (*cd36*) expression in early differentiating and mature adipocytes. Thus, leptin seems to have an anti-adipogenic function in differentiating preadipocytes of gilthead seabream and in mature adipocytes, but ghrelin did not seem to influence adipogenesis progression.

KEYWORDS

Anorexigenic/orexigenic hormones, Digestive enzymes, Endocrine regulation, Fishmeal, Ghrelin, Histomorphology, Immunohistochemistry, Immune status; Leptin, Microbiota, Oxidative stress, Plant-feedstuffs, Protein/carbohydrates ratio.

SUMÁRIO

A aquacultura é uma das indústrias com maior taxa de crescimento dentro do sector da produção animal. No entanto, neste sector, a alimentação dos animais representa a maioria dos custos produção. Para reduzir os custos com a alimentação, a otimização da composição da dieta e da frequência de alimentação (FA) assumem um papel importante, assegurando o crescimento e a sustentabilidade da indústria. A farinha de peixe (FP) ainda é considerada a fonte de proteína mais adequada para peixes carnívoros. Contudo, a inclusão de FP precisa de ser reduzida e substituída por alternativas mais sustentáveis, disponíveis e económicas. As matérias-primas vegetais (MPV) estão amplamente disponíveis no mercado a preços acessíveis, e têm uma composição nutricional relativamente constante, por isso são uma das alternativas mais usadas na substituição de FP. Outra opção, apesar de os peixes não precisarem de hidratos de carbono (HC) para o seu desenvolvimento, é o uso de uma quantidade apropriada de HC nas dietas, uma vez que estes podem ser usados como fonte de energia, poupando o uso de proteína exclusivamente para crescimento. Ambas as opções, quer o uso de MPV quer a inclusão de HC na dieta, e por isso a alteração do rácio de proteína (P)/HC das dietas, estão exploradas em dourada (*Sparus aurata*), uma das espécies marinhas mais importantes produzidas na Europa, mas os seus efeitos integrados permanecem pouco explorados. Outra forma de garantir a sustentabilidade e crescimento da aquacultura, pode ser pela otimização da FA, que pode melhorar o crescimento, a saúde e o bem-estar do animal, assim como aumentar o lucro económico para a indústria. Contudo, é necessário um maior conhecimento sobre efeitos da manipulação da FA na dourada, e possivelmente até um maior conhecimento sobre os efeitos desta manipulação em conjugação com a alteração da composição da dieta.

A presente tese usa uma abordagem holística para explorar as estratégias acima mencionadas. Esta abordagem inclui a performance de crescimento do peixe, a utilização de ração, metabolitos plasmáticos, técnicas de histomorfologia e imunohistoquímica, caracterização da microbiota, atividade enzimática de algumas enzimas relacionadas aos processos digestivos e stress oxidativo, e expressão génica de alguns genes envolvidos em diferentes vias metabólicas, tais como regulação do apetite, metabolismo intermediário, imunologia, e stress oxidativo.

Os Capítulos 2 e 5 investigaram os efeitos integrados do uso de diferentes fontes proteicas (FP ou MPV) e diferentes rácios de P/HC (P50/HC10 and P40/HC20) na regulação do apetite, metabolismo intermediário, e funcionalidade e saúde intestinal de

dourada (140 g). Para avaliar o mecanismo de regulação de apetite foram recolhidos diferentes tecidos do peixe, nomeadamente tecido adiposo, cérebro, intestino, fígado e estômago. Adicionalmente, também foram avaliados os efeitos do jejum de curta duração em alguns dos genes de regulação de apetite, pela análise da expressão génica às 5 e às 24 h após a alimentação.

No final, as interações entre a fonte proteica e os diferentes rácios de P/HC foram apenas observadas no peso corporal final (PCF), conteúdo lipídico do fígado, glucose plasmática, atividade proteolítica nos cecos pilóricos (CP), e expressão génica de *colecistoquinina* (*ccq*) no intestino (24 h após alimentação), *recetor da hormona de crescimento-i* (*rhc-i*), e *fator de crescimento semelhante à insulina tipo-1* (*fci-1*) no fígado. Os restantes efeitos observados foram devido à fonte proteica ou aos rácios de P/HC, de forma independe.

O uso de dietas à base de FP levou a um aumento do colesterol e dos níveis totais de lípidos plasmáticos, da atividade da α-amílase nos CP e intestino, e da expressão génica do *transcrito regulado por cocaína e anfetamina* (*trca*) e da *leptina* (24 h após alimentação) no cérebro, do *rhc-ii* no fígado, e da *glutationa redutase* (*gr*) e *glutationa peroxidase* (*gp*) no intestino. Já, as dietas à base de MPV promoveram um maior conteúdo de glicogénio no fígado, número e tamanho dos adipócitos, alterações histomorfológicas no intestino, número de unidades taxonómicas operacionais (UTOs), e índices de riqueza e diversidade da microbiota intestinal autóctone, e expressão de *leptina* (24 h após a alimentação), *ácido gordo sintase* (*ags*), *glucoquinase* (*gq*), e do *alvo mecanístico da rapamicina* (*amr*) no fígado.

Em relação aos rácios de P/HC da dieta, os peixes que consumiram a dieta P50/HC10 apresentaram maior eficiência alimentar (EA), triglicerídeos plasmáticos, atividade de α-amílase nos CP, e expressão génica de *ccq* (5 h após a alimentação), *ciclo-oxigenase-2* (*cox2*), e *superoxide dismutase* (*sod*) no intestino, e *recetor de grelina* (*rg*)-*b* (24 h após a alimentação), *glutamato desidrogenase* (*gdd*) e *rhc-ii* no fígado. Já os peixes que consumiram a dieta P40/HC20 apresentaram um maior rácio de eficiência proteica (REP), índices hépato-somático (IHS) e visceral (IVS), níveis de glucose plasmática, e expressão do gene *receptor de leptina* (*rl*) (5 h após a alimentação) no cérebro. Para além disto, os rácios de P/HC não tiveram efeitos relevantes, nem na histomorfologia de intestino nem na composição da microbiota.

Assim, de acordo com os resultados em cima parece que, as dietas à base de MPV promoveram uma sensação de saciedade mais longa, um aumento da lipogénese, da

glicogénese, e da hipocolesterolemia, e afetaram significativamente a aparência histomorfológica do intestino e a composição da microbiota da dourada. Por outro lado, rácios de P/HC mais baixos, pareceram promover uma menor sensação de saciedade, uma inibição do catabolismo de aminoácidos (AA), e um aumento da lipogénese.

Os efeitos integrados dos rácios de P/HC da dieta (P50/HC10 and P40/HC20) e FA (1, 2, ou 3 refeições por dia) na regulação de apetite, metabolismo intermediário, e funcionalidade e saúde intestinal de dourada (9.1 g) foram avaliados nos Capítulos 3, 4, e 6. Interações entre os rácios de P/HC e a FA só foram observadas na glucose plasmática, no colesterol plasmático, nos lípidos totais plasmáticos, na atividade intestinal da gr, e na expressão génica do *rl* no cérebro, e do *rhc-ii* e do *fci-1* no fígado. Os restantes efeitos observados foram devido aos rácios de P/HC da dieta ou à FA, de forma independe.

As dietas P50/HC10 levaram a um aumento da EA, atividade da α-amílase, e expressão hepática do gene *gdd*. Enquanto, que as dietas P40/HC20 levaram a um maior consumo de ração (CR), REP, conteúdo hepático de lípidos e glicogénio, área coberta por vacúolos lipídicos no fígado, número de UTOs, índice de riqueza e diversidade na microbiota intestinal autóctone, e expressão hepática de *leptina* e *gq*, 5 h após a alimentação.

Em relação à FA, os peixes que comeram mais refeições por dia apresentaram maior CR, PCF, e expressão dos gene *rg-b* no fígado, 5 h após a alimentação. Enquanto que os peixes que comeram apenas 1 refeição por dia apresentaram maior EA, REP, triglicerídeos e níveis totais de proteína plasmática, atividade de α-amílase, e expressão dos genes *rhc-i*, *gq*, e *ags* no fígado. A atividade da glucose-6-fosfato desidrogenase e da catálase no intestino também foi maior em peixes que comeram 2 refeições por dia, em comparação com aqueles que apenas 1 ou 3 refeições por dia, respetivamente, e a expressão do gene *ccq* no cérebro também foi maior em peixes que comeram 2 refeições por dia do que aqueles que consumiram 3 refeições por dia.

Através dos resultados acima, concluiu-se que rácios de P/HC mais baixos promoveram uma menor sensação de saciedade e um aumento da glicogénese e da glicólise, enquanto que o catabolismo de AA foi reduzido. Um aumento da FA também pareceu promover uma menor sensação de saciedade, um aumento do crescimento, e uma redução da glicólise e da lipogénese.

No geral, não existiram interações consistentes entre o uso das diferentes fontes proteicas (FP ou MPV) e os rácios de P/HC diatéticos, nem entre os rácios P/HC diatéticos e os protocolos de FA testados na regulação do apetite, metabolismo intermediário, e funcionalidade e saúde intestinal de dourada. Por isso não foi possível retirar nenhuma conclusão sobre o potencial efeito interativo entre estes fatores. Contudo, a presente tese concluí que as dietas com menor rácio de P/HC (P40/HC20) distribuídas em 2 refeições por dia podem ser a melhor estratégia para a espécie, uma vez que não existiu nenhum comprometimento do crescimento e apenas alguns parâmetros do apetite e do metabolismo foram ligeiramente afetados. Já as dietas à base de MPV devem ser usadas com precaução para evitar anomalias nas funções digestivas e absortivas.

A presente tese também teve o objetivo de melhorar o conhecimento sobre o mecanismo de regulação do apetite na dourada, focando particularmente as funções da grelina e da leptina. Assim, foram detetadas, pela primeira vez, células imunpositivas de grelina no estômago de dourada através de uma técnica de imunohistoquímica (Capítulo 3). As células imunopositivas de grelina apresentaram-se pequenas e com uma forma redonda, e foram encontradas principalmente na base das vilosidades gástricas da camada mucosa do estômago.

A presente tese também explorou os efeitos da leptina e da grelina no processo adipogénico da dourada, usando uma abordagem *in vitro* (Capítulo 7). A grelina promoveu uma diminuição da expressão de *recetor ativado por proliferadores de peroxissoma-γ* (*rapp-γ*) na fase inicial de diferenciação dos adipócitos, mas não influenciou o conteúdo lipídico intracelular. Enquanto a leptina inibiu a acumulação de lípidos e reduziu a expressão do *rapp-γ* e do *cluster de diferenciação-36* (*cd36*), tanto na fase inicial de diferenciação como nos adipócitos maduros. Assim, a leptina pareceu ter uma função anti-adipogénica quer na diferenciação de pré-adipócitos quer nos adipócitos maduros de dourada, mas a grelina não pareceu influenciar a progressão da adipogénese.

PALAVRAS-CHAVE

Enzimas digestivas, Estado imune, Farinha de peixe, Grelina, Histomorfologia, Hormonas anorexigénicas/orexigénicas, Imunohistoquímica, Ingredientes vegetais, Leptina, Microbiota, Rácio proteína/hidrato de carbono, Regulação endócrina, Stress oxidativo.

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ABBREVIATIONS LIST

AA	Amino acids
AF	After feeding
ANF	Antinutritional factors
BW	Body weight
CART	Cocaine-amphetamine-related transcript
CAT	Catalase
CCK	Cholecystokinin
CH	Carbohydrates
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
EPA/DHA	Eicosapentaenoic acid/docosahexaenoic acid
FAS	Fatty acid synthase
FBW	Final body weight
FE	Feed efficiency
FF	Feeding frequency
FI	Feed intake
FM	Fishmeal
FO	Fish oil
G6Pase	Glucose-6-phosphatase
G6PD	Glucose-6-phosphate dehydrogenase
GDH	Glutamate dehydrogenase
GH	Growth hormone
GI	Gastrointestinal tract
GHRR	Ghrelin receptor
GK	Glucokinase
GPX	Glutathione peroxidase
GR	Glutathione reductase
HSI	Hepatosomatic index
HUFA	n-3 highly unsaturated fatty acid
IBW	Initial body weight
ICV	Intracerebroventricular
IGF	Insulin-like growth factor
IL1β	Interleukin 1β
IP	Intraperitoneal

LEPR	Leptin receptor
LPL	Lipoprotein lipase
mTOR	Mechanistic target of rapamycin
NPY	Neuropeptide y
OTUs	Operational taxonomic units
P	Protein
PC	Pyloric caeca
PER	Protein efficiency ratio
PF	Plant feedstuffs
PPAR	Peroxisome proliferator- activated receptor
SOD	Superoxide dismutase
TG	Triglycerides
VSI	Visceral somatic index

CHAPTER 1| GENERAL INTRODUCTION

1.1. Global aquaculture production

It is estimated that by 2037 nine billion people in the world will need to be fed (Worldometer 2021). Fish is a good candidate to fulfill animal protein needs since it is easily digested; is rich in essential amino acids (AA); is rich in vitamins and minerals, such as vitamin D and A, calcium, iodine, zinc, iron, and selenium; and is rich in omega-3 fatty acids. In addition, a healthy diet might also prevent some diseases of the 21st century, such as obesity, cardiovascular diseases, and malformations of the nervous system during fetal and infant development (FAO 2020). In 2017, fish accounted for about 17% of the total animal protein and 7% of all proteins consumed globally (FAO 2020).

Aquaculture had an annual average growth of 6% between 1990 and 2020, having the biggest annual growth rate compared with the other livestock industries, such as beef, veal, pig, poultry, and sheep (**Figure 1**). In 2020, aquaculture represented 48% of total fish production in the world, while fisheries completed the remaining 52% (FAO 2020; FIGIS 2021a; b). The majority of the global fish production was used for human consumption (87%), while the remaining production was intended for non-food uses, mainly to produce fishmeal (FM) and fish oil (FO) (FAO 2020). However, as capture fisheries have not been able to keep up with population growth over the past two decades, aquaculture will be probably the only real solution to supply the increase of global market needs (Tacon and Metian 2018).

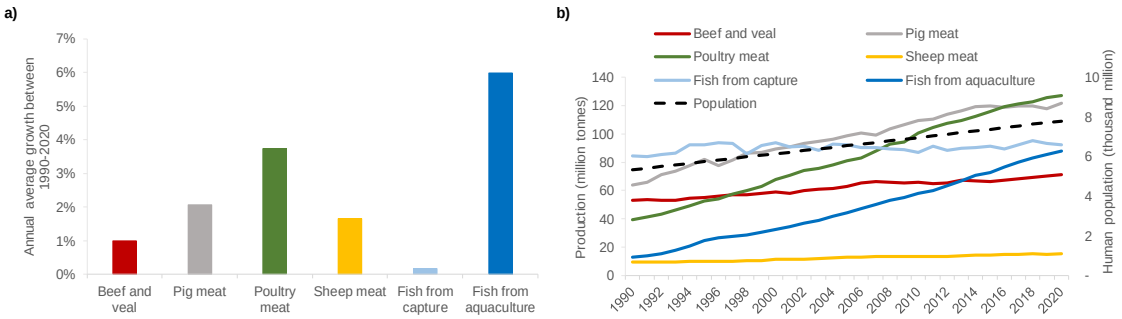

Figure 1. (a) Annual average growth of livestock between 1990 and 2020. (b) world livestock production and human population between 1990 and 2020. Data was collected from OECD-FAO (2021) and Worldometer (2021).

In 2019, Europe contributed with only 3% for the world aquaculture production, being Asia the highest producer, mainly due to China's production volumes (FAO 2020; FIGIS 2021a; b). Regarding the economic value, European aquaculture generated 6% of all economic value, being the 3rd major contributor, behind China and America (FIGIS 2021a). The majority of the companies (80%) in the European aquaculture sector between 2017-2018 were micro-companies, with less than 10 employees, usually family-owned and using extensive production systems (Nielsen et al. 2021).

In 2019, diadromous fishes represented 64% of European aquaculture production and were the ones that generated greater economic value, contributing 76% of the total value (FIGIS 2021a). Marine fishes were the 2nd most important group producing value, representing 10% of the total economic value generated (FIGIS 2021a). The main fish species produced in Europe regarding economic value were Atlantic salmon (*Salmo salar,* representing 71%), rainbow trout (*Oncorhynchus mykiss*, 11%), gilthead seabream (*Sparus aurata*, 4%), and European seabass (*Dicentrarchus labrax*, 4%), while concerning total quantity produced were Atlantic salmon (67%), rainbow trout (13%), common carp (*Cyprinus carpio*, 7%), and gilthead seabream (4%) (FIGIS 2021a).

In the European context, in 2019 Portugal occupied the 21st position by weight and the 15th position by value, producing 13 691 tonnes and making 115 045 USD of value. Moreover, between 1965 and 2018, the annual growth rate of Portuguese aquaculture was 12%, reflecting a positive and progressive evolution (FIGIS 2021a). Following the European trend, in 2018 the Portuguese aquaculture sector was dominated by small companies (96%) with less than 5 employees. Indeed, the aquaculture sector in Portugal comprised 846 companies with 1 652 employees, of which 348 were women and 1 304 were men, in a proportion of 1:4 (Nielsen et al. 2021). The main aquaculture production companies in Portugal are located in the central and south areas and produce oysters, mussels, and clams, using mainly long lines systems in estuaries areas and coastal lagoons. The second most important segment is the marine production of turbot (*Psetta maxima*) and Senegalensis sole (*Solea senegalensis*) in tanks and recirculation aquatic systems, in the central region of Portugal. Other marine fishes, as European seabass and gilthead seabream, are produced in ponds and cages located both near the coast or in the open sea, in the central and south region of Portugal, and also in the Autonomous Region of Madeira (Nielsen et al. 2021).

1.1.1. Aquafeeds – Fish meal vs Plant feedstuffs

One of the major concerns of modern aquaculture is the formulation of compound feeds (Edwards 2015). FM and FO are highly digestible and have good palatability (Oliva-Teles et al. 2015) and, due to their nutritional composition, they are considered the most adequate protein and lipid sources to be used in aquaculture, mainly for carnivorous fish (Tacon and Metian 2008; 2015). In 2019, approximately 78% of FM and 68% of FO production worldwide were used in aquafeeds. Marine fishes were the third higher users of FM, consuming 17% of overall production allocated to aquaculture, just after crustaceans and freshwater species with respectively 25% and 21% of consumption. While, regarding FO, marine fishes were the second higher consumers, just after salmonids, with 17% and 71% consumption respectively (EUMOFA 2021). Nonetheless, FM and FO inclusion on aquaculture diets decreased in the last years due to: (i) reduction and/or stagnation of wild fisheries stocks available for FM and FO production; (ii) increase of small pelagic fish prices, due to increased fishing costs and high fish demand for direct human consumption; (iii) increase of FM and FO prices in the global market; (iv) increased market and social pressure on feed manufactures to replace FM and FO on aquafeeds by more sustainable alternatives (Tacon and Metian 2008; Olsen and Hasan 2012; Naylor et al. 2021). These constraints lead to an increased research effort to find alternative protein and lipid sources to the use of FM and FO for aquafeeds (Olsen and Hasan 2012).

Plant feedstuffs (PF) are highly available on the market and have also a relatively constant chemical composition (Enes et al. 2011). Hence, over the past 20 years, they have been studied as feasible alternatives to FM on aquafeeds for several fish species (Carter and Hauler 2000; Lee et al. 2002; Fournier et al. 2004; Kaushik et al. 2004; Kissil and Lupatsch 2004; Hansen et al. 2007; Dias et al. 2009; Estévez et al. 2011; Cabral et al. 2013; Monge-Ortiz et al. 2016; Niu et al. 2016; Hua et al. 2019; Naylor et al. 2021). However, PF have some disadvantageous characteristics, as the presence of antinutritional factors (ANF), lower nutrient digestibility, and lower palatability (Francis et al. 2001; Hua et al. 2019; Glencross et al. 2020; Naylor et al. 2021). These characteristics seem to affect intestine morphology, microbiota composition, absorptive and digestive processes, and the immune and oxidative status of fish, mainly carnivorous species (Sitjà-Bobadilla et al. 2005; Bonaldo et al. 2008; Santigosa et al. 2008; Green et al. 2013; Estruch et al. 2015; Batista et al. 2016; Estruch et al. 2018; Miao et al. 2018; Naylor et al. 2021). Efforts have been made to overcome some of the undesirable characteristics present in PF, such as the use of biotechnology processes to surpass the ANF problems,

use of attractants to enhance diet palatability, or use functional ingredients to improve immune status, reduce oxidative stress and enhance disease resistance (Dias et al. 1997; Francis et al. 2001; Guerreiro et al. 2015; Jiang et al. 2016; Niu et al. 2016; Hua et al. 2019; Glencross et al. 2020; Naylor et al. 2021). However, PF effects seem to be species-specific and dependent on several factors, as age, health status, selective breeding, and/or dietary macronutrients balance (Tocher et al. 2003; Figueiredo-Silva et al. 2010; Le Boucher et al. 2011; Oliva-Teles 2012; Bonacic et al. 2017; Castro et al. 2019).

1.1.2. Aquafeeds – carbohydrates inclusion level

It is well-known that fish do not have carbohydrates (CH) requirements since they efficiently synthesize glucose through gluconeogenesis, especially using AA as glucose precursors (NRC 2011). However, the supply of an appropriate amount of digestible CH in aquafeeds has some advantages, such as sparing the use of protein as an energy source; reducing dietary costs; improving pellet binding, stability, and floatability; reducing nitrogen load in effluent discharges; and providing bulk, therefore facilitating feces evacuation (NRC 2011; Kamalam et al. 2017). Thus, several studies were performed on the potential of CH to spare protein for plastic purposes, and define the best dietary protein (P)/CH ratio in several fish species (Shiau and Lan 1996; Sanz et al. 2000; Lupatsch et al. 2001; Azevedo et al. 2002; Kim et al. 2004; Grisdale-Helland et al. 2008; Ye et al. 2009; Webb et al. 2010; Li et al. 2012; García-Meilán et al. 2013).

CH digestibility depends on the molecule composition, processing technology applied, and dietary inclusion level (NRC 2011). Generally, the apparent digestibility coefficient of CH decreases with the increasing complexity of the molecule (glucose>dextrin>starch) (Enes et al. 2010; NRC 2011; Kamalam et al. 2017). Nonetheless, fish CH utilization is also affected by biological (as fish trophic level and genetic characteristics), environmental (as stress and temperature), and nutritional (as dietary inclusion level and interaction with other nutrients) factors (Kamalam et al. 2017). For instance, herbivorous and omnivorous species can successfully use diets with up to 50% of dietary CH inclusion, while for carnivorous fishes the maximum recommended level of dietary CH is 15-25% (Kamalam et al. 2017). When higher levels are used, fish growth, intermediary metabolism, digestive and absorptive capacity, and immune and oxidative status could be compromised (Couto et al. 2008; Pérez-Jiménez et al. 2009;

Enes et al. 2011; Castro et al. 2012; Figueiredo-Silva et al. 2012; Coutinho et al. 2016; Ma et al. 2019; García-Meilán et al. 2020; Tian et al. 2020).

As dietary CH utilization is fish species-dependent, effects of its dietary inclusion level and potential interactions with PF on growth, appetite regulation, metabolism, and intestine functionality and immune status will be further ahead discussed.

1.1.3. Feeding frequency – a strategy to a sustainable aquaculture

Feed represents about 50-70% of the total variable production costs in commercial aquaculture (Rana et al. 2009; White 2013). Hence, feeding frequency (FF) optimization is crucial for a more sustainable and profitable industry, avoiding dietary losses and environmental pollution and promoting fish growth (Aderolu et al. 2010; Amirkolaie 2011; White 2013).

The effect of FF on appetite regulation, growth, feed utilization, metabolism, and intestine functionality and health was already evaluated in several fish species (Dwyer et al. 2002; Seo and Lee 2008; Küçük et al. 2014; Li et al. 2014; Guo et al. 2018; Oh et al. 2018; Busti et al. 2020; Sherif et al. 2020; Silva et al. 2020; Gilannejad et al. 2021; Pham et al. 2021), and its optimization seems to be species-specific. For instance, in Korean rockfish (*Sebastes schlegeli*), one meal per day is the recommended FF (Seo and Lee 2008) while in yellowtail flounder (*Limanda ferruginea*), dark-banded rockfish (*Sebastes inermis*), and flounder (*Platichthys flesus luscus*) the recommended FF are two meals per day (Dwyer et al. 2002; Küçük et al. 2014; Oh et al. 2018). Differently, for blunt snout bream (*Megalobrama amblycephala*) and dolly varden char (*Salvelinus malma*) the recommendation is 4 meals per day (Li et al. 2014; Guo et al. 2018), and for Lebranche mullet (*Mugil liza*) is between 3 to 5 meals per day (Silva et al. 2020). Regarding gilthead seabream, recent studies concluded that regardless of the number of meals (1, 2, or 3 meals per day) no significant changes were noticed in growth, feed utilization, plasmatic metabolites, and in the activity of digestive enzymes (Busti et al. 2020; Gilannejad et al. 2021).

FF might also affect dietary CH utilization, since in hybrid tilapia (*Oreochromis niloticus x O. aureus*) and rainbow trout dietary CH utilization was improved when FF was optimized, leading to improved feed utilization and growth performance (Tung and Shiau 1991; Hung and Storebakken 1994; Lin et al. 1997). However, in white seabream (*Diplodus sargus*), white sturgeon (*Acipenser transmontanus*), Korean rockfish, and

common carp, no interactions were observed between dietary P/CH ratio and FF on growth performance, feed utilization, and CH metabolism (Lin et al. 1997; Seo and Lee 2008; Enes et al. 2015; Cheng et al. 2019).

1.2. Appetite regulation mechanisms

Vertebrates' survival and growth depend on the balance between energy intake and energy expenditure (Volkoff 2011). Under this balance, the endocrine system assumes great importance in feed intake (FI) regulation by secreting hormones and regulating the activity of cells by transferring information between organs (Bertucci et al. 2019). At normal conditions, when energy intake exceeds expenditure, anorexigenic signals are produced inhibiting fish appetite; and when energy expenditure exceeds intake, orexigenic signals are produced inducing fish appetite, as described in **Figure 2** (Volkoff 2011). This appetite regulation is reached through a circular pathway where the feeding center areas in the hypothalamus receive and send both orexigenic and anorexigenic signals from/to peripheral organs (Le Bail and Boeuf 1997; Volkoff 2011). Overall, neural information circulates through the vagus nerve, and endocrine (e.g., hormones) and chemical (e.g., glucose) signals are released into the bloodstream.

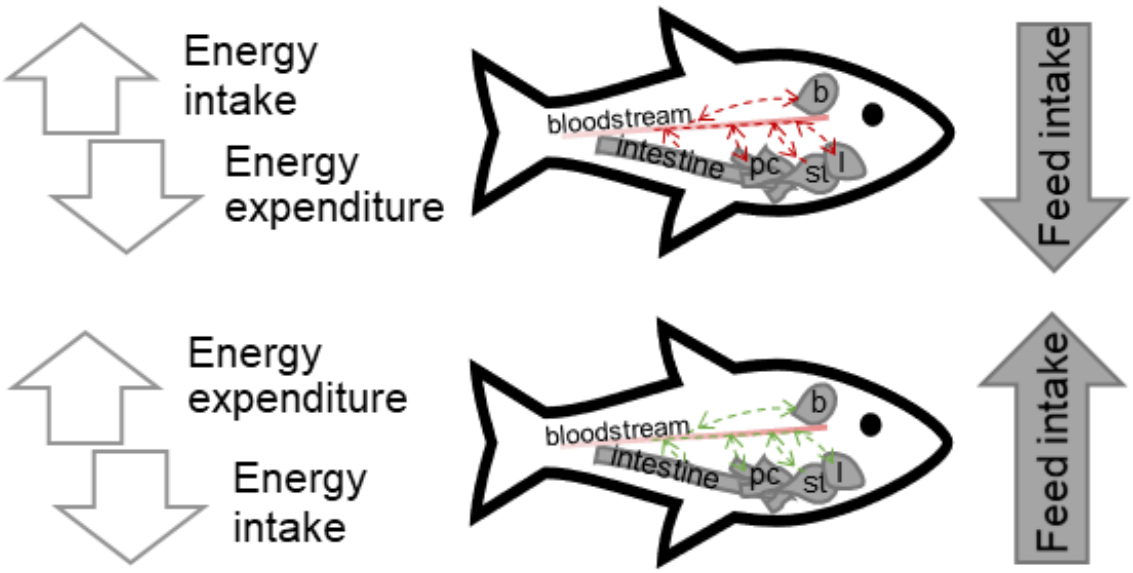

Figure 2. Simplified scheme of appetite regulation. Appetite is controlled by the brain, which integrates information on nutritional status relayed by the blood from/to the peripheral organs. When energy intake exceeds expenditure, anorexigenic signals are produced, and fish appetite is inhibited, and when energy expenditure exceeds intake, orexigenic signals are produced, inducing fish appetite. Anorexigenic signals are marked as red color and orexigenic as green. b: brain; l: liver; pc: pyloric caeca; st: stomach.

Fish are the most diversified group of vertebrates, with 34 300 species identified so far (FishBase 2020). Although the basic mechanisms of appetite regulation appear to be relatively well conserved between mammals and fish (Volkoff 2016), some of the appetite-related hormones seem to have a species-specific function. **Table 1** summarizes available data for several fish species of the effects on FI of intracerebroventricular (icv) and intraperitoneal (ip) injections or oral administration of some appetite-regulating hormones. Fasting effects on gene expression of the main hormones involved in fish appetite regulation mechanisms in different tissues and species are presented in **Table 2**. Hereafter, some of the main hormones involved in fish appetite regulation mechanisms are briefly characterized, with leptin and ghrelin being presented in more detail as they are the two hormones which received the greatest focus in this thesis.

Cocaine-amphetamine-related transcript (cart) was characterized for the first time in fish by Volkoff and Peter (2000). This neuropeptide is composed of ~100 AA, and in some fish species, such as in goldfish (*Carassius auratus*), appears to have two isoforms (Volkoff and Peter 2001). However, independently of the isoform found, cart is mainly expressed in the brain and also to a lesser extent in some peripheral organs, such as gonads and kidney (Volkoff and Peter 2001; MacDonald and Volkoff 2009a; b; Murashita et al. 2009a; Babichuk and Volkoff 2013; Gomes et al. 2015; Volkoff et al. 2016; Pitts and Volkoff 2017; Volkoff et al. 2017). This widespread distribution might suggest that cart has several physiological roles in fish, besides being involved in FI regulation.

After icv injections in goldfish, cart seemed to have a potent satiety role and to inhibit neuropeptide y (npy) and orexin-a signals (Volkoff and Peter 2000; 2001). However, cart answers to short- or long-term fasting periods seem to be species-specific. In channel catfish (*Ictalurus punctatus*), *cart* gene expression in the brain increased at 1, 2, and 4 h after feeding (AF) (Peterson et al. 2012) but decreased after 30 days of fasting (Kobayashi et al. 2008), confirming cart anorexigenic role either during short- and long-term fasting. However, in dorado (*Salminus brasiliensis*), this anorexigenic role of cart was only confirmed in short-fasting (1 h AF), and not in longer-term fasting (5 days) where no effects on *cart* expression in the brain were reported (Volkoff et al. 2016). Similarly, unaffected *cart* expression was reported in cunner (*Tautogolabrus adspersus*) after 1, 2, or 3 weeks of fasting (Babichuk and Volkoff 2013), and in winter skate (*Raja*

ocellata) after 2 weeks of fasting (MacDonald and Volkoff 2009b). However, opposite effects seemed to be true for Atlantic cod (*Gadus morhua*) and pacu (*Piaractus mesopotamicus*) since *cart* expression in the brain was lower after 7 days of fasting but was not affected by short-term fasting of up to 22 h (Kehoe and Volkoff 2007; Volkoff et al. 2017). Long-term fasting of 6, 7, or 10 days also decreased *cart* expression in the brain of Atlantic salmon, red-bellied piranha (*Pygocentrus nattereri*), and platyfish (*Xiphophorus maculatus*), respectively, evidencing the anorexigenic role of this peptide (Murashita et al. 2009a; Volkoff 2014; Pitts and Volkoff 2017). Somehow unexpectedly, in Siberian sturgeon (*Acipenser baerii*) cart in the brain seems to act as a satiety signal in short-term fasting (24 h AF), but to act as a starvation signal after long-term fasting (3 and 15 days) (Zhang et al. 2018). Long-term fasting effects on c*art* expression were also measured in the intestine of platyfish, but no effects were found after 10 of fasting (Pitts and Volkoff 2017).

Moreover, cart regulation also seems to be influenced by temperature (Kehoe and Volkoff 2008) and dietary composition (Li et al. 2017a). The effects of dietary composition on *cart* expression will be discussed later.

Cholecystokinin (cck) was first related to the digestive function since it promotes the release of pancreatic enzymes, as trypsin or chymotrypsin, and the gallbladder contraction (Aldman et al. 1992; Einarsson et al. 1997). Only later it was demonstrated the role of cck in appetite regulation in fish (Himick and Peter 1994). This peptide is composed of ~120 AA, and its mRNA sequence has been described for several fish species (Murashita et al. 2006; MacDonald and Volkoff 2009a; b; Murashita et al. 2009b; Babichuk and Volkoff 2013; Yuan et al. 2014; Volkoff et al. 2016; Pitts and Volkoff 2017; Volkoff et al. 2017). Some of these fish seem to have two cck isoforms, as channel catfish and Atlantic salmon, confirming the multi-function of this hormone (Murashita et al. 2009b; Peterson et al. 2012). Independently of the isoforms, cck is mainly expressed in the brain and the digestive tract of fish (Murashita et al. 2006; MacDonald and Volkoff 2009a; b; Murashita et al. 2009b; Babichuk and Volkoff 2013; Yuan et al. 2014; Volkoff et al. 2016; Pitts and Volkoff 2017; Volkoff et al. 2017).

Concerning the appetite regulation function of cck, studies involving icv and ip injections pointed to an anorexigenic role of this peptide, inhibiting appetite in several fish species, such as goldfish, cavefish (*Astyanax fasciatus mexicanus*), coho salmon (*Oncorhynchus kisutch*), and platyfish (Himick and Peter 1994; Volkoff et al. 2003; Kang et al. 2010;

Penney and Volkoff 2014; White et al. 2016; Pitts and Volkoff 2017). Further, in coho salmon, the decrease of FI after cck ip injections was observed together with a decrease of swimming activity and foraging behavior and an increase of spitting behavior, which is consistent with rapid satiety feeling promoted by cck (White et al. 2016). Similar evidence was observed in platyfish, where feed searching also decreased after ip injections with cck (Pitts and Volkoff 2017).

Nevertheless, the anorexigenic responses to short- and long-term fasting were not similar for all fish species studied nor in all tissues of the same species. For instance, in *Schizothorax prenanti*, cck seems to have an anorexigenic role both in short- and long-term fasting and both in brain and intestine, since its expression increased at 1 or 3 h AF, and decreased at 1, 3, 5, and 7 days of fasting (Yuan et al. 2014). However, in the dorado brain and channel catfish and yellowtail (*Seriola quinqueradiata*) intestine, cck only seemed to respond to short-term fasting, since it promoted an increase of *cck* expression only at 3 or 4 h AF, being unaffected by long-term fasting of 23 h, 3 or 5 days (Murashita et al. 2006; Peterson et al. 2012; Volkoff et al. 2016). Differently, in the pacu intestine, *cck* gene expression was not affected during the postprandial period but decreased after 7 days of fasting (Volkoff et al. 2017).

The majority of the studies available on *cck* expression focus on long-term fasting effects. For instance, in cunner, 1, 2, or 3 weeks of fasting seemed to decrease *cck* expression in the brain (Babichuk and Volkoff 2013). Similar results were observed in the brain and intestine of blunt snout bream, grass carp (*Ctenopharyngodon idella*), and platyfish up to 15 days of fasting (Feng et al. 2012; Ji et al. 2015; Pitts and Volkoff 2017). In Atlantic salmon, 6 days of fasting promoted a decrease of the *cck* expression in the brain but not in the intestine (Murashita et al. 2009b) while in yellowtail, 3 days or 2 weeks of fasting did not affect *cck* expression either in the brain or intestine (Murashita et al. 2006; Hosomi et al. 2014), and similar results were observed in red-bellied piranha after 7 days of fasting (Volkoff 2014). Contrary to what was expected, in winter skate, intestinal cck seemed to have an orexigenic role since its expression increased after 2 weeks of fasting (MacDonald and Volkoff 2009b). Recently, Babaei et al. (2017) observed that 23 days of fasting did not affect *cck* expression in the gilthead seabream intestine.

cck expression also seems to be affected by external factors, such as season (MacDonald and Volkoff 2009a) and dietary composition (Hevrøy et al. 2008; Van Nguyen et al. 2013; Babaei et al. 2017; Li et al. 2017a; Volkoff et al. 2017). The influence of dietary composition on *cck* expression will be further explored in the present thesis.

Corticotropin-releasing hormone (crh) or corticotropin-releasing factor (crf)-related peptide was first discovered in fish by Okawara et al. (1988). This hormone is composed of ~160 AA, is expressed mainly in the brain, and can present one or two isoforms, depending on fish species (Okawara et al. 1988; Ando et al. 1999; Van Enckevort et al. 2000; Doyon et al. 2003; Huising et al. 2004; Chandrasekar et al. 2007; Martos-Sitcha et al. 2014; Wang et al. 2014). For instance, in white sucker (*Catostomus commersonii*), *S. prenanti*, Mozambique tilapia (*Oreochromis mossambicus*), zebrafish (*Danio rerio*), and gilthead seabream was found only one crh isoform (Okawara et al. 1988; Van Enckevort et al. 2000; Chandrasekar et al. 2007; Martos-Sitcha et al. 2014; Wang et al. 2014), but in sockeye salmon (*Oncorhynchus nerka*), rainbow trout, and common carp, two isoforms were detected (Ando et al. 1999; Doyon et al. 2003; Huising et al. 2004).

The crh responses have been highly explored in fish under stressful conditions, such as crowding, handling, hypoxic or salinity changes (Rotllant et al. 2000; 2001; Doyon et al. 2003; Bernier et al. 2004; Pepels et al. 2004; Bernier and Craig 2005; Wunderink et al. 2011; Martos-Sitcha et al. 2014). Nevertheless, little is known about crh relevance on fish appetite regulation. The influence of crh on appetite regulation was demonstrated for the first time in goldfish (De Pedro et al. 1993). The authors showed that icv injections decreased FI during the first 2 h of treatment. Similar results were observed in other studies with goldfish and rainbow trout (Bernier and Peter 2001; Matsuda et al. 2008; Ortega et al. 2013). This suggests a potent anorexigenic role for crh. However, in *S. 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). In gilthead seabream, long-term fasting of 21 days did not affect brain *crh* expression, suggesting that in this, and eventually other species, crh may not be involved in appetite regulation (Martos-Sitcha et al. 2014).

Ghrelin was discovered for the first time in rats by Kojima et al. (1999), and the name originated from the Proto-Indo-European word: “ghre” which means “grow” since it stimulates the release of growth hormone (gh). In fish, ghrelin was described for the first time in goldfish by Unniappan et al. (2002). This peptide is composed of ~100 AA, and only one genomic sequence was described in most fish species (Terova et al. 2008; Amole and Unniappan 2009; Xu and Volkoff 2009; Frøiland et al. 2010; Feng et al. 2013;

Volkoff 2015a; b; Song et al. 2017; Perelló-Amorós et al. 2018). However, two ghrelin isoforms were described in gibel carp (*Carassius auratus gibelio*), goldfish, Mozambique tilapia, and Atlantic salmon (Unniappan et al. 2002; Kaiya et al. 2003; Murashita et al. 2009b; Zhou et al. 2016). Ghrelin is mainly expressed in the stomach, but it is also present in other tissues, such as the brain, gastrointestinal tract (GI), spleen, kidney, heart, muscle, and adipose tissue (Unniappan et al. 2002; Kaiya et al. 2003; Amole and Unniappan 2009; Murashita et al. 2009b; Xu and Volkoff 2009; Feng et al. 2013; Volkoff 2015a; b; Zhou et al. 2016; Song et al. 2017; Perelló-Amorós et al. 2018).

Ghrelin icv and ip injections seemed to promote an increase of the FI in the majority of fish species studied, such as brown trout (*Salmo trutta*), cavefish, goldfish, orange-spotted grouper (*Epinephelus coioides*), and Senegalese sole, suggesting an orexigenic role for this hormone (Unniappan et al. 2002; 2004; Matsuda et al. 2006; Miura et al. 2006; 2007; Gao et al. 2012; Penney and Volkoff 2014; Tinoco et al. 2014a; Navarro-Guillén et al. 2017). However, in channel catfish and rainbow trout, FI decreased after the ip or icv ghrelin injections (Jönsson et al. 2010; Schroeter et al. 2015), and in grass carp, ip ghrelin injections did not affect FI (Yuan et al. 2015).

Data regarding short or long-term fasting on ghrelin response seem to be species- and tissue-specific. Goldfish is one of the most well-studied fish species regarding ghrelin responses, but results do not seem consistent, with responses being different between tissues and even for the same tissue, depending on the study. For instance, in the study by Unniappan et al. (2004), *ghrelin* expression in the brain and intestine of goldfish seemed to follow an orexigenic pattern, decreasing 1 and 3 h AF but increasing after 7 days of fasting. However, in the study of Blanco et al. (2016), although long-term fasting of 7 and 30 days also promoted an increase of brain and stomach *ghrelin* expression, no effects were reported at least during the first 21 h AF. Postprandial *ghrelin* expression in goldfish was also explored by Sánchez-Bretaño et al. (2015) in the brain, GI tract, and pituitary gland. While brain *ghrelin* expression was not affected by a postprandial period between 4 and 20 h, in the GI tract and pituitary gland *ghrelin* was highly expressed at 20 h AF, supporting the orexigenic function for this hormone (Sánchez-Bretaño et al. 2015). Matsuda et al. (2006) also explored the long-term fasting effects on ghrelin expression in goldfish and concluded that in the brain it was not affected by 7 days of fasting, but the intestine presented a higher *ghrelin* expression. These diverse results in goldfish can be due to the different experimental conditions between the different studies, but also can be due to the different initial body weights (IBW) of the animals. For instance, Unniappan et al. (2004) used goldfish with 40-50 g, Blanco et al. (2016), fish with 20-30

g, Sánchez-Bretaño et al. (2015), fish with ~22 g, and Matsuda et al. (2006), goldfish between 3 and 10 g.

An orexigenic function of ghrelin was also reported in other fish species, such as the European seabass, where stomach *ghrelin* expression increased after 35 days of fasting (Terova et al. 2008), and in blunt snout bream, grass carp, and zebrafish, where a fasting period of up to 15 days also promoted an increase of *ghrelin* expression in the brain and intestine (Amole and Unniappan 2009; Feng et al. 2013; Ji et al. 2015). A similar orexigenic pattern was also observed in the intestine of gibel carp, since *ghrelin* expression decreased 1 and 3 h AF, but increased after 7 days of fasting (Zhou et al. 2016). However, in red-bellied piranha, although 7 days of fasting also promoted an increase of *ghrelin* expression in the intestine, in the brain *ghrelin* expression was not affected (Volkoff 2015b). Differently, no differences in *ghrelin* expression were reported in the brain and stomach of gilthead seabream after a postprandial period of 2, 5, and 24 h, and a long-term fasting period of 7 and 23 days (Babaei et al. 2017; Perelló-Amorós et al. 2018), and in the intestine and stomach of channel catfish after a postprandial period of 4, 22, and 23 h (Peterson et al. 2012). Also in Mozambique tilapia, intestine *ghrelin* expression was not affected by the postprandial period or long-term fasting between 4 days and 4 weeks (Fox et al. 2009; Peddu et al. 2009) but in the brain, *ghrelin* expression was increased at 1 h AF and also after 3 days of fasting but, contrary to other studies, decreased after 5 days of fasting, and was not affected after 7 days of fasting (Riley et al. 2008; Peddu et al. 2009). Unexpected results were also reported for Chinese perch (*Siniperca chuatsi*), Atlantic cod, and Atlantic salmon. In Chinese perch, brain *ghrelin* expression decreased at 1, 3, and 12 h AF, and also after 2 days of fasting, but stomach *ghrelin* expression was only decreased 1 h and 3 h AF (Song et al. 2017). In Atlantic cod, *ghrelin* expression in the stomach was neither affected at 2 h AF nor 10 or 30 days of fasting, but a decrease was observed at 22 h AF (Xu and Volkoff 2009). In Atlantic salmon, decreased *ghrelin* expression in the stomach was observed after 2 days of fasting but not after 14 days of fasting (Hevrøy et al. 2011). These unexpected results suggest that, at least in some fish species, ghrelin may not be acting only as an appetite-regulating hormone.

Ghrelin also interacts with other central appetite regulators, although interaction results seem to be inconsistent. For example, ghrelin ip injections inhibited *cart* gene expression in the brain of grass carp (Yuan et al. 2015), but in cavefish, no effects on *cart* expression were observed (Penney and Volkoff 2014). Also, ghrelin treatment (ip injections or oral administration) stimulated brain *npy* expression in grass carp and orange-spotted

grouper (Gao et al. 2012; Yuan et al. 2015), but no effects were reported after ip ghrelin injections in goldfish, brown trout, and channel catfish (Nisembaum et al. 2014; Tinoco et al. 2014a; Schroeter et al. 2015). In orange-spotted grouper, an *in vitro* ghrelin treatment led to a decrease in the expression of *ghrelin receptor (ghrr)-a* and *-b* (Chen et al. 2008) in the pituitary gland, while in grass carp an increase of *ghrr-a* expression was observed in the same tissue after ip ghrelin injection (Cai et al. 2015). A consistent effect of ghrelin on *cck* expression was observed on grass carp and cavefish since *cck* expression was not affected by ip ghrelin injections (Penney and Volkoff 2014; Yuan et al. 2015).

Besides the effects on feeding behavior, ghrelin also seems to have a role on brain glucose metabolism (Polakof et al. 2011), locomotor activity (Matsuda et al. 2006; Nisembaum et al. 2014; Tinoco et al. 2014a), gh release (Kojima et al. 1999; Fox et al. 2007; Picha et al. 2009), plasma insulin-like growth factor-1 (igf-1) levels, and expression of *igf-1* (Fox et al. 2007), *glucago*n (Cruz et al. 2010), and *mechanistic target of rapamycin* (*mtor*) (Penney and Volkoff 2014).

External factors, such as acute stress (Upton and Riley 2013), water temperature (Picha et al. 2009; Hevrøy et al. 2012a; Song et al. 2017), photoperiod (Song et al. 2017), and dietary composition (Johnsen et al. 2011; Ettore et al. 2012; Wu et al. 2016; Babaei et al. 2017) also affect ghrelin responses, although effects seem to be species-specific. For instance, in Chinese perch, higher water temperatures increased *ghrelin* expression in the stomach (Song et al. 2017), while in Atlantic salmon, stomach *ghrelin* expression was reduced with the increase of water temperature (Hevrøy et al. 2012b). The present thesis will further explore the effects of dietary composition on ghrelin responses.

Growth hormone secretagogue-receptor also named **ghrelin receptor (ghrr)**, is the endogenous receptor of ghrelin. Two receptor genes, with ~380 and 290 AA, respectively, have been described in several fish species (Chan and Cheng 2004; Fox et al. 2007; Chen et al. 2008; Kaiya et al. 2009a; b; Small et al. 2009; Kaiya et al. 2010; Hevrøy et al. 2011; Eom et al. 2014; Kaiya et al. 2014; Cai et al. 2015; Perelló-Amorós et al. 2018), being their expression widespread in different tissues, such as the brain, gill, stomach, liver, kidney, and muscle (Chan and Cheng 2004; Fox et al. 2007; Chen et al. 2008; Kaiya et al. 2009a; b; Small et al. 2009; Kaiya et al. 2010; Eom et al. 2014; Kaiya et al. 2014; Cai et al. 2015). The majority of tissue distribution studies indicate that the two receptors are mainly expressed by the fish central nervous system (Chan and

Cheng 2004; Kaiya et al. 2009a; b; Small et al. 2009; Kaiya et al. 2014), however, in goldfish, *ghrr-a* was mainly expressed in testis (Kaiya et al. 2010), and in gilthead seabream, *ghrr-b* was mainly expressed in the liver (Perelló-Amorós et al. 2018).

Little is known about the physiological relevance of ghrr on appetite regulation, but the metabolic reaction seems to be dependent on the species and the period of fasting applied. For instance, short-term fasting up to 24 h did not affect *ghrr-a* expression in the brain of gilthead seabream, goldfish, and Mozambique tilapia, nor in the GI tract or pituitary gland of goldfish (Peddu et al. 2009; Sánchez-Bretaño et al. 2015; Blanco et al. 2016; Perelló-Amorós et al. 2018). However, in the gilthead seabream pituitary gland, a decrease in the expression of this receptor was observed 5 h AF, possibly suggesting an orexigenic effect (Perelló-Amorós et al. 2018).

Regarding long-term fasting, although in goldfish was reported an increase of *ghrr-a* expression in the brain after 7 and 30 days of fasting (Blanco et al. 2016), in general, brain *ghrr-a* expression was not affected by fasting, namely by 2 or 14 days in Atlantic salmon, 7 days in gilthead seabream, up to 7 days in Mozambique tilapia, and 15 days in zebrafish (Riley et al. 2008; Hevrøy et al. 2011; Eom et al. 2014; Perelló-Amorós et al. 2018). A similar unaffected pattern was reported for *ghrr-a* expression in the intestine of zebrafish fasted for 15 days (Eom et al. 2014), or in the pituitary gland of gilthead seabream after fasting of 1 or 7 days (Perelló-Amorós et al. 2018). However, different results were reported for *ghrr-a* expression in grass carp and goldfish (Kaiya et al. 2010; Cai et al. 2015; Blanco et al. 2016). In the pituitary gland of grass carp, although 14 days of fasting did not affect *ghrr-a* expression, 21 and 28 days of fasting increased its expression (Cai et al. 2015). In goldfish, an upregulation of *ghrr-a* was observed in the liver of goldfish after 7 days of fasting (Kaiya et al. 2010), and in the stomach after 30 days of fasting, but not in fish fasted for 7 days (Blanco et al. 2016).

Although little is known about ghrr-b in gilthead seabream, this receptor expression was not affected by short- or long-term fasting of up to 24 h and 7 days, respectively in the brain and pituitary gland (Perelló-Amorós et al. 2018). Similar results were also reported in the brain of goldfish up to 23 h AF (Blanco et al. 2016), and in the brain and intestine of zebrafish after 15 days of fasting (Eom et al. 2014). However, in Mozambique tilapia, a decrease in *ghrr-b* expression was observed in the brain at 3 h AF (Peddu et al. 2009), which suggests an orexigenic role for *ghrr-b* in this species. However, this orexigenic role might be questioned when evaluating the response to longer fasting periods since in another study in the same species, although 3 days fasting increased brain *ghrr-b*

expression, 5 days of fasting led to a decrease of this receptor expression, and 7 days fasting did not affect it (Riley et al. 2008).

Sexual dimorphism and reproduction effects on *ghrr* expression were explored by Eom et al. (2014) and Bertucci et al. (2016). In the study by Eom et al. (2014), female zebrafish presented significantly lower *ghrr* expression in ventral skin than males, suggesting that these receptors might be involved in pigmentation regulation during sexual dimorphism, with males being darker than females. Bertucci et al. (2016) observed that pituitary *ghrr* expression increased after estradiol and testosterone administration in goldfish, concluding on a positive relationship between sex steroids and the ghrr.

Leptin in fish was first identified in pufferfish (*Takifugu rubripes*) by Kurokawa et al. (2005). It is composed of ~160 AA, with the precise AA number depending on fish species (Kurokawa et al. 2005; Murashita et al. 2008; Kurokawa and Murashita 2009; Frøiland et al. 2010; Li et al. 2010; Rønnestad et al. 2010; Won et al. 2012; Zhang et al. 2013; Yuan et al. 2014; Han et al. 2016; Yuan et al. 2016). The majority of fish species only have one leptin isoform (Murashita et al. 2008; Frøiland et al. 2010; Li et al. 2010; Won et al. 2012; Yuan et al. 2014; Volkoff 2015a; b; Han et al. 2016). However, two leptin paralog genes were described in some fish species, such as Atlantic salmon, goldfish, Japanese medaka (*Oryzias latipes*), mandarin fish (*Siniperca chuatsi*), orange-spotted grouper, and zebrafish (Gorissen et al. 2009; Kurokawa and Murashita 2009; Rønnestad et al. 2010; Tinoco et al. 2012; Zhang et al. 2013; Yuan et al. 2016). Although in mammals the adipose tissue seems to be the major producer of leptin (Harris 2014), in fish, this hormone is found mainly in the liver, and to a lesser extent in other tissues, such as the brain, pituitary gland, intestine, gonads, kidney, gills, heart, and eye (Kurokawa et al. 2005; Murashita et al. 2008; Gorissen et al. 2009; Tinoco et al. 2012; Trombley et al. 2012; Won et al. 2012; Zhang et al. 2013; Yuan et al. 2014; Volkoff 2015a; b; Han et al. 2016). Leptin is also expressed in the adipose tissue of rainbow trout and gilthead seabream (Salmerón et al. 2015; Babaei et al. 2017).

Leptin ip or icv injections inhibited FI in several fish species, such as goldfish, grass carp, rainbow trout, and striped bass (*Morone saxatilis*), suggesting a strong anorexigenic role for this hormone (Volkoff et al. 2003; De Pedro et al. 2006; Murashita et al. 2008; Aguilar et al. 2010; Li et al. 2010; Won et al. 2012). However, this anorexigenic function does not seem so clear when evaluating short- and long-term fasting effects on *leptin* expression across different fish species and tissues. For instance, in gilthead seabream,

23 days of fasting did not affect *leptin* expression in the adipose tissue (Babaei et al. 2017), and similar results were reported in the brain of goldfish, pacu, and red-billed piranha, and in the intestine of pacu, submitted to short-term fasting of up to 24 h or long-term fasting of 3 or 7 days (Tinoco et al. 2012; Tinoco et al. 2014b; Volkoff 2015b; Volkoff et al. 2017). However, in orange-spotted grouper, 7 days of fasting promoted an increase of *leptin* expression in the brain (Zhang et al. 2013), while in the red-bellied piranha intestine *leptin* expression decreased after 7 days of fasting (Volkoff 2015b). Regarding hepatic *leptin* expression, the same pattern was observed in different species, with short-term fasting not affecting expression up to 3 h AF in goldfish, orange-spotted grouper, and *S. prenanti*, while increasing expression 9-12 h AF (Tinoco et al. 2012; Tinoco et al. 2014b; Yuan et al. 2014). However, these consistent responses across species were not maintained when fish were subjected to long periods of fasting. For instance, 23 days of fasting did not affect hepatic *leptin* expression in gilthead seabream (Babaei et al. 2017) nor in goldfish fasted for 1 week (Tinoco et al. 2012), but promoted an increase of expression in orange-spotted grouper fasted for 1, 2, or 3 weeks (Zhang et al. 2013). Further, in *S. prenanti* and striped bass, a decrease of *leptin* expression was reported up to 7 days or between 10 and 20 days of fasting, respectively (Won et al. 2012; Yuan et al. 2014).

Leptin was also reported to interact with other central appetite regulators, and a strong functional interaction between leptin and npy in the central regulation of appetite of several species was already described in both *in vivo* and *in vitro* studies, with leptin treatment inducing inhibition of *npy* expression (Volkoff et al. 2003; Murashita et al. 2008; Li et al. 2010; Aguilar et al. 2011).

Leptin also plays a role in metabolic and physiologic processes such as in glucose and lipid metabolism and reproduction (De Pedro et al. 2006; Kim et al. 2008; Aguilar et al. 2010; Li et al. 2010; Lu et al. 2012; 2015; Salmerón et al. 2015; Song et al. 2015). For example, in grass carp hepatocytes, an *in vitro* leptin treatment led to an increase of glucokinase (GK) and pyruvate kinase activities (two key glycolytic enzymes), and a decrease of glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase activities (two key gluconeogenesis enzymes), which suggests a role of leptin in the increase of glycolysis and decrease of gluconeogenesis (Lu et al. 2015). Hyperglycemia and glycogenolysis were also induced by icv leptin injections in rainbow trout (Aguilar et al. 2010). In goldfish, ip leptin injections promoted lipolysis and hepatic and muscle glycogen storage (De Pedro et al. 2006). Lipolysis was also enhanced in rainbow trout adipocytes by an *in vitro* leptin treatment, supporting the anti-adipogenic role of this

hormone (Salmerón et al. 2015). Similar results were observed in grass carp and yellow catfish (*Pelteobagrus fulvidraco*) hepatocytes after an *in vitro* leptin treatment, which stimulated both hepatic lipolysis and β-oxidation while inhibiting lipogenesis (Lu et al. 2012; Song et al. 2015). In both studies, leptin treatment increased the release of glycerol, reduced hepatic lipid content, decreased *peroxisome proliferator-activated receptor* (*ppar)-γ* protein and expression levels, and upregulated key β-oxidation-related genes, such as *pparα*, and *carnitine palmitoyl transferase-1*. In an *in vivo* study with grass carp, injection of leptin also seemed to promote lipolysis since it was observed a decrease of hepatic *lipoprotein lipase* (*lpl*) and an increase of *fatty acid elongase* and *bile salt-activated lipase* expression (all genes participating in lipid metabolism) (Li et al. 2010).

In addition, external factors such as photoperiod, water temperature, and dietary composition also seem to affect leptin responses (Vivas et al. 2011; Kullgren et al. 2013; Cai et al. 2018). Regarding photoperiod, it was observed in goldfish a reduction of FI when leptin was injected during the light phase (at 10 h), but not when injected at scotophase (at 22 h) (Vivas et al. 2011). Concerning water temperature, it was observed in Atlantic salmon that higher temperatures promoted simultaneously an increase of plasma leptin levels and a decrease of FI, which agrees with the strong anorexigenic function of leptin (Kullgren et al. 2013). The effects of dietary composition on leptin will be further explored in the present thesis.

In fish, the **leptin receptor (lepr)** gene was first identified in medaka (*Oryzias melastigma*) by Wong et al. (2007). This receptor is composed of ~1200 AA and usually, only one isoform has been described for each fish species (Kurokawa and Murashita 2009; Liu et al. 2010; Rønnestad et al. 2010; Zhang et al. 2013; Shpilman et al. 2014; Han et al. 2016), but in some species, two or even three lepr isoforms have been found, such as in crucian carp (*Carassius carassius*) and European and Japanese eel (*Anguilla anguilla*, and *Anguilla japonica*) (Cao et al. 2011; Morini et al. 2015). This receptor was detected in a variety of tissues, such as muscle, gonads, gills, skin, heart, kidney, intestine, liver, brain, and adipose tissue of several fish species (Kurokawa and Murashita 2009; Liu et al. 2010; Rønnestad et al. 2010; Tinoco et al. 2012; Trombley et al. 2012; Zhang et al. 2013; Shpilman et al. 2014; Morini et al. 2015; Han et al. 2016; Ohga et al. 2017). Probably due to this widespread distribution, lepr seem to participate in several physiological processes in fish, like development and growth (Liu et al. 2010), sensory development (Liu et al. 2010), hypoxia (Wong et al. 2007; Cao et al. 2011), and reproduction (Rønnestad et al. 2010; Morini et al. 2015; Ohga et al. 2017). However, lepr

function on fish appetite regulation is not yet clear. For instance, He et al. (2013) showed that PF-based diets up-regulated *lepr* expression in grass carp and increased FI, and Chisada et al. (2014) confirmed that lepr had a strong effect in FI control in Japanese medaka, since lepr-deficient fish consumed significantly more feed than fish with functional lepr. However, in goldfish, Nile tilapia (*Oreochromis niloticus*), and orange-spotted grouper, neither short-term fasting of up to 24 h nor long-term fasting of 1 week or 26 days affected *lepr* expression (Tinoco et al. 2012; Zhang et al. 2013; Shpilman et al. 2014; Tinoco et al. 2014b).

Furthermore, lepr can be also influenced by others factors, as photoperiod (Chi et al. 2019) and satiation level (Rønnestad et al. 2010; Gong et al. 2017). Regarding photoperiod, it was shown that 24 h light seemed to reduce *lepr* expression in Atlantic salmon and consequently increased FI and growth rate (Chi et al. 2019). Also in Atlantic salmon, a satiation level of 60% did not affect *lepr* expression (Rønnestad et al. 2010). Similar results were found for grass carp with rationed feeding of 40, 60 or 80% in comparison with fish fed *ad libitum* (Gong et al. 2017).

Neuropeptide y (npy) was first identified in fish by Kimmel et al. (1986) in coho salmon. This peptide is composed of ~36 AA, and is predominantly expressed in the brain, but has also been detected in the GI tract, pituitary gland, spleen, kidney, muscle, and gonads (Kehoe and Volkoff 2007; MacDonald and Volkoff 2009a; b; Murashita et al. 2009a; Campos et al. 2010; Babichuk and Volkoff 2013; Van Nguyen et al. 2013; Zhou et al. 2013; Hosomi et al. 2014; Tang et al. 2014; Wei et al. 2014; Ji et al. 2015; Pitts and Volkoff 2017). The majority of fish species have only one npy isoform, but in a few cases, two isoforms were described, such as in tiger puffer (*Takifugu rubripes*) and Jian carp (*Cyprinus carpio* var. Jian) (Kamijo et al. 2011; Tang et al. 2014).

López-Patiño et al. (1999) showed for the first time in fish, that icv injections of npy increased FI in goldfish, confirming the orexigenic function of this hormone, as already described in mammals (Stanley and Leibowitz 1984; 1985; Morley 1987). Thereafter, more studies using icv and ip injections confirmed the orexigenic function of npy in several fish species, such as grass carp, olive flounder (*Paralichthys olivaceus*), and rainbow trout (Narnaware et al. 2000; Volkoff et al. 2003; Aldegunde and Mancebo 2006; Zhou et al. 2013; Li et al. 2017b).

However, npy does not seem to always behave as an orexigenic hormone, its effects depending on species and fasting duration. For instance, *npy* expression in the brain was not affected in Atlantic salmon, platyfish, and gilthead seabream fasted for 6, 10, or

23 days, respectively (Murashita et al. 2009a; Babaei et al. 2017; Pitts and Volkoff 2017). Similar results were observed in the brain of Atlantic cod fasted for 7 days, although a decrease in *npy* expression was observed at 22 h AF (Kehoe and Volkoff 2007). In Mozambique tilapia, *npy* expression was also unaffected by long-term fasting of up to 7 days (Riley et al. 2008), but a postprandial period of 1 or 3 h decreased its expression in the brain (Peddu et al. 2009). Differently, in Brazilian flounder (*Paralichthys orbignyanus*), a postprandial period of up to 24 h did not affect *npy* expression, but 2 weeks of fasting increased its expression (Campos et al. 2010). In blunt snout bream and *S. prenanti*, fasting up to 15 days increased brain *npy* expression (Wei et al. 2014; Ji et al. 2015), and in goldfish, *npy* expression was also higher up to 3 days of fasting than in fed fish (Narnaware et al. 2000). However, in cunner, although 1 or 2 weeks of fasting did not affect *npy* expression in the brain, this neuropeptide expression decreased after 3 weeks of fasting (Babichuk and Volkoff 2013). Moreover, in channel catfish, *npy* expression was higher at 4 h AF, but at 22 and 23 h AF *npy* expression was similar to that of 0 h (= feeding time) (Peterson et al. 2012).

In the intestine, *npy* expression was not affected by a fasting up to 15 days in blunt snout bream or up to 10 days in platyfish (Ji et al. 2015; Pitts and Volkoff 2017), suggesting that in this tissue this hormone does not participate in long-term appetite regulation mechanism.

As with other appetite-regulating hormones, *npy* expression can be influenced by external factors, as diet composition (Narnaware and Peter 2002; Figueiredo-Silva et al. 2012; Jin et al. 2015; Wu et al. 2016; Li et al. 2017a) or year season (MacDonald and Volkoff 2009a; Babichuk and Volkoff 2013), but not by temperature (Kehoe and Volkoff 2008), salinity (Luz et al. 2008), or hypoxia (Burt et al. 2013). Both dietary composition and year season effects seem to be species-specific. Regarding year season effects, MacDonald and Volkoff (2009a) observed that in the brain of winter flounder (*Pseudopleuronectes americanus*) *npy* expression was higher in the winter than in summer, which corresponds to a period when fish eat less and thus have emptied gut. However, opposite results were found in cunner, since *npy* expression was lower in the winter than in summer (Babichuk and Volkoff 2013). The differences between the two species may be explained by different survival strategies. While winter flounder remains active during winter, cunner seems to become completely dormant, decreasing its oxygen consumption and metabolic rate (Babichuk and Volkoff 2013). Dietary composition effects will be further explored in the present thesis.

Table 1. Intracerebroventricular (icv) and intraperitoneal (ip) injections or oral administration (oa) of some appetite-regulating hormones and their effects on fish feed intake (FI).

Hormone	Fish species	IBW (g)	Admin. via	Dosage	FI	Reference
cart	Goldfish	30-55	icv	1, 5, 10, 50 ng gBW^{-1}	↓	(Volkoff and Peter 2000; Volkoff and Peter 2001)
		35-75		5 ng gBW^{-1}	↓	(Volkoff and Peter 2001)
cck	Cavefish	1.2	ip	50 ng gBW^{-1}	↓	(Penney and Volkoff 2014)
	Channel catfish	55	ip	50, 100, 200 ng gBW^{-1}	→	(Schroeter et al. 2015)
	Coho salmon	10-30	ip	50, 100, 300 ng gBW^{-1}	↓	(White et al. 2016)
	Goldfish	25-45	icv	50 ng gBW^{-1}	↓	(Himick and Peter 1994)
		30-45		5 ng gBW^{-1}	↓	(Volkoff et al. 2003)
		25-45	ip	50, 500 ng gBW^{-1}	↓	(Himick and Peter 1994)
		30-45		50 ng gBW^{-1}	↓	(Volkoff et al. 2003)
		6-10		100 pmol gBW^{-1}	↓	(Kang et al. 2010)
	Platyfish	n.a.	ip	50 ng gBW^{-1}	↓	(Pitts and Volkoff 2017)
crh	Goldfish	5-9	icv	1, 2 µg gBW^{-1}	↓	(De Pedro et al. 1993)
		47.3		2, 20, 200 ng gBW^{-1}	↓	(Bernier and Peter 2001)
		3-10		20 pmol gBW^{-1}	↓	(Matsuda et al. 2008)
		5-9	ip	1 µg gBW^{-1}	→	(De Pedro et al. 1993)
	Rainbow trout	68.3	icv	5, 25,125 ng gBW^{-1}	↓	(Ortega et al. 2013)
ghrelin	Brown trout	3.5-5.5	ip	475 ng gBW^{-1}	↑	(Tinoco et al. 2014a)
	Cavefish	1.2	ip	100 ng gBW^{-1}	↑	(Penney and Volkoff 2014)
	Channel catfish	55	ip	50, 100, 200 ng gBW^{-1}	↓	(Schroeter et al. 2015)
	Goldfish	40	icv	1, 5 ng gBW^{-1}	↑	(Unniappan et al. 2002)
		40-50		1-10 ng gBW^{-1}	↑	(Unniappan et al. 2004)
		3-10		1, 2 pmol gBW^{-1}	↑	(Matsuda et al. 2006)
		3-10		1 pmol gBW^{-1}	↑	(Miura et al. 2006)
		3-10		1 pmol gBW^{-1}	↑	(Miura et al. 2007)
		40-50	ip	10, 100 ng gBW^{-1}	↑	(Unniappan et al. 2004)
		3-10		8, 16 pmol gBW-1	↑	(Matsuda et al. 2006)
		3-10		16 pmol gBW-1	↑	(Miura et al. 2006)

Hormone	Fish species	IBW (g)	Admin. via	Dosage	FI	Reference
	Grass carp	43.9	ip	100 ng gBW^{-1}	→	(Yuan et al. 2015)
	Orange-spotted grouper	84.8	oa	4 mg $kg\ diet^{-1}$	↑	(Gao et al. 2012)
	Rainbow trout	130	icv	2 ng gBW^{-1}	↓	(Jönsson et al. 2010)
	Senegalese sole	0.0015	oa	0.06 ng $mgBW^{-1}$	↑	(Navarro-Guillén et al. 2017)
leptin	Goldfish	30-45	icv	100 ng gBW^{-1}	↓	(Volkoff et al. 2003)
		30-45	ip	300, 400 ng gBW^{-1}	↓	(Volkoff et al. 2003)
		n.a.		1 µg gBW^{-1}	↓	(De Pedro et al. 2006)
	Grass carp	100	ip	2.1 µg gBW^{-1}	↓	(Li et al. 2010)
	Rainbow trout	n.a.	icv	5 µg gBW^{-1}	↓	(Aguilar et al. 2010)
		58.3	ip	720 ng gBW^{-1}	↓	(Murashita et al. 2008)
	Striped bass	30.8	ip	100 ng, 1 µg gBW^{-1}	↓	(Won et al. 2012)
npy	Goldfish	7.9	icv	1 µg gBW^{-1}	↑	(López-Patiño et al. 1999)
		25-45		0.5, 1, 2, 4, 5 ng gBW^{-1}	↑	(Narnaware et al. 2000)
				7, 8 ng gBW^{-1}	↓	
		30-45		3, 5 ng gBW^{-1}	↑	(Volkoff et al. 2003)
		7.9	ip	0.1, 0.33 µg gBW^{-1}	→	(López-Patiño et al. 1999)
	Grass carp	n.a.	icv	0.5, 1.0 µg gBW^{-1}	↑	(Zhou et al. 2013)
	Olive flounder	13-23	ip	1 µg gBW^{-1}	↑	(Li et al. 2017b)
	Rainbow trout	85.8-112	icv	4, 8 µg gBW^{-1}	↑	(Aldegunde and Mancebo 2006)

Symbols represent an increase (↑), no effect (→), or decrease (↓) of the FI relative to the control treatment. Admin.: Administration; IBW: initial body weight; *cart*: *cocaine-amphetamine-related transcript*; *cck*: *cholecystokinin*; *crh*: *corticotropin-releasing factor*; n.a.: not available; *npy*: *neuropeptide y.*

Table 2. Fasting effects on gene expression (GE) of the main hormones involved in fish appetite regulation mechanisms, listed by tissue and species.

Gene	Tissue	Fish species	IBW (g)	Fasting	GE	Reference
cart	Brain	Atlantic cod	100	2 h	↓	(Kehoe and Volkoff 2007)
				22 h	→	
				7 days	↓	
		Atlantic salmon	44.7	6 days	↓	(Murashita et al. 2009a)
		Channel catfish	17.6	0.5, 1, 2, 4 h	↑	(Peterson et al. 2012)
				22, 23 24 h	→	
			73.4	30 days	↓	(Kobayashi et al. 2008)
		Cunner	19.6	1, 2, 3 weeks	→	(Babichuk and Volkoff 2013)
		Dorado	63.4-65.0	1h	↑	(Volkoff et al. 2016)
				5 days	→	
		Pacu	62.4-67.2	1h	→	(Volkoff et al. 2017)
				7 days	↓	
		Platyfish	1.5-3	10 days	↓	(Pitts and Volkoff 2017)
		Red-bellied piranha	0.54	7 days	↓	(Volkoff 2014)
		Siberian sturgeon	29	24h	↓	(Zhang et al. 2018)
				3,6,10,15 days	↑	
		Winter skate	1860	2 weeks	→	(MacDonald and Volkoff 2009b)
	Intestine	Platyfish	1.5-3	10 days	→	(Pitts and Volkoff 2017)
cck	Brain	Atlantic salmon	44.3	6 days	↓	(Murashita et al. 2009b)
		Blunt snout bream	10	4, 7, 15 days	↓	(Ji et al. 2015)
		Channel catfish	17.6	4 h	↓	(Peterson et al. 2012)
				22, 23 h	→	
		Cunner	19.6	1, 2, 3 weeks	↓	(Babichuk and Volkoff 2013)
		Dorado	63.4-65.0	1h	↑	(Volkoff et al. 2016)
				5 days	→	
		Grass carp	5	2, 7, 15 days	↓	(Feng et al. 2012)
		Pacu	62.4-67.2	1h	→	(Volkoff et al. 2017)
				7 days	→	
		Platyfish	1.5-3	10 days	↓	(Pitts and Volkoff 2017)
		Red-bellied piranha	0.54	7 days	→	(Volkoff 2014)
		Schizothorax prenanti	39.4	1, 3 h	↑	(Yuan et al. 2014)
				1, 3, 5, 7 days	↓	

Gene	Tissue	Fish species	IBW (g)	Fasting	GE	Reference
		Winter skate	1860	2 weeks	→	(MacDonald and Volkoff 2009b)
		Yellowtail	514	2 weeks	→	(Hosomi et al. 2014)
	Intestine	Atlantic salmon	44.3	6 days	→	(Murashita et al. 2009b)
		Blunt snout bream	10	1, 4, 7, 15 days	↓	(Ji et al. 2015)
		Channel catfish	17.6	4 h	↑	(Peterson et al. 2012)
				22, 23 h	→	
		Dorado	63.4-65.0	1h	→	(Volkoff et al. 2016)
				5 days	→	
		Gilthead seabream	16.9	23 days	→	(Babaei et al. 2017)
		Grass carp	5	2, 7, 15 days	↓	(Feng et al. 2012)
		Pacu	62.4-67.2	1h	→	(Volkoff et al. 2017)
				7 days	↓	
		Platyfish	1.5-3	10 days	↓	(Pitts and Volkoff 2017)
		Red-bellied piranha	0.54	7 days	→	(Volkoff 2014)
		Schizothorax prenanti	39.4	3 h	↑	(Yuan et al. 2014)
				1, 3, 5, 7 days	↓	
		Winter skate	1860	2 weeks	↑	(MacDonald and Volkoff 2009b)
		Yellowtail	619	3 h	↑	(Murashita et al. 2006)
				3 days	→	
crh	Brain	Gilthead seabeam	213	21 days	→	(Martos-Sitcha et al. 2014)
		Schizothorax prenanti	254	1, 3 h	→	(Wang et al. 2014)
				1, 3, 5 days	→	
				7 days	↓	
ghrelin	Brain	Blunt snout bream	10	1, 4, 7, 15 days	↑	(Ji et al. 2015)
		Chinese perch	120	1, 3, 12 h	↓	(Song et al. 2017)
				2 days	↓	
		Gilthead seabream	50	2, 5, 24 h	→	(Perelló-Amorós et al. 2018)
				7 days	→	
			16.9	23 days	→	(Babaei et al. 2017)
		Goldfish	40-50	1, 3 h	↓	(Unniappan et al. 2004)
				3, 5 days	→	
				7 days	↑	
			20-30	1, 3, 21, 23 h	→	(Blanco et al. 2016)
				7, 30 days	↑	
			3-10	7 days	→	(Matsuda et al. 2006)
			22	4, 8, 12, 16, 20 h	→	(Sánchez-Bretaño et al. 2015)
		Grass carp	5	5, 7, 15 days	↑	(Feng et al. 2013)
		Mozambique tilapia	60-70	1h	↑	(Peddu et al. 2009)
				3h	→	
			80-100	3 days	↑	(Riley et al. 2008)

Gene	Tissue	Fish species	IBW (g)	Fasting	GE	Reference
				5 days	↓	
				7 days	→	
		Red-bellied piranha	0.54	7 days	→	(Volkoff 2015b)
		Zebrafish	n.a.	3, 5, 7 days	↑	(Amole and Unniappan 2009)
	GI tract	Goldfish	22	4, 8, 12 h	→	(Sánchez-Bretaño et al. 2015)
				16, 20 h	↑	
	Intestine	Blunt snout bream	10	1, 4, 7, 15 days	↑	(Ji et al. 2015)
		Channel catfish	17.6	4, 22, 23 h	→	(Peterson et al. 2012)
		Gibel carp	107.4-116.1	1, 3 h	↓	(Zhou et al. 2016)
				1, 3, 5 days	→	
				7 days	↑	
		Goldfish	40-50	3h	↓	(Unniappan et al. 2004)
				3, 5 days	→	
				7 days	↑	
			3-10	7 days	↑	(Matsuda et al. 2006)
		Grass carp	5	5, 7, 15 days	↑	(Feng et al. 2013)
		Red-bellied piranha	0.54	7 days	↑	(Volkoff 2015b)
		Zebrafish	n.a.	3, 5, 7 days	↑	(Amole and Unniappan 2009)
	Pituitary gland	Goldfish	22	4, 8, 12, 16 h	→	(Sánchez-Bretaño et al. 2015)
				20	↑	
	Stomach	Atlantic cod	35	2 h	→	(Xu and Volkoff 2009)
				22 h	↓	
				10, 30 days	→	
		Atlantic salmon	44.3	6 days	↑	(Murashita et al. 2009b)
			128	2 days	↓	(Hevrøy et al. 2011)
				14 days	→	
		Channel catfish	17.6	1, 2, 4, 22, 23 h	→	(Peterson et al. 2012)
		Chinese perch	120	1, 3 h	↓	(Song et al. 2017)
				6, 12 h	↑	
		European seabass	117.6-120.1	4 days	→	(Terova et al. 2008)
				35 days	↑	
		Gilthead seabream	50	2, 5, 24 h	→	(Perelló-Amorós et al. 2018)
				7 days	→	
		Goldfish	20-30	1, 3, 21 h	→	(Blanco et al. 2016)
				23 h	↑	
				7, 30 days	↑	
		Mozambique tilapia	60-70	1, 3 h	→	(Peddu et al. 2009)
			30-100	2, 10, 24 h	→	(Fox et al. 2009)
				4, 8 days	→	
				2, 4 weeks	→	
ghrr-a	Brain	Atlantic salmon	128	2, 14 days	→	(Hevrøy et al. 2011)
		Gilthead seabream	50	2, 5, 24 h	→	(Perelló-Amorós et al. 2018)
				7 days	→	

Gene	Tissue	Fish species	IBW (g)	Fasting	GE	Reference
		Goldfish	22	4, 8, 12, 16, 20 h	→	(Sánchez-Bretaño et al. 2015)
			20-30	1, 3, 21, 23 h	→	(Blanco et al. 2016)
				7, 30 days	↑	
		Mozambique tilapia	60-70	1, 3 h	→	(Peddu et al. 2009)
			80-100	1,3,5, 7 days	→	(Riley et al. 2008)
		Zebrafish	n.a.	15 days	→	(Eom et al. 2014)
	GI tract	Goldfish	22	4, 8, 12, 16, 20 h	→	(Sánchez-Bretaño et al. 2015)
	Intestine	Zebrafish	n.a.	15 days	→	(Eom et al. 2014)
	Liver	Goldfish	3-10	7 days	↑	(Kaiya et al. 2010)
	Pituitary gland	Gilthead seabream	50	2 h	→	(Perelló-Amorós et al. 2018)
				5 h	↓	
				1, 7 days	→	
		Goldfish	22	4, 8, 12, 16, 20 h	→	(Sánchez-Bretaño et al. 2015)
		Grass carp	43.9	14 days	→	(Cai et al. 2015)
				21, 28 days	↑	
	Stomach	Goldfish	20-30	1, 3, 21, 23 h	→	(Blanco et al. 2016)
				7 days	→	
				30 days	↑	
ghrr-b	Brain	Gilthead seabream	50	2, 5, 24 h	→	(Perelló-Amorós et al. 2018)
				7 days	→	
		Goldfish	20-30	1, 3, 21, 23 h	→	(Blanco et al. 2016)
		Mozambique tilapia	60-70	1 h	→	(Peddu et al. 2009)
				3 h	↓	
			80-100	3 days	↑	(Riley et al. 2008)
				5 days	↓	
				7 days	→	
		Zebrafish	n.a.	15 days	→	(Eom et al. 2014)
	Intestine	Zebrafish	n.a.	15 days	→	(Eom et al. 2014)
	Pituitary gland	Gilthead seabream	50	2, 5, 24 h	→	(Perelló-Amorós et al. 2018)
				7 days	→	
leptin	Adipose tissue	Gilthead seabream	16.9	23 days	→	(Babaei et al. 2017)
	Brain	Goldfish	10-17	3, 6, 9, 12, 15, 18, 21, 24 h	→	(Tinoco et al. 2014b)
			15-20	3, 6, 9, 12 h	→	(Tinoco et al. 2012)
				1 week	→	
		Orange-spotted grouper	2000-2200	3 days	→	(Zhang et al. 2013)
				7 days	↑	
		Pacu	62.4-67.2	1h	→	(Volkoff et al. 2017)
				7 days	→	
		Red-bellied piranha	0.54	7 days	→	(Volkoff 2015b)
	Liver	Gilthead seabream	16.9	23 days	→	(Babaei et al. 2017)

Gene	Tissue	Fish species	IBW (g)	Fasting	GE	Reference
		Goldfish	10-17	3, 6, 9 h	→	(Tinoco et al. 2014b)
				12 h	↑	
				15, 18, 21, 24 h	→	
			15-20	3, 6 h	→	(Tinoco et al. 2012)
				9, 12 h	↑	
				1 week	→	
		Orange-spotted grouper	2000-2200	3, 6 h	→	(Zhang et al. 2013)
				9h	↑	
				3 days	→	
				7 days	↑	
				2, 3 weeks	↑	
		Schizothorax prenanti	39.4	1, 3 h	→	(Yuan et al. 2014)
				1, 3, 5, 7 days	↓	
		Striped bass	71.1	10, 20 days	↓	(Won et al. 2012)
	Intestine	Pacu	62.4-67.2	1h	→	(Volkoff et al. 2017)
				7 days	→	
		Red-bellied piranha	0.54	7 days	↓	(Volkoff 2015b)
lepr	Brain	Goldfish	10-17	3, 6, 9, 12, 15, 18, 21, 24 h	→	(Tinoco et al. 2014b)
			15-20	3, 6, 9, 12 h	→	(Tinoco et al. 2012)
				1 week	→	
		Nile tilapia	41.7	26 days	→	(Shpilman et al. 2014)
		Orange-spotted grouper	2000-2200	3, 7 days	→	(Zhang et al. 2013)
npy	Brain	Atlantic cod	100	2 h	→	(Kehoe and Volkoff 2007)
				22 h	↓	
				7 days	→	
		Atlantic salmon	44.7	6 days	→	(Murashita et al. 2009a)
		Blunt snout bream	10	1, 4, 7, 15 days	↑	(Ji et al. 2015)
		Brazilian flounder	250	1, 2, 6, 12, 24 h	→	(Campos et al. 2010)
				2 weeks	↑	
		Channel catfish	17.6	4 h	↑	(Peterson et al. 2012)
				22, 23 h	→	
		Cunner	19.6	1, 2 weeks	→	(Babichuk and Volkoff 2013)
				3 weeks	↓	
		Gilthead seabream	16.9	23 days	→	(Babaei et al. 2017)
		Goldfish	25-45	1, 3 h	↓	(Narnaware et al. 2000)
				1, 2, 3 days	↑	
		Mozambique tilapia	60-70	1, 3 h	↓	(Peddu et al. 2009)
			80-100	1, 3, 5, 7 days	→	(Riley et al. 2008)

Gene	Tissue	Fish species	IBW (g)	Fasting	GE	Reference
		Platyfish	1.5-3	10 days	→	(Pitts and Volkoff 2017)
		Schizothorax prenanti	500	14 days	↑	(Wei et al. 2014)
	Intestine	Blunt snout bream	10	1, 4, 7, 15 days	→	(Ji et al. 2015)
		Platyfish	1.5-3	10 days	→	(Pitts and Volkoff 2017)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the GE relative to fed fish. *cart*: *cocaine-amphetamine-related transcript*; *cck*: *cholecystokinin*; *crh*: *corticotropin-releasing factor*; *ghrr*: *ghrelin receptor*; GI tract: gastrointestinal tract (which included all digestive system); IBW: initial body weight; *lepr*: *leptin receptor*; n.a.: not available; *npy*: *neuropeptide y.*

1.3. Feeding frequency effects

As previously mentioned, although FF optimization seems to be crucial for a more sustainable and profitable industry, avoiding dietary losses and environmental pollution and promoting fish growth (Aderolu et al. 2010; Amirkolaie 2011; White 2013), the effects of FF on appetite regulation, growth, feed utilization, metabolism, and intestine functionality and health are still poorly explored. The present chapter aims to report the FF effects on appetite regulation, growth, and intermediary metabolism, and in the intestine functionality and health of fish.

1.3.1. Appetite regulation

The few studies focusing on the influence of feeding strategies on appetite regulation emphasize the relevance of feeding rates (% satiation) (Pfundt et al. 2016; Xu et al. 2016; Gong et al. 2017) and not of the FF protocol. In all these studies was observed an increase of hepatic *leptin* expression with the feeding rate increase, which suggests that increasing feeding rate reduce fish appetite (Pfundt et al. 2016; Xu et al. 2016; Gong et al. 2017).

Until now, only two studies evaluated FF protocol effects on fish appetite regulation. Pham et al. (2021) that fed clown anemonefish (*Amphiprion ocellaris*) to satiety 1 or 3 times per day and observed that some neuropeptides in the brain, such as agouti-related protein (already known as appetite regulator), seem to have a role in fish appetite regulation associated to FF since its expression decreased in fish fed 3 times per day.

However, in gilthead seabream fed a fixed daily amount of feed distributed by different FF protocols (1, 3, or 5 meals per day, or continuous feeding) stomach *ghrelin* and intestine *cck* expressions were not affected (Gilannejad et al. 2021). These observations suggest that appetite control mechanisms are species-specific and can be modulated by other factors as the amount of feed provided. An interaction between FF and dietary composition was also previously reported in gibel carp, where the increase of FF together with a higher dietary P/CH ratio led to an increase in FI (Zhao et al. 2016). However, the causes of this interaction on fish appetite regulation mechanisms were not yet evaluated.

1.3.2. Growth and intermediary metabolism

Overall, an increase of FF seems to promote an increase of FI and growth in several fish species (Murai et al. 1983; Tung and Shiau 1991; Lee et al. 2000a; Basçinar et al. 2001; Lee and Pham 2010; Zolfaghari et al. 2011; Sun et al. 2014; Oh and Maran 2015; Tian et al. 2015; Daudpota et al. 2016; Zhao et al. 2016; Rahman and Lee 2017; Guo et al. 2018; Oh et al. 2018; Silva et al. 2020), while intermediary metabolic responses are only slightly affected (Oh et al. 2018; Cheng et al. 2019; Silva et al. 2020) (**Table 3**). For instance, common carp fed 4 meals per day presented lower plasmatic glucose and gh levels, and higher GK activity in the liver than those fed 2 meals per day, suggesting an enhancement of glycolysis with the FF increase (Cheng et al. 2019). Similarly, in blunt snout bream, *gh* expression also decreased with the increase of the FF (Tian et al. 2015). Regarding other metabolic responses, Lebranche mullet fed more meals per day presented higher levels of plasmatic glucose, triglycerides (TG), and cholesterol than those fed only once a day (Silva et al. 2020). Oh et al. (2018) also reported an increase of the plasmatic cholesterol levels in dark-banded rockfish fed more than 1 meal per day.

However, different results were also reported (Lee et al. 2000b; Costa-Bomfim et al. 2014; Enes et al. 2015; Pedrosa et al. 2019). For instance, feeding arapaima (*Arapaima gigas*) juveniles 2 or 3 times per day did not affect FI, growth performance, feed utilization, or plasmatic metabolites responses (Pedrosa et al. 2019). Similarly in white seabream and Korean rockfish, the increase of FF also did not affect FI, growth, feed utilization, or intermediary metabolism responses (Lee et al. 2000b; Enes et al. 2015). These different results between fish species may be related to differences in the experimental protocols but might also suggest that FF effects are species-specific.

Some studies also reported that FF manipulation can enhance the use efficiency of dietary CH, thus improving feed utilization and growth (Tung and Shiau 1991; Hung and

Storebakken 1994). However, this effect needs to be better explored, since recent studies in gibel carp and common carp found no relation between dietary P/CH ratio and FF on feed utilization and CH metabolism (Zhao et al. 2016; Cheng et al. 2019).

In gilthead seabream, until now only two studies are available regarding FF effects on growth and intermediary metabolism, and no major effects were reported comparing fish fed 1, 2, or 3 meals per day (Busti et al. 2020), or 2, 4, or 6 meals per day (Yilmaz and Eroldogan 2011). However, Busti et al. (2020) provided the same amount of feed per day distributed by the different meals, which may not allow a clear evaluation of the effects of FF on growth performance, feed utilization, or metabolic responses.

Table 3. Feeding frequency (FF) effects on fish growth, feed utilization, and intermediary metabolism.

			Higher FF promoted:															
									Liver comp.		Time	Enzymatic activity or mRNA levels				Plasmatic metabolites			
Fish species	**FF tested** (meals/day)	**IBW** (g)	**FI**	**FBW**	**FE**	**PER**	**HSI**	**VSI**	**LIP**	**GLY**	**AF**	**gh**	**gdh**	**gk/hk**	**g6pase**	**GLU**	**TG**	**CHO**	**Reference**
Arapaima	2, or 3	500	→	→	→	→					24h					→		→	(Pedrosa et al. 2019)
Atlantic salmon	2, or 4	195	*	↑	→		→												(Sun et al. 2014)
Blunt snout bream	1, 2, 3, 4, or 5	9	*	↑	↑		→				24h	↓							(Tian et al. 2015)
Cobia	1, 2, 3, 4 or 6	110	→	→	→														(Costa-Bomfim et al. 2014)
Common carp	2, 4 or 6	2	↑	↑	↓					→									(Murai et al. 1983)
	2 or 4	56									24h	↓		↑		↓			(Cheng et al. 2019)
Dark-banded rockfish	1, 2, or 3	14	↑	↑	→	→					24h					→		↑	(Oh et al. 2018)
Dolly varden char	1, 2, 3, 4, 5 or 6	9	↑	↑			↑				24h					→	→	→	(Guo et al. 2018)
Gibel carp	2, 4, or 6	4	↑	↑	↑						-								(Zhao et al. 2016)
Gilthead seabream	2, 4, or 6	10	→	→	→	→													(Yilmaz and Eroldogan 2011)
	1, 2, or 3	88	*	→	→	→	→	→			5h					→	→	→	(Busti et al. 2020)
Hybrid striped bass	1, 2, 3, or 4	13	→	→	↑		→	↓											(Liu and Liao 1999)
Hybrid tilapia	2 or 6	8	*	↑	↓	↑					n.a.			→	→				(Tung and Shiau 1991)
Korean rockfish	1, or 2	6	→	→	→	→	→		→										(Lee et al. 2000b)
Lebranche mullet	1, 3, 5, or 7	14	↑	↑	↑						24h					↑	↑	↑	(Silva et al. 2020)
Nile tilapia	2, 3, 4 or 5	1	↑	↑	↑	↑													(Daudpota et al. 2016)
Olive flounder	1, 2, or 3	4	↑	↑	→	→													(Lee et al. 2000a)
		11	↑	↑	→	→													(Lee and Pham 2010)
Persian sturgeon	3, 4, or 5	0.9	↑	↑	↑	→					24h					→			(Zolfaghari et al. 2011)
Rainbow trout	2, 3, or 4	9	n.a.	↑															(Basçinar et al. 2001)

			Higher FF promoted:															
									Liver comp.		Time	Enzymatic activity or mRNA levels				Plasmatic metabolites			
Fish species	**FF tested** (meals/day)	**IBW** (g)	**FI**	**FBW**	**FE**	**PER**	**HSI**	**VSI**	**LIP**	**GLY**	**AF**	**gh**	**gdh**	**gk/hk**	**g6pase**	**GLU**	**TG**	**CHO**	**Reference**
	4 or continuous feeding†	6	*	↑	↑		↑	→		↑									(Hung and Storebakken 1994)
Rock bream	1, 2, 3, or 4	12	↑	↑	→														(Oh and Maran 2015)
Spotted seabass	1, 2, or 3	6	↑	↑	→	→													(Rahman and Lee 2017)
White seabream	2, 3, or 4	55	→	→		→	→		→	→	4h		→	↓		→	→	→	(Enes et al. 2015)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in feed intake (FI), feed utilization indices, or in the specific intermediary metabolism parameters. *, fish fed with a daily fixed amount of feed. †, continuous feeding using automatic feeders.

AF: after feeding; CHO: plasmatic cholesterol; FBW: final body weight; FE: feed efficiency; FF: feeding frequency; FI: feed intake; g6pase: glucose-6-phosphatase; gdh: glutamate dehydrogenase; gh: growth hormone; GLU: plasmatic glucose; GLY: glycogen content; gk: glucokinase; hk: hexokinase; HSI: hepatosomatic index; IBW: initial body weigh; LIP: lipid content; n.a.: not available; PER: protein efficiency ratio; TG: plasmatic triglycerides; VSI: Visceral somatic index.

1.3.3. Intestine functionality and health

FF manipulation may modulate intestine feed transit, affecting intestinal functionality and health parameters, such as histomorphology, microbiota composition, or digestive enzymes activities, which can compromise digestion efficiency and nutrient utilization (Enes et al. 2015; Cheng et al. 2019; Imsland et al. 2019; Salger et al. 2020). However, the way this modulation occurs is not clear, as described by **Table 4**, which summarizes FF effects on fish intestine histomorphology, microbiota composition, and digestive enzymes in several fish species. For instance, in gilthead seabream, Gilannejad et al. (2021) observed that changing daily FF affected the gut filling rate and some digestive enzymes activities, such as pepsin, but did not modify the evacuation rate or trypsin activity. In another study with gilthead seabream, FF modification significantly affected the gastric pH and pepsin activity pattern, with 2 meals and continuous feeding allowing a better and prolonged gastric digestion and consequently increasing juvenile's growth (Yúfera et al. 2014). However, in the study of Busti et al. (2020), despite no effect being reported for growth performance of on-growing gilthead seabream, an increase in the daily amylase, lipase, and protease activities was observed when FF increased from 1 to 2-3 meals per day, though those differences tended to disappear when the activities were reported in measured activity per meal.

Also, for other fish species, the digestive enzyme responses are not clear. For instance, in Nile tilapia and arapaima juveniles, changing the FF protocol did not affect the activity of the digestive enzymes evaluated, namely amylase, lipase, and protease (Thongprajukaew et al. 2017; Pedrosa et al. 2019). However, in Lebranche mullet, white seabream, and blunt snout bream juveniles, modification of the FF protocol affected some of those enzyme activities (Enes et al. 2015; Tian et al. 2015; Silva et al. 2020). In Lebranche mullet, changing the daily FF from 1 to 3 meals per day promoted an increase of amylase, lipase, and protease activities, but further increasing FF to 5 or 7 meals per day led to a decrease in the enzymatic activity (Silva et al. 2020). The decrease of the intestine amylase activity in fish fed 3 meals per day was also reported in white seabream (Enes et al. 2015). However, in blunt snout bream, a decrease of amylase activity was only observed when fish were fed more than 5 meals per day, while lipase and protease activities were not affected by the FF protocol (Tian et al. 2015).

Regarding FF effects on intestine histomorphology, Imsland et al. (2019) observed that lumpfish (*Cyclopterus lumpus*), despite presenting intestine inflammation in all FF tested, the inflammation severity increased in fish fed daily in comparison with those fed only 3

or 4 days per week, presenting those fish that fed daily a higher lamina propria width. On the other hand, the distal intestine of Nile tilapia fed 1 or 2 meals per day did not suffer any histomorphological changes (Sherif et al. 2020).

Regarding intestine microbiota, Nile tilapia fed in an alternate-day feeding regime presented the highest intestine microbial biodiversity compared with fish fed every third day, or fasted fish (Salger et al. 2020). Similar observations were made also for Nile tilapia, where alternate weekly exchange of feeding regimes also affected the intestine microbiota composition (Sherif et al. 2020).

More studies must be performed regarding the effects of FF on the oxidative and immune intestine status since the few available studies do not focus on the intestine, but rather on the liver or head kidney. For instance, regarding oxidative stress status, blunt snout bream juveniles fed 3 or 4 meals per day presented lower liver malondialdehyde content than those fed 1, 2, 5, or 6 meals per day (Li et al. 2014). Regarding the immune status, *interleukin 1β* (*il1β*) and *tumor necrosis factor-α* expression was significantly increased in Nile tilapia that fed 1 time per day than those fed 2 meals per day (Sherif et al. 2020).

Table 4. Feeding frequency (FF) effects on fish intestine histomorphology, microbiota composition, and digestive enzymes.

				Higher FF promoted:					
			Time	Morpho.	Micro.	Digestive enzymes activity			
Fish species	**FF tested** (meals/day)	**IBW** (g)	**AF**	**changes**	**changes**	**Amy.**	**Lip.**	**Prot.**	**References**
Arapaima	2, or 3	500	24h			I: →	I: →	I: →	(Pedrosa et al. 2019)
Commom carp	2, or 4	56				I: ↓			(Cheng et al. 2019)
Gilthead seabream	1, 3, 5, continuos feeding	18	n.a.					GI: →	(Gilannejad et al. 2021)
	1, 2, or 3	88	5h			I: ↑	I: ↑	I: ↑	(Busti et al. 2020)
Lebranche mullet	1, 3, 5, or 7	14	24h			I: Inc	I: Inc.	I: Inc	(Silva et al. 2020)
Lumpfish	†	22	n.a.	I, PC: Yes					(Imsland et al. 2019)
Nile tilapia	‡	4	n.a.		D: Yes				(Salger et al., 2020)
	1, 2, or 3	12	24h			I: →	I: →	I: →	(Thongprajukaew et al. 2017)
	1, or 2	50	n.a.	DI: No	M: Yes				(Sherif et al. 2020)
White seabream	2, 3, or 4	55	4h			I, PC: ↓			(Enes et al. 2015)
Blunt snout bream	1, 2, 3, 4, or 5	9	24h			I: ↓	I: →	I: →	(Tian et al. 2015)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in intestine functionality or health parameters. †, fish were fed 3, 4, or 7 days per week. ‡, fish were fed daily, every other day, every third day, or not at all. Letters indicate the tissue where the gene expression or enzymatic activity was analyzed. D: digesta; DI: distal intestine; GI: gastrointestinal tract (included all digestive system); I: intestine; M: mucosa; PC: pyloric caeca.

AF: after feeding; Amy.: amylase; D: digesta; FF: feeding frequency; IBW: initial body weight; Inc.: inconclusive; Lip.: lipase; M: mucosa; Micro.: microbiota; Morpho.: morphological; n.a.: not available; Prot.: proteases

1.4. Gilthead seabream (*Sparus aurata*)

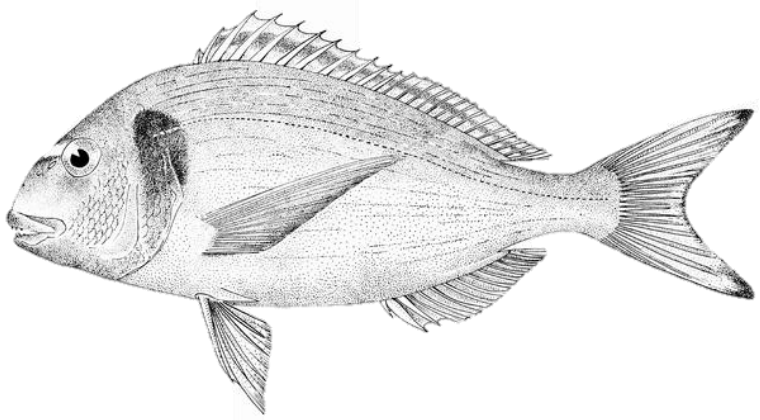

Figure 3. Gilthead seabream, *Sparus aurata* (Colloca and Cerasi 2005).

Sparus aurata (Linnaeus, 1758), or gilthead seabream as the common name, belongs to the class Actinopterygii, order Perciformes, and family Sparidae (**Figure 3**). According to the International Union for Conservation of Nature (IUCN) red list (2014), the species is listed as "Least concern" and can be found in the western and southern Black Sea, Mediterranean Sea, and in the eastern Atlantic Ocean, around Canary Islands, British Isles, Strait of Gibraltar to Cape Verde (Russell et al. 2014). It is an euryhaline species, reproduces in the open sea during October-December and juveniles live in coastal lagoons, seagrass beds, and sandy bottoms usually until 30 meters depth, but can go up to 150 meters depth (Colloca and Cerasi 2005; Russell et al. 2014; Froese and Pauly 2019). Gilthead seabream is a protandrous hermaphrodite species, being male in the first and second year of life, changing to female in the third year (Colloca and Cerasi 2005; Froese and Pauly 2019). Gilthead seabream is a solitary fish or establishes small fish schools (Colloca and Cerasi 2005; Froese and Pauly 2019).

Gilthead seabream was first produced by the ancient Egyptians or Italians, through extensive aquaculture. These civilizations took advantage of the natural trophic migration of juveniles to shallow waters to catch and keep them enclosed in coastal lagoons until having enough commercial value (Colloca and Cerasi 2005). Intensive aquaculture production was developed and implemented during the 1980s. First, successful artificial breeding was achieved in Italy, and thereafter large-scale production systems were implemented in Spain, Italy, Greece, and Portugal (Colloca and Cerasi 2005). Nowadays, gilthead seabream is mainly farmed intensively in sea cages at an average

of 15-25 kg m^{-3}, with a food conversion ratio of 1.5-2.0, and needs 18-24 months from eclosion to 400 g of body weight (Pavlidis and Mylonas 2011).

In 2019, 267 012 tonnes of gilthead seabream were obtained, with 97% originating from the aquaculture industry and the remaining 3% from fisheries. Egypt (25%), Tunisia (22%), and France (14%) were the countries with the highest volumes of captured gilthead seabream, while Portugal only contributed with about 3% of the world caught gilthead seabream (FIGIS 2021b). Regarding aquaculture production, Turkey (39%), Greece (21%), and Egypt (14%) were the main producers of gilthead seabream, while Portugal only produced 2 316 tonnes, representing less than 1% of the global gilthead seabream production (FIGIS 2021a). Globally gilthead seabream production generated 1 275 882 000 USD, and Portugal contributed 17 577 000 USD, which represented almost 1.4% of the world economic value of gilthead seabream (FIGIS 2021a).

1.4.1. Nutritional requirements

In the wild, gilthead seabream is mainly a carnivorous fish, feeding mostly on shellfish, like mussels and oysters (Froese and Pauly 2019). In aquaculture, the nutritional requirements must be completely satisfied to promote adequate growth and feed utilization, health, and welfare status. **Table 5** presents a summary of the dietary macronutrient and micronutrient recommendations for gilthead seabream. In this chapter, fish are divided into the following classes: fingerlings (from metamorphosis up to 3 g), juveniles (from 3 to 200 g), and on-growing (more than 200 g).

Protein and AA are essential for all living organisms' cell structure and metabolism. Fish cannot synthesize essential AA, thus must acquire them through diet (NRC 2011). Overall, the dietary protein requirement decreases with gilthead seabream growth. For fingerlings, the dietary protein requirement ranges between 51-55% (Vergara et al. 1996a; Lupatsch et al. 2003; Fountoulaki et al. 2005a), for juveniles, it ranges between 42-55% (Vergara and Jauncey 1993; Santinha et al. 1996; Lupatsch et al. 2003), and for on-growing fish, it is estimated to be around 40% (Lupatsch et al. 2003). The dietary AA requirement for gilthead seabream fingerlings and juveniles was estimated to be, respectively (g/16 g N): 3.08-5.55, arginine; 5.05-5.13, lysine; 1.35-2.98, threonine; 1.89-3.54, histidine; 1.12-2.55, isoleucine; 4.75-5.32, leucine; 2.42-2.60, methionine; 3.17-5.76, phenylalanine + tyrosine; 2.7-3.21, valine; and 0.75-0.94, tryptophan (Kaushik

1998; Peres and Oliva-Teles 2009; Gaber et al. 2016). These AA requirements were estimated based on the ideal protein concept strategy.

Other studies were performed to estimate the essential AA requirements based on dose-response studies. For instance, Luquet and Sabaut (1974) estimated that gilthead seabream need 5 g/16 g N of lysine, 4 g/16 g N of methionine + cystine, 0.6 g/16 g N of tryptophan, and less than 2.6 g/16 g N of arginine; and Marcouli et al. (2005) estimated that gilthead seabream juveniles need 4.88 g/16 g N of lysine and 2.77g/16g N of methionine.

Lipids have important structural and energy functions, besides being involved in other physiological functions (NRC 2011). The optimal dietary lipid level for fingerlings, juveniles, and on-growing gilthead seabream range between 15-16% (Fountoulaki et al. 2005a), 16-21% (Vergara and Jauncey 1993; Santinha et al. 1999), and 22-28% (Vergara et al. 1999), respectively, meaning that dietary lipid content for this species can be increased with fish growth. Lipids are also a source of n-3 highly unsaturated fatty acids (HUFAs), which are required for marine fish and participate in several functions, such as membrane permeability and plasticity, enzymatic activation, and prostaglandin production (Ibeas et al. 1994). The HUFAs dietary requirements seem to vary with fish size. For instance, in 3-day-old larvae, the HUFAs requirement is about 5.5% (included in rotifers), with an eicosapentaenoic acid/docosahexaenoic acid (EPA/DHA) ratio of about 2.6 (Rodriguez et al. 1994), and in juveniles, it was estimated to range between 0.9-1.9% of the dry diet (Kalogeropoulos et al. 1992; Ibeas et al. 1994; 1996), depending on the EPA/DHA ratio (range between 0.5-2.2) (Kalogeropoulos et al. 1992; Ibeas et al. 1996).

Although fish do not have dietary CH requirements the provision of an appropriate amount of digestible CH in aquafeeds is important to spare the use of protein as an energy source (NRC 2011). Independently of life stage, an dietary inclusion of up to 20% of starch is well accepted by gilthead seabream, without affecting fish growth or physiologic responses (Fountoulaki et al. 2005b; Fernández et al. 2007; Couto et al. 2008; Enes et al. 2008; Couto et al. 2012; Castro et al. 2016a; b; 2019; García-Meilán et al. 2020). Higher starch levels were also tested without compromising growth performance but affecting lipid and glucose metabolism (Bou et al. 2014).

Data on vitamins and minerals requirements of gilthead seabream is scarce. The importance of the vitamin B complex was described by Morris et al. (1995) since diets deficient in vitamin B complex compromised growth, feed efficiency, and apparent net

protein utilization. According to the authors, the recommended amount of vitamin B complex for juveniles is 5 g kg^{-1} of diet, including 69.9 mg kg^{-1} of thiamin, 208.3 mg kg^{-1} of riboflavin, 48.6 mg kg^{-1} of pyridoxine, 800 mg kg^{-1} of niacin, 305.3 mg kg^{-1} of pantothenic acid, 300 mg kg^{-1} of biotin, and 16.9 mg kg^{-1} diet of folic acid (Morris et al. 1995). Ascorbic acid, also known as vitamin C, requirements are less than 25 mg kg^{-1} diet for juveniles (Henrique et al. 1998) and the recommended amount of vitamin D_3 is 0.3 mg kg^{-1} of diet for juveniles fed diets containing high levels of plant ingredients (Domínguez et al. 2021). Regarding vitamin E, juveniles seem to require at least 150 mg kg^{-1} of diet, since vitamin E-deficient diets compromised the immune and oxidative stress status increasing fish mortality (Montero et al. 2001).

Regarding minerals, only dietary requirements of selenium, copper, manganese, and zinc requirements were studied to date, being the requirement levels highly dependent on fish age. In fingerlings, selenium, copper, manganese, and zinc requirements were estimated to be 11.65 (Saleh et al. 2014), 21.0, 4.0, and 119 mg kg^{-1} of diet (Eryalçın et al. 2020), respectively. For juveniles, dietary selenium and copper inclusion levels should be 0.94-1.1 and 5.5 mg kg^{-1} of diet, respectively (Domínguez et al. 2019; Mechlaoui et al. 2019; Domínguez et al. 2020a), and zinc level should range between 60 and 300 mg kg^{-1} of diet depending on the year season (Serra et al. 1996; Carpenè et al. 1999). Regarding manganese, up to 19 mg kg^{-1} of diet seems to be enough to cover requirements of juveniles fed PF-based diets; however, dietary supplementation levels up to 30 mg kg^{-1} of the diet should be considered for fish under stressful conditions (Domínguez et al. 2020b). The dietary phosphorous requirement was estimated for juveniles, as being 0.75% of the diet (Pimentel-Rodrigues and Oliva-Teles 2001).

Table 5. Summary of the dietary macronutrient and micronutrient recommendations for gilthead seabream.

Nutrient	Life stage	Recommendation level	References
Protein	Fingerling	51-55%	(Vergara et al. 1996a; Lupatsch et al. 2003; Fountoulaki et al. 2005a)
	Juvenile	42-55%	(Vergara and Jauncey 1993; Santinha et al. 1996; Lupatsch et al. 2003)
	On-growing	40%	(Lupatsch et al. 2003)
Lipids	Fingerling	15-16%	(Fountoulaki et al. 2005a)
	Juvenile	16-21%	(Vergara and Jauncey 1993; Santinha et al. 1999)
	On-growing	22-28%	(Vergara et al. 1999)

Nutrient	Life stage	Recommendation level	References
Carbohydrates	Fingerling	≤20%	(Fernández et al. 2007)
	Juvenile	≤20%	(Couto et al. 2008; Enes et al. 2008; Castro et al. 2016a, b; García-Meilán et al. 2020; Magalhães et al. 2021)
Essential amino acids			
Arginine	Fingerling and juvenile	2.6-5.55 g/16 g N	(Luquet and Sabaut 1974; Kaushik 1998; Marcouli et al. 2005; Peres and Oliva-Teles 2009; Gaber et al. 2016)
Lysine		5.05-5.13 g/16 g N	
Threonine		1.35-2.98 g/16 g N	
Histidine		1.89-3.54 g/16 g N	
Isoleucine		1.12-2.55 g/16 g N	
Leucine		4.75-5.32 g/16 g N	
Methionine		2.42-2.60 g/16 g N	
Methionine + Cystine		4 g/16 g N	
Phenylalanine +Tyrosine		3.17-5.76 g/16 g N	
Valine		2.7-3.21 g/16 g N	
Tryptophan		0.6-0.94-g/16 g N	
Essential fatty acids			
EPA/DHA ratio	Fingerling	~2.6	(Rodriguez et al. 1994)
	Juvenile	0.5-2.2	(Kalogeropoulos et al. 1992; Ibeas et al. 1996)
Vitamins			
Vitamin B complex	Juvenile	5 g kg^{-1} diet	(Morris et al. 1995)
Vitamin C	Juvenile	<25 mg kg^{-1} diet	(Henrique et al. 1998)
Vitamin D_3	Juvenile	0.3 mg kg^{-1} diet	(Domínguez et al. 2021)
Vitamin E	Juvenile	150 mg kg^{-1} diet	(Montero et al. 2001)
Minerals			
Copper	Fingerling	21 mg kg^{-1} diet	(Eryalçın et al. 2020)
	Juvenile	5.5 mg kg^{-1} diet	(Domínguez et al. 2019)
Manganese	Fingerling	4 mg kg^{-1} diet	(Eryalçın et al. 2020)
	Juvenile	19-30 mg kg^{-1} diet	(Domínguez et al. 2020b)
Phosphorus	Juvenile	0.75% on diet	(Pimentel-Rodrigues and Oliva-Teles 2001)
Selenium	Fingerling	11.65 mg kg^{-1} diet	(Saleh et al. 2014)
	Juvenile	0.94-1.1 mg kg^{-1} diet	(Mechlaoui et al. 2019; Domínguez et al. 2020a)
Zinc	Fingerling	119 mg kg^{-1} diet	(Eryalçın et al. 2020)
	Juvenile	60-300 mg kg^{-1} diet	(Serra et al. 1996; Carpenè et al. 1999)

EPA/DHA ratio: Eicosapentaenoic acid/docosahexaenoic acid.

1.4.2. Plant feedstuffs as a dietary protein source

As already mentioned in section 1.1.1., FM and FO are still considered the most adequate protein and lipid sources to be used in aquaculture diets (Tacon and Metian 2008; 2015). However, their inclusion in the diets should be reduced due to: (i) reduction

and/or stagnation of wild fisheries stocks available for FM and FO production; (ii) increase of FM and FO prices in the global market; (iii) increased market and social pressure on feed manufactures to replace FM and FO on aquafeeds by more environmentally sustainable alternatives (Tacon and Metian 2008; Olsen and Hasan 2012; Naylor et al. 2021).

The most studied alternatives to FM are PF, which are highly available on the market, have a relatively constant chemical composition, and are cost-effective (Enes et al. 2011). However, PFs have some disadvantages, as the presence of ANF or the lower nutrient digestibility and palatability (Francis et al. 2001; Hua et al. 2019; Glencross et al. 2020; Naylor et al. 2021). These characteristics may affect FI, feed utilization, growth, intestine morphology, microbiota composition, absorptive and digestive processes, and the immune and oxidative status of several fish species, including gilthead seabream (Gómez-Requeni et al. 2003; 2004; Sitjà-Bobadilla et al. 2005; De Francesco et al. 2007; Bonaldo et al. 2008; Santigosa et al. 2008; Green et al. 2013; Estruch et al. 2015; Izquierdo et al. 2015; Batista et al. 2016; Benedito-Palos et al. 2016; Estruch et al. 2018; Miao et al. 2018; Naylor et al. 2021).

The study by Kissil and Lupatsch (2004) was the first one to demonstrate a successful total substitution of FM by PFs on gilthead seabream juveniles' diets without affecting fish growth or feed efficiency (FE). However, most studies with gilthead seabream juveniles indicate that fish tolerate well up to 50% of PFs in their diets, while higher dietary inclusion levels may bring some negative effects on growth, feed utilization, intermediary metabolism, and intestine functionality and health (Gómez-Requeni et al. 2003; 2004; Sitjà-Bobadilla et al. 2005; De Francesco et al. 2007; Izquierdo et al. 2015; Benedito-Palos et al. 2016). For instance, in the studies of Gómez-Requeni et al. (2004) and Sitjà-Bobadilla et al. (2005), dietary incorporation of above 50% PFs decreased FI and growth, as well as decreased the plasmatic cholesterol level, although the hepatosomatic index (HSI) was not affected and the FE was increased. De Francesco et al. (2007) also observed a decrease in FI of gilthead seabream juveniles fed diets including 75% PFs, besides an increase of HSI and hepatic lipid content, and a lack of effect on growth. Furthermore, high dietary inclusion of PFs can also promote histomorphological changes, such as a decrease of intestine fold height, enlargement of submucosa and lamina propria, increase in the number of inflammatory cells, and modification on enterocytes vacuolization (Sitjà-Bobadilla et al. 2005; Bonaldo et al. 2008; Santigosa et al. 2008; Kokou et al. 2015; Monge-Ortiz et al. 2016; Kokou et al. 2017; Estruch et al. 2018).

The absence of negative effects when PF-based diets were used for on-growing gilthead seabream suggests that bigger fish have a higher tolerance to PFs, and thus, seem to be able to successfully use diets with a full replacement of FM by PFs, without negative effects on growth and feed utilization (Dias et al. 2009; Monge-Ortiz et al. 2016; Estruch et al. 2018).

A deeper evaluation of the effects of the use of PF-based diets on appetite regulation, growth and intermediary metabolism, and intestine functionality and health of gilthead seabream will be done in the present thesis.

1.4.3. Dietary composition effects

This section will focus on the effects of dietary composition, namely the use of PF vs. FM and the P/CH ratio, in appetite regulation, growth, intermediary metabolism, and intestine functionality and health of gilthead seabream, the species studied in the present thesis.

Appetite regulation

As previously mentioned, the dietary composition can affect fish appetite regulation mechanisms and consequently FI, compromising the economic and environmental sustainability of aquaculture. However, little is known about appetite regulation in fish and its connection with diet composition; and even less information is available for gilthead seabream. To our knowledge, there are only two studies that focus on the effects of dietary composition on gilthead seabream appetite regulation (Babaei et al. 2017; Pulido-Rodriguez et al. 2021). One regarding the effects of PF-based diets (Pulido-Rodriguez et al. 2021), and the other evaluating the effects of dietary P/CH ratios (Babaei et al. 2017). Pulido-Rodriguez et al. (2021) evaluated the appetite regulation-related genes effects on gilthead seabream fed different protein sources. The authors concluded that PF-based diets did not affect the endocrine appetite regulation mechanisms, since none of the appetite regulation-related genes were affected in comparison with fish fed FM-based diets. This is in agreement with what was previously reported for other fish species, such as Atlantic salmon, pacu, and pearl gentian grouper (*Epinephelus fuscoguttatus*♀ × *E. lanceolatus*♂) (Sissener et al. 2013; Volkoff et al. 2017; He et al. 2021).

Regarding dietary P/CH ratios, Babaei et al. (2017) observed that changing the dietary P/CH ratio from 58/15 to 39/37 promoted a decrease of *cck* and *ghrelin* gene expression

in the intestine and an increase of *ghrelin* expression in the brain of gilthead seabream. This was the first evidence that dietary P/CH ratio can affect the appetite regulation mechanisms in gilthead seabream, but the physiologic mechanisms for this effect remain utmost unexplored. Furthermore, in the previous studies, gilthead seabream was fed with a daily fixed amount of feed, and not *ad libitum*, and this can affect the mechanism of FI control by fish.

Growth and intermediary metabolism

In general, a dietary inclusion of more than 50% of PFs affects growth, feed utilization, and intermediary metabolism of gilthead seabream juveniles (**Table 6**) (Gómez-Requeni et al. 2003; 2004; Sitjà-Bobadilla et al. 2005; De Francesco et al. 2007; Izquierdo et al. 2015; Benedito-Palos et al. 2016). These effects include the decrease of FI (Gómez-Requeni et al. 2004; Sitjà-Bobadilla et al. 2005; De Francesco et al. 2007), growth (Gómez-Requeni et al. 2004; Sitjà-Bobadilla et al. 2005; Izquierdo et al. 2015; Benedito-Palos et al. 2016), and plasmatic cholesterol level (Gómez-Requeni et al. 2004; Sitjà-Bobadilla et al. 2005; Benedito-Palos et al. 2016), and an increase of FE (Gómez-Requeni et al. 2004; Sitjà-Bobadilla et al. 2005; De Francesco et al. 2007), PER (Gómez-Requeni et al. 2004; De Francesco et al. 2007), HSI (De Francesco et al. 2007) and hepatic lipid content (Sitjà-Bobadilla et al. 2005; De Francesco et al. 2007).

Nevertheless, these effects were not observed when PFs were used in diets for on-growing fish, suggesting that bigger fish have a higher tolerance to PF-based diets, successfully using diets with a full replacement of FM by PFs (Dias et al. 2009; Monge-Ortiz et al. 2016; Estruch et al. 2018).

Table 6. Plant feedstuffs (PF)-based diets effects on growth, feed utilization, and intermediary metabolism of gilthead seabream, in comparison with fish fed FM-based diets.

			The PFs use promoted:																
									Liver comp.	**Time**	**Enzymatic activity or mRNA levels**					**Plasmatic metabolites**			
Blend of PFs used	**Inclusion level** (%)*	**IBW** (g)	**FI**	**FBW**	**FE**	**PER**	**HSI**	**VSI**	**LIP**	**AF**	**gh**	**gdh**	**gk**	**g6pase**	**fas**	**GLU**	**TG**	**CHO**	**References**
SPC, CG; WG, RM, WM	94	4	→	↓	↓														(Izquierdo et al. 2015)
SBM, PM, WG, WM	35	14	→	→	→	→				6h	→	→	→	→	→	→	→		(Gómez-Requeni et al. 2003)
SPC, CG, WG, RM, WM	96	15	→	↓	→		→	→		24h						→	→	↓	(Benedito-Palos et al. 2016)
CG, WG, PM, RM	50, 75, 100	16	↓	↓	↑	↑	→	→		6h	→	→				→	↓	↓	(Gómez-Requeni et al. 2004)
CG, WG, PM, RM	50, 75	16	↓	↓	↑		→		→	24h						→		↓	(Sitjà-Bobadilla et al.
white lupin meal	100		↓	↓	→		→		↑							→		↓	2005)
SBM, WM, CG, WG	47, 56	18	→	→	→	→	→	→											(Bonaldo et al. 2008)
WG, SPC, CG,	25	41	**	→	→														(Kissil and Lupatsch
WM	50, 75			→	↑														2004)
	100			→	→														
CG, WG, PM, RM WM	75	99	↓	→	↑	↑	↑	→	↑										(De Francesco et al. 2007)
WG, BBM, SBM, PM, SFM	100	129	→	→	→														(Estruch et al. 2018)
WG, SBM, RM	75	131	→	↑	→		→	↑											(Monge-Ortiz et al.
	100	131	→	→	→		→	→											2016)
PPC, WG, WM, CG	40, 60	140	→	→	→	↓													(Dias et al. 2009)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in feed intake (FI), feed utilization indices, or in the specific intermediary metabolism parameter relative to fish fed FM-based diets. *, inclusion level (%) which replace the FM protein source; **, fish fed with a daily fixed amount of feed.

AF: after feeding; BBM: broad bean meal; CG: corn gluten; CHO: plasmatic cholesterol; fas: fatty acid synthase; FBW: final body weight; FE: feed efficiency; FI: feed intake; g6pase: glucose-6-phosphatase; gdh: glutamate dehydrogenase; gh: growth hormone; gk: glucokinase; GLU: plasmatic glucose; HSI: hepatosomatic index; IBW: initial body weight; LIP: lipid content; PER: protein efficiency ratio; PF: plant feedstuffs; PM: pea meal; PPC: pea protein concentrate; RS: rapeseed meal; SBM: soybean meal; SFM: sunflower meal; SPC: soybean protein concentrate; TG: plasmatic triglycerides; VSI: visceral somatic index; WG: wheat gluten; WM: wheat meal.

Regarding dietary P/CH ratio, gilthead seabream juveniles seem to tolerate up to 20% dietary CH with no negative effects on growth and feed utilization, independently of the protein or CH source used (Vergara et al. 1996a; b; Fernández et al. 2007; Couto et al. 2008; Enes et al. 2008; García-Meilán et al. 2013; Castro et al. 2016a; Magalhães et al. 2021), while higher inclusion levels compromise fish growth, feed utilization and intermediary metabolism responses (Vergara et al. 1996a; b; Fernández et al. 2007; Couto et al. 2008; García-Meilán et al. 2020) (**Table 7**). For instance, a diet with a P/CH ratio of P42/CH28 promoted a decrease in juveniles growth, but no effects were reported when fish were fed diets with P/CH ratios of 58/11, 52/18, or even 46/26 (Vergara et al. 1996b). Couto et al. (2008) also reported a decrease in growth, and in FE, PER, and glutamate dehydrogenase (gdh) activity, and an increase in HSI, visceral somatic index (VSI), hepatic glycogen, and plasmatic glucose levels when the dietary P/CH ratios decreased from 58/8 to 47/26. Castro et al. (2016a) and Magalhães et al. (2021), did not observe any decrease in FI and final body weight (FBW) with changes in dietary P/CH ratio, but lower dietary P/CH ratios affected CH metabolism, lipogenesis, and long-chain polyunsaturated fatty acids biosynthesis, promoting an increase of HSI, VSI, hepatic glycogen, fatty acid synthase (fas) activity, gk activity and gene expression, and *g6pase* expression. However, different results were reported by Fernández et al. (2007), since dietary P/CH ratios of P63/CH5 or P47/CH26 did not affect fish growth, but an intermediary dietary P/CH ratio (P54/CH18) promoted fish growth performance.

Data regarding gilthead seabream fingerlings are scarce, but available results suggest that fish of this life stage might tolerate up to 30% of dietary CH since growth performance decreased when fish were fed P39/CH39 diet compared when they were fed P67/CH7 or P50/CH28 diets (Vergara et al. 1996a).

Results with on-growing gilthead seabream are also few. In the study by Bou et al. (2014), changing the dietary P/CH ratio from P46/CH11 to P46/CH28 did not affect growth nor feed utilization, although slight effects were reported in the intermediary metabolism, such as an increase of hepatic *gk* expression. However, García-Meilán et al. (2020) reported a decrease in FI, growth, and feed utilization in fish fed with a P40/CH39 diet compared with those fed a P46/CH19 diet. It must be kept in mind that when given nutritionally balanced diets fish feed to meet their energy needs (Bureau et al. 2002); therefore, the results described above may not be directly related to the dietary P/CH ratios, but also the different amounts of available digestible energy.

Table 7. Dietary protein/carbohydrate (P/CH) ratios effects on growth, feed utilization, and intermediary metabolism of gilthead seabream.

				Low protein and higher dietary CH content promoted:																
										Liver comp.		Time	Enzymatic activity or mRNA levels				Plasmatic metabolites			
P/CH ratio	Main protein source	Main CH source	IBW (g)	FI	FBW	FE	PER	HSI	VSI	LIP	GLY	AF	gdh	gk	g6pase	fas	GLU	TG	CHO	Reference
67/7 50/28 39/39	FM	CS and dextrin	0.79	*	↓	→														(Vergara et al. 1996a)
63/5 54/18 47/26	FM	Gelatinized CS	2	n.a.	→ ↑ →	→ → →	↑ ↑ ↑	→ → ↑												(Fernández et al. 2007)
58/11 52/18 46/26 42/28	FM	CS and dextrin	5.3	*	↓	→	↑	→												(Vergara et al. 1996b)
47/0 47/10 47/20	FM	CS	20	→	→	→	→	→	→		↑	6h	↓	↑			→			(Enes et al. 2008)
58/8 53/19 47/26	FM	Gelatinized CS	31	↑	↓	↓	↓	↑	↑		↑	6h	↓	→			↑			(Couto et al. 2008)
47/5 47/20	FM, CG, WG	Gelatinized CS	48	→	→	↑	↑	↑	↑	→	↑	4h		↑		↑	↑	↑	↓	(Magalhães et al. 2021)
53/10 44/15 35/21	FM, WM, WG, SPC	WM	70	↓		↓														(García-Meilán et al. 2013)
63/0 50/18	FM	Gelatinized CS	71	→	→	→	↑	↑	↑	→	↑	18h		↑	↑	↑	↓	↑	↓	(Castro et al. 2016a)
46/11 46/19 46/28	FM, CG, WG, SPC	WM	115	n.a.	→	→		↑				24h		↑	→	→	→	→		(Bou et al. 2014)
46/19 40/39	WG, CG, SPC	WM	115	↓	↓	↓														(García-Meilán et al. 2020)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in feed intake (FI), feed utilization indices, or in the specific intermediary metabolism parameters relative to fish fed control diets. *, fish fed with a daily fixed amount of feed.
AF: after feeding; CG: corn gluten; CH: carbohydrates; CHO: plasmatic cholesterol; CS: corn starch; fas: fatty acid synthase; FBW: final body weight; FE: feed efficiency; FI: feed intake; FM: fishmeal; g6pase: glucose-6-phosphatase; gdh: glutamate dehydrogenase; GLU: plasmatic glucose; GLY: glycogen content; gk: glucokinase; HSI: hepatosomatic index; IBW: initial body weigh; LIP: lipid content; n.a.: not available; P: protein; PER: protein efficiency ratio; SPC: soybean protein concentrate; TG: plasmatic triglycerides; VSI: Visceral somatic index; WG: wheat gluten; WM: wheat meal.

Intestine functionality and health

It was already established that one of the most important factors to maintain intestine health is the use of balanced diets which fulfill nutritional requirements (Dawood 2021). Thus, the dietary composition has an important impact on intestine health and functionality through several pathways, including intestine oxidative and immune status, morphology, microbiota composition, and digestive enzymes activities (Oliva-Teles 2012). **Tables 8** and **9** summarize the effects of PF-based diets and dietary P/CH ratios on gilthead seabream intestine histomorphology, microbiota composition, digestive enzymes, and immunological and oxidative stress status.

Overall, independently of the PF source or dietary inclusion level, PF-based diets promote histomorphological changes in the intestine of gilthead seabream. These changes include a decrease of intestine fold height, enlargement of submucosa and lamina propria, increase in the number of inflammatory cells, and modification on enterocytes vacuolization (Sitjà-Bobadilla et al. 2005; Bonaldo et al. 2008; Santigosa et al. 2008; Kokou et al. 2015; Monge-Ortiz et al. 2016; Kokou et al. 2017; Estruch et al. 2018). These histomorphological changes might trigger immune- and inflammatory responses and might also influence fish antioxidant status. For instance, in Kokou et al. (2017) study, the histomorphological changes in the intestine were accompanied by some antioxidant answers, since hepatic activity of superoxide dismutase (SOD) and glutathione reductase (GR) were affected. However, these responses are not always evident. For instance, Estruch et al. (2018) found significant histomorphological changes in the intestine of gilthead seabream fed 100% PF-based diets, but some immune-related genes analyzed, such as *cox* and *IL1β*, were not affected by the consumption of these diets in comparison with fish fed FM-based diets.

On the other hand, digestive enzymes activities, namely amylase, lipase, and proteases, were not affected by the use of PF-based diets, independently of the dietary inclusion level (Santigosa et al. 2008; Busti et al. 2020).

Regarding microbiota composition, Dimitroglou et al. (2010) observed that gilthead seabream fed PF-based diets had a higher number of operational taxonomic units (OTUs), richness, and diversity indices when compared with fish fed FM-based diets. This is in agreement with what was reported in other fish species, such as Atlantic salmon and Senegalese sole (Bakke-McKellep et al. 2007; Green et al. 2013; Batista et al. 2016), and supports the idea that the non-digestible CH in PF provides the required substrates for intestine bacteria proliferation.

The effects on intestine function and health of dietary P/CH ratio are well-explored in gilthead seabream. For instance, Castro et al. (2016b) and Castro et al. (2019) did not observe any effect on the intestine histomorphology, microbiota composition, digestive enzymes activity, and oxidative stress-related enzymes, when the dietary P/CH ratio was changed from P66/CH0 to P50/CH20. Similar unaffected results in digestive enzymes activities were also reported in other studies with gilthead seabream, such as in García-Meilán et al. (2013), and Couto et al. (2012) studies, where the dietary P/CH ratio was changed from 50/12 and 58/8 to 35/21 and 46/25, respectively. Differently, in the study of Fountoulaki et al. (2005b), changing the dietary P/CH ratio from P50/CH24 to P40/CH36, led to an increase in amylase activity in the whole intestine and pyloric caeca (PC), but did not affect the proteolytic activity in these tissues. Also, in García-Meilán et al. (2020) study, it was observed that gilthead seabream fed a P40/CH39 diet presented lower amylase activity in the PC, but not in the foregut, and higher proteolytic activity in both tissues, than those fish fed P46/CH19 diet.

It is important to mention that none of the available studies regarding the effects of dietary P/CH ratios focused on the fish immune responses, and regarding the effects on the oxidative status, the available studies are limited and focus mainly on the liver and not the intestine (Sitjà-Bobadilla et al. 2005; Kokou et al. 2015; 2017). The only available study focusing on the intestine was performed by Castro et al. (2016b). The authors observed that none of the oxidative stress enzymes measured in the intestine of gilthead seabream, namely catalase (CAT), GR, glutathione peroxidase (GPX), and SOD, were affected when the dietary P/CH ratio changed from P66/CH0 to P50/CH20. Differently, in the liver, CAT activity was decreased and SOD activity increased, suggesting that the intestine might have a limited capacity to deal with oxidative stress (Castro et al. 2016b).

Table 8. Plant feedstuffs (PF)-based diets effects on intestine histomorphology, microbiota composition, digestive enzymes, and immunological markers of gilthead seabream, in comparison with fish fed FM-based diets.

				The PFs use promoted:							
						Enzymatic activity or mRNA levels					
						Digestive			Immunology		
Blend of the PFs used	**Inclusion level** (%)*	**IBW** (g)	**Time AF**	**Morpho. changes**	**Micro. changes**	**Amy.**	**Lip.**	**Prot.**	**COX**	**IL1β**	**References**
SBM, WM	56, 72	16	24h	DI: Yes							(Kokou et al. 2015)
	87			DI: Yes							
CG; WG; PM; RM; LM	50, 75	16	24h	DI Yes							(Sitjà-Bobadilla et al. 2005)
	100			DI: Yes							
CG, WG, PM, RM	50, 75, 100	17	6h	I: Yes		I: →		I: →			(Santigosa et al. 2008)
SBM, WM, CG, WG	47, 56	18	n.a.	DI: Yes							(Bonaldo et al. 2008)
SBM, CG, dextrin	53	24	24h	I: No	M, D: Yes						(Dimitroglou et al. 2010)
SPC, WM	52	27	24h	DI: No							(Kokou et al. 2017)
	72			DI: Yes							
	94			DI: Yes							
SBM, SPC, WG, CG, WM, RM, SFM	88	88	5h			GI: ↓	GI: →	GI: ↓			(Busti et al. 2020)
WG, BBM, SBM, PM, SFM	100	129	40h	FG: Yes					I: →	I: →	(Estruch et al. 2018)
WG, SBM, RM	75, 100	131	n.a.	DI: Yes							(Monge-Ortiz et al. 2016)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in intestine functionality and health parameters relative to fish fed FM-based diets. *, inclusion level (%) which replace the FM protein source. Letters indicate the tissue where the gene expression or enzymatic activity was analyzed. D: digesta; DI: distal intestine; FG: foregut; GI: gastro-intestinal tract (included all digestive system); I: intestine; M: mucosa.

AF: after feeding; Amy.: amylase; BBM: broad bean meal; CG: corn gluten; COX: cyclooxygenase; IL1β: interleukin 1β; IBW: initial body weight; Lip.: lipase; LM: lupin meal; Micro.: microbiota; Morpho.: morphological; n.a.: not available; PM: pea meal; Prot.: proteases; RM: rapeseed meal; SBM: soybean meal; SFM: sunflower meal; SPC: soybean protein concentrate; WG: wheat gluten; WM: wheat meal.

Table 9. Dietary protein/carbohydrate (P/CH) ratios effects on intestine histomorphology, microbiota composition, digestive enzymes, and oxidative stress-markers of gilthead seabream.

					Low protein and higher dietary CH content promoted:									
							Enzymatic activity or mRNA levels							
							Digestive			Oxidative Stress				
P/CH	**Main protein source**	**Main CH source**	**IBW** (g)	**Time AF**	**Morpho. changes**	**Micro. changes**	**Amy.**	**Lip.**	**Prot.**	**CAT**	**GR**	**GPX**	**SOD**	**References**
50/12 44/15 35/21	FM + PF (WG, SPC)	WM	70	5h			FG, PC: →	FG, PC: →	FG: ↓ PC: →					(García-Meilán et al. 2013)
66/0	FM	Gelatinized corn starch	71	6h	DI, FG: No	M: No	I: →	I: →	I: →					(Castro et al. 2019)
50/20				18h						I: →	I: →	I: →	I: →	(Castro et al. 2016b)
58/8 50/19 46/25	FM	Gelatinized corn starch	104	24h			I: →		I: →					(Couto et al. 2012)
50/24 40/36	FM	Dextrin	115	5h			I, PC: ↑		I, PC: →					(Fountoulaki et al. 2005b)
46/19 40/39	PF (WG, CG, SPC)	Wheat starch	115	7h			FG: → PC: ↓	FG, PC: →	FG, PC: ↑					(García-Meilán et al. 2020)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in intestine functionality or health parameters relative to fish fed control diets. Letters indicate the tissue where the gene expression or enzymatic activity was analyzed. DI: distal intestine; FG: foregut; I: intestine; M: mucosa; PC: pyloric caeca.

AF: after feeding; Amy.: amylase; CAT: catalase; CH: carbohydrates; CG: corn gluten; GPX: glutathione peroxidase; GR: glutathione reductase; IBW: initial body weight; Lip.: lipase; Micro.: microbiota; Morpho.: morphological; P: Protein; PF: plant feedstuffs; Prot.: proteases; SOD: superoxide dismutase; SPC: soybean protein concentrate; WG: wheat gluten; WM: wheat meal.

1.4.4. Feeding frequency effects

The FF effects were already explored in section 1.3. of the present thesis. In this section, it will be presented a summary of the effects of FF protocols in appetite regulation, growth, intermediary metabolism, and intestine functionality and health of gilthead seabream, the species studied in the present thesis.

Appetite regulation

To our knowledge, only one study is available in gilthead seabream, focusing on appetite regulation. In that study, none of the appetite regulation-related genes evaluated were affected by FF protocols (1, 3, or 5 meals per day, or continuous feeding) (Gilannejad et al. 2021). In that study, however, the fish were fed with a fixed daily amount of feed, distributed by the different FF protocols, and this may limit the fish´s physiological responses to the FF and voluntary FI.

Growth and intermediary metabolism

Busti et al. (2020) and Yilmaz and Eroldogan (2011) evaluated the growth and intermediary metabolism of gilthead seabream fed 1, 2, or 3 meals per day, or 2, 4, or 6 meals per day, respectively, and did not observe any significant differences between the groups. However, Busti et al. (2020) provided the same amount of feed distributed by the different meals per day, and this may not allow a clear evaluation of the effects of FF on growth performance, feed utilization, or metabolic responses.

Intestine functionality and health

Gilannejad et al. (2021) observed that changing the daily FF affected gilthead seabream gut filling rate and some digestive enzymes activities, such as pepsin, but did not modify the evacuation rate or trypsin activity. In another study, FF modifications significantly affected the rhythm of gastric pH and pepsin activity pattern, with 2 meals and continuous feeding allowing a better and prolonged gastric digestion and consequently increasing juvenile's growth (Yúfera et al. 2014). Busti et al. (2020), despite reporting no effect on growth performance of on-growing gilthead seabream, observed an increase in the daily estimated amylase, lipase, and protease activities when FF increased from 1 to 2-3 meals per day, although those differences tended to disappear when enzyme activities were reported as activity per meal.

1.5. Aims and thesis overview

Feed and feeding practices influence fish growth and feed utilization and have economic, environmental, and social implications, which may compromise aquaculture profitability and sustainability (Kaushik 2013). Overall, the dietary composition, namely the use of PF-based diets and different P/CH ratios, and FF protocols may affect FI, fish growth performance, and intestine functionality and health, which consequently may also compromise fish intermediary metabolism. Despite the importance of understanding and improving FI and utilization, the influence of the dietary composition and FF on appetite regulation mechanism remains largely unknown in fish.

Gilthead seabream (*Sparus aurata*) is one of the main species produced in Europe and seems able to cope with a total replacement of dietary FM by PF (Monge-Ortiz et al. 2016), and with the inclusion of up to 20% of dietary CH to spare the use of protein as an energy source, reduce nitrogen waste, and dietary costs (Fernández et al. 2007; Enes et al. 2011; NRC 2011). However, the integrated effects of diet manipulation, focusing on the dietary protein source and dietary P/CH ratio, and FF on appetite regulation, growth performance, feed utilization, intermediary metabolism, and intestine functionality and health, remain utmost unexplored in gilthead seabream, as well as in fish in general. Hence, the present thesis aimed to explore these topics, increasing the knowledge on gilthead seabream appetite regulation.

To accomplish the aims of the present thesis, two dietary P/CH ratios were used to feed the fish. One with 50% protein and 10% CH (P50/CH10 diet), and the other with 40% protein and 20% CH (P40/CH20 diet). The FF evaluated was 1, 2, or 3 meals per day.

Thus, Chapter 2 aimed to evaluate the integrated effects of using PF-based diets compared to FM-based diets, and dietary P/CH ratios on gilthead seabream appetite regulation, growth, feed utilization, body and liver composition, plasma metabolites indicators of nutrient metabolism, adipose and liver histomorphology, and gene expression of intermediary metabolism-related enzymes. Chapter 3 focused on the effects of FF and dietary P/CH ratio on gilthead seabream appetite regulation-related genes expression and FI. Chapter 4 explored the effects of diets used in Chapter 3 on growth, feed utilization, economic efficiency, body and liver composition, plasma metabolites indicators of nutrient metabolism, and gene expression of intermediary metabolism-related enzymes in gilthead seabream juveniles. Chapters 5 and 6 focus on the intestine's functionality and health. Chapter 5 evaluated the effects of diets used in Chapter 2 on gilthead seabream intestine histomorphology, microbiota composition,

digestive enzymes activity, immunological and oxidative stress-related genes expression. Chapter 6 assessed the effects of diets used in Chapter 3 on gilthead seabream intestine histomorphology, microbiota characterization, digestive and oxidative stress enzymes activities.

Besides the *in vivo* trials, the present thesis included an *in vitro* experiment. Thus, Chapter 7 aimed to increase the knowledge on gilthead seabream adipogenesis characterization, and evaluated leptin, ghrelin, and insulin effects in the adipogenic process.

The integrated discussion and main conclusions of the present thesis are presented in Chapters 9 and 10, respectively.

CHAPTER 2| DIETARY PROTEIN SOURCE AND PROTEIN/CARBOHYDRATE RATIO AFFECTS APPETITE REGULATION-RELATED GENES EXPRESSION IN GILTHEAD SEABREAM (*Sparus aurata*)

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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 5 h after feeding (AF) compared to 24 h 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 140 g gilthead seabream for 41 days. The expression of appetite regulation genes was assessed at 5 and 24 h AF, while other evaluated parameters were assessed only at 5 h AF. Liver *leptin* expression was higher at 5 h AF, and brain *leptin receptor* (*lepr*) expression was higher at 24 h 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 24 h 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 24 h 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 5 h AF), liver *ghrr-b* (at 24 h AF), *glutamate dehydrogenase* and *ghr-ii*. The protein efficiency ratio, hepatosomatic and visceral indices, plasmatic glucose level, and brain *lepr* expression (at 5 h 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.

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

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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 5 h 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 (5 h compared to 24 h 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 2 mm diameter. Pellets were dried in an oven for 48 h and then stored in plastic containers at 4 °C 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 ± 0.7 °C, and supplied with a continuous flow (6.0 l min^{-1}) of filtered seawater with 36.0 ± 1.0 g l^{-1} of salinity, and a dissolved oxygen level near saturation (6.0 ± 0.5 mg 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 ± 0.1 g 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

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 12,018. 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. 18,000 (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.

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 0.3 ml 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 5 h 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 3000 ×*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 24 h 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 −80 °C until analysis. Histology samples were immediately fixed in phosphate-buffered formalin (4%, pH 7.4) for 24 h 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 grey-scale, 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 10× 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 −20 °C 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 2.5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (presented in Table 2), 1 μL of each cDNA sample and autoclaved water until a final volume of 5 μL. 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 2
Genes and primers used for qPCR.

Gene	ID primer	Sequence (5′- 3′)	Accession n°	Tm (°C)	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
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		SEM	PF		SEM	*Two-way ANOVA*		
P/CH ratio	P50/CH10	P40/CH20		P50/CH10	P40/CH20		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; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.
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		SEM	PF		SEM	*Two-way ANOVA*		
P/CH ratio	P50/CH10	P40/CH20		P50/CH10	P40/CH20		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, 5 h after feeding.

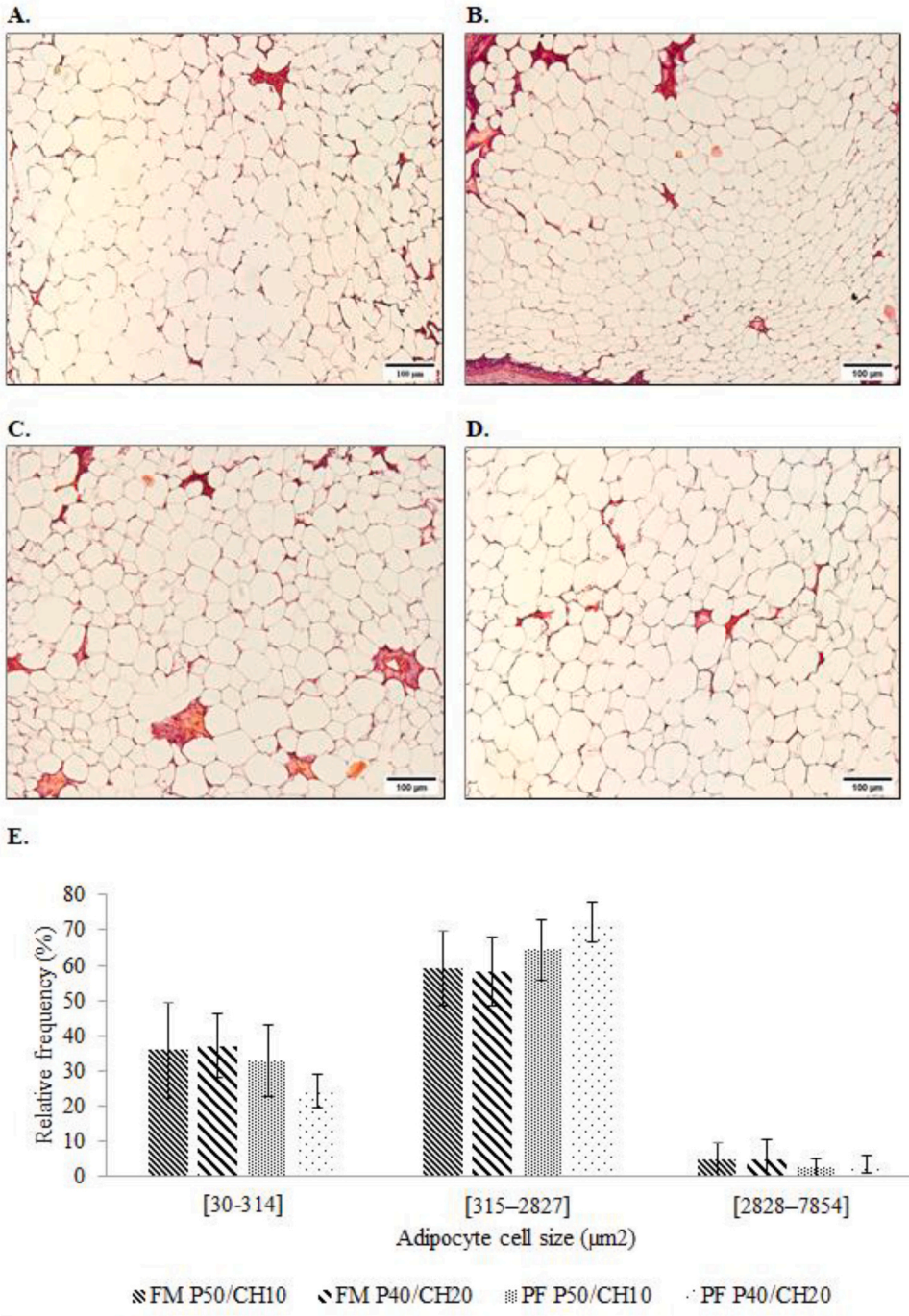
Protein source	FM			PF			*Two-way ANOVA*		
P/CH ratio	P50/CH10	P40/CH20	SEM	P50/CH10	P40/CH20	SEM	PS	P/CH	I
Glucose (mg dL^{-1})	71.9[a]	156.9[Bb]	11.6	70.4[a]	113.3[Ab]	6.2	***	***	***
Cholesterol (mg dL^{-1})	231.5	218.3	8.2	160.9	142.2	5.5	***	ns	ns
Triglycerides (mg dL^{-1})	636.4	517.2	31.8	580.3	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 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 (Fig. 1). Thus, fish fed the FM-based diets had a higher number of smaller adipocytes cells (30-314 μm^2), while fish fed the PF-based diets had a higher amount of medium-size adipocytes (315-2827 μm^2). The liver area covered by lipid vacuoles was not affected by dietary composition (Fig. 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 5 h than at 24 h AF in all dietary treatments, while the opposite was true for brain *leptin receptor* (*lepr*). Brain *leptin* expression was higher at 24 h AF than at 5 h 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 24 h AF in fish fed the P50/CH10 diets and PF-P50/CH10 diet, respectively. The *cart* expression in the brain was higher at 24 h than at 5 h AF, only in fish fed the FM-P50/CH10 diet.



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	-	-	-	-

Fig. 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; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. CH: Carbohydrate; FM: Fishmeal; PF: Plant-feedstuffs; P: Protein.

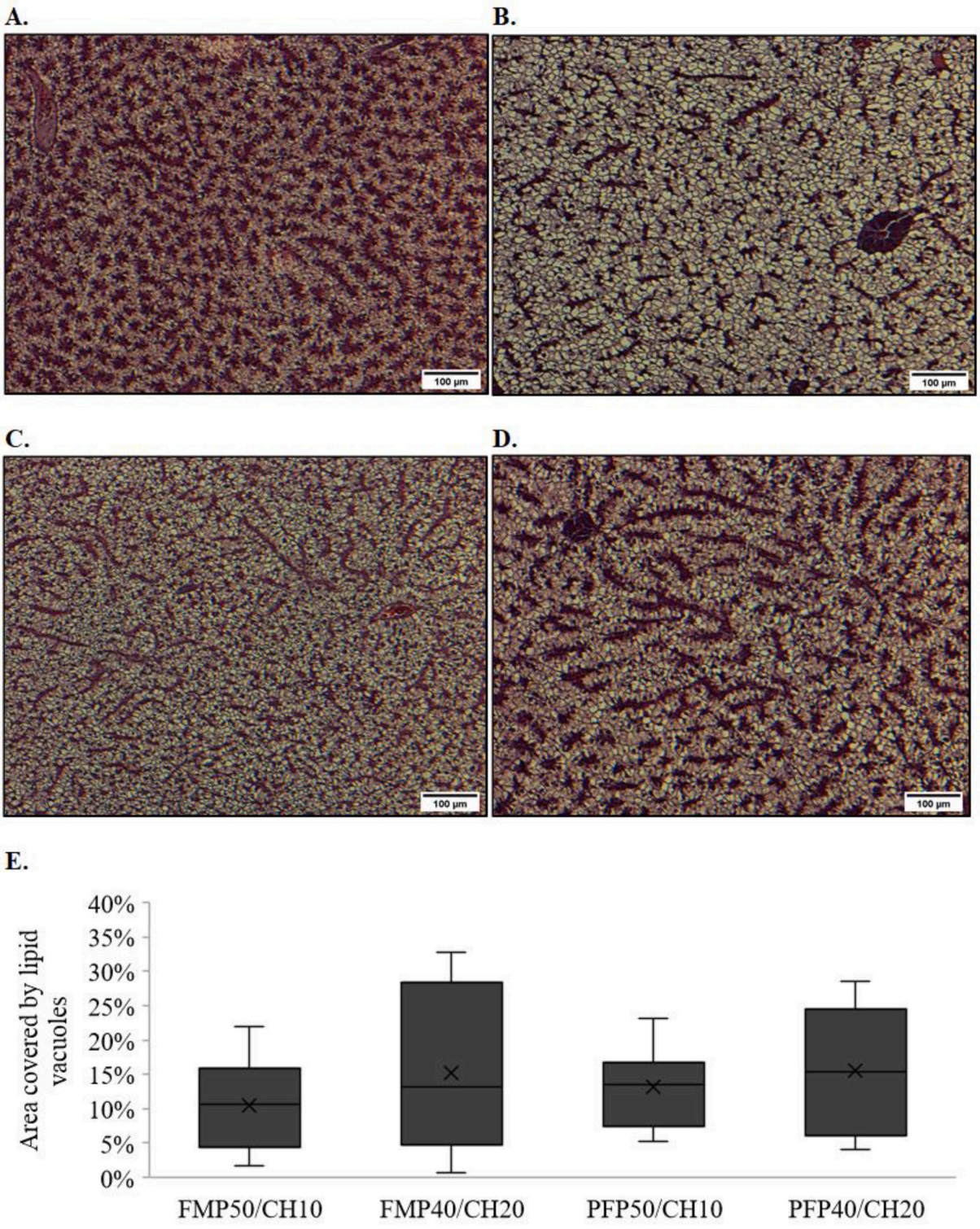


Fig. 2. Representative hematoxylin and eosin-stained histological sections of liver from fish fed FM-P50/CH10 (A), FM-P40/CH20 (B), PF-P50/CH10 (C), and PF-P40/CH20 (D); and area covered by lipid vacuoles (%) in the liver of gilthead seabream fed the experimental diets (E). Images captured at 10× magnification. 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.

At 24 h AF, but not at 5 h, 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 5 h AF, but not at 24 h, 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 5 h AF, while at 24 h 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 24 h AF, but not at 5 h, in fish fed the P50/CH10 diets. In the intestine, the *cck* expression, at 5 h AF, was higher in fish fed the P50/CH10 than the P40/CH20 diets. At 24 h 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*

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

Sampling time	5 h								24 h							
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

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

(*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

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	*
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	**	**
mtor	0.93	0.82	0.04	1.05	0.96	0.05	*	ns	ns

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

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 (5 h compared to 24 h 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 24 h 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 3 h 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, 24 h 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 24 h AF, while plasma ghrelin concentration followed an orexigenic role, decreasing significantly its concentration 5 h AF. As in the present study, a lack of variation in stomach *ghrelin* expression at 24 h 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 24 h 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 5 h 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 3 h 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 9 h 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 24 h than at 5 h AF, liver *leptin* expression was higher at 5 h than at 24 h 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 24 h 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 24 h 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 24 h 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 24 h AF, none being detected at 5 h 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 30 min 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 24 h 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 5 h 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, 5 h 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., 2013). 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 6 h 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 24 h 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 5 h and 24 h 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 24 h 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.

Credit author statement

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

Declaration of Competing Interest

None.

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CHAPTER 3| 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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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.

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

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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 (*C. 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 °C and then stored in plastic containers at 4 °C 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 (*S. 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 (54% protein, 21% nitrogen free extract, 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 °C, 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

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. 18,000 (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).

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 °C 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 °C, 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 °C 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

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

Gene	ID primer	Sequence (5′- 3′)	[1]Accession n°	Tm (°C)	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

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

were not affected by the experimental treatments. Since some of the expressed genes did not have optimum efficiency curves (between 95 and 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

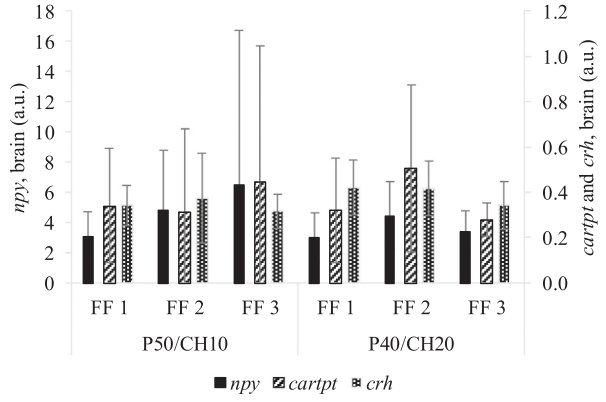
Table 3
Growth performance, feed intake, and feed utilization efficiency of gilthead seabream fed the experimental diets at different feeding frequencies.

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

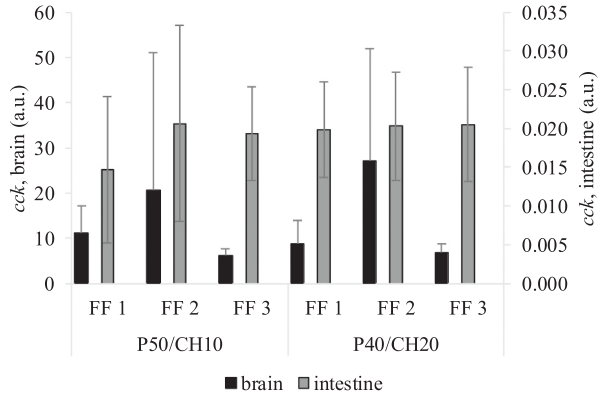
Values presented as means ($n = 3$) and standard deviation. Different upper-case letters denote for significant differences between dietary P/CH ratio and different lower-case letters denote for significant differences between feeding frequencies.
ns: not significant; **$P \leq 0.01$; ***$P \leq 0.001$.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

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



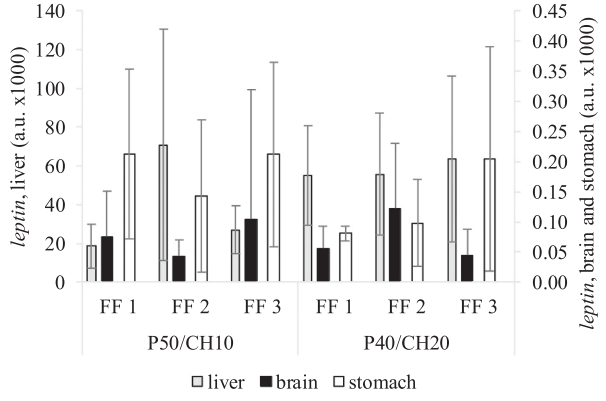
		FF	P/CH ratio	FF x P/CH
Two-way *p*-value	*npy*	0.164	0.607	0.890
	cartpt	0.718	0.329	0.172
	crh	0.340	0.117	0.794

(b) *cck*



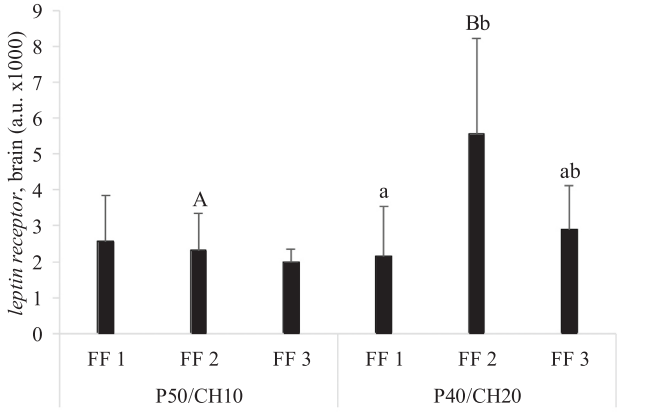
		FF	P/CH ratio	FF x P/CH	† FF		
					1	2	3
Two-way *p*-value	*cck*, brain	≤ 0.01†	0.792	0.329	ab	b	a
	cck, intestine	0.514	0.410	0.631	-	-	-

(c) *leptin*



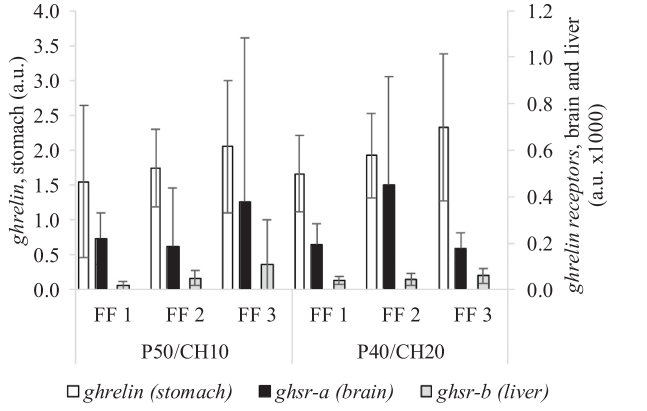
		FF	P/CH ratio	FF x P/CH	‡ P/CH	
					50/10	40/20
Two-way *p*-value	*leptin*, liver	0.256	≤ 0.05‡	0.194	A	B
	leptin, brain	0.366	0.474	0.328	-	-
	leptin, stomach	0.347	0.181	0.764	-	-

(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

(caption on next page)

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.

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., 2002; 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

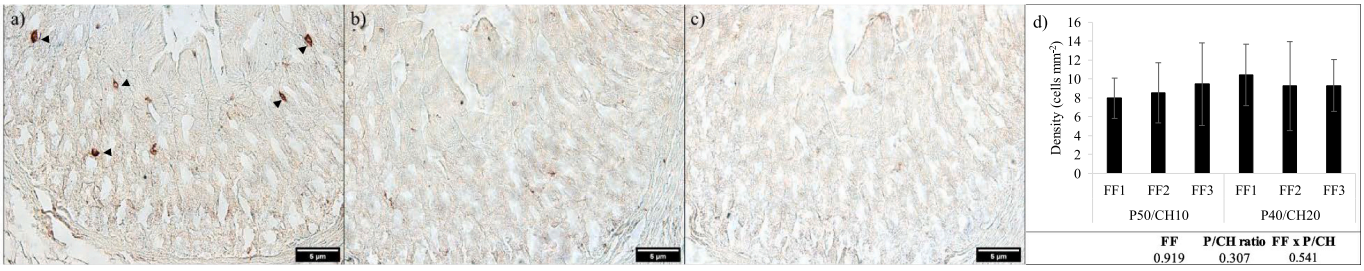


Fig. 2. Representative immunopositive ghrelin cells (▶) in the middle part of the stomach (a), negative control without primary antibody (b), negative control without secondary antibody (c), density of immunopositive ghrelin cells (cells mm^{-2}) in the stomach of gilthead seabream fed the experimental 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.

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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CHAPTER 4| DIETARY PROTEIN/CARBOHYDRATE RATIO AND FEEDING FREQUENCY AFFECT FEED UTILIZATION, INTERMEDIARY METABOLISM, AND ECONOMIC EFFICIENCY OF GILTHEAD SEABREAM (*Sparus aurata*) JUVENILES

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Dietary protein/carbohydrate ratio and feeding frequency affect feed utilization, intermediary metabolism, and economic efficiency of gilthead seabream (*Sparus aurata*) juveniles

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ABSTRACT

To evaluate the effects of dietary protein/carbohydrate (P/CHO) ratio and feeding frequency (FF) on growth, intermediary metabolism, and economic efficiency of gilthead seabream (*Sparus aurata*) juveniles, two practical isolipidic (17%) diets were formulated to include high protein (50%)/ low starch (10%) (diet P50/CHO10) or low protein (40%)/ high starch (20%) (diet P40/CHO20). Triplicate groups of fish with 9.1 ± 0.01 g were fed for 60 days with these diets until visual satiation at three FF: one (9:00), two (9:00 and 17:00), or three (9:00, 13:00, and 17:00) meals per day. Dietary P/CHO ratios did not affect growth performance while feeding 2 or 3 meals per day improved fish growth. Fish fed diet P40/CHO20 had increased feed intake (FI), protein efficiency ratio (PER), and nitrogen retention (NR), and lower feed efficiency (FE), nitrogen intake (NI), and economic conversion ratio (ECR). Feeding 2 or 3 meals per day increased FI, NI, ECR, and economic profit index, and decreased FE, PER, and NR. Fish fed diet P40/CHO20 presented increased hepatic lipid and glycogen content, hepatocyte area covered by lipid vacuoles, and *glucokinase* (*gk*) gene expression, and decreased *glutamate dehydrogenase* expression. Fish fed 3 meals per day had decreased plasma triglycerides and total protein levels, while fish fed 2 or 3 meals per day presented decreased hepatic *growth hormone receptor-i* (*ghr-i*), *gk,* and *fatty acid synthase* gene expression. Interaction between P/CHO ratio and FF was only observed in plasmatic glucose, cholesterol, and total lipids levels, and *insulin-like growth factor-1*, and *ghr-ii* gene expression. Overall, glycogenesis, glycolysis, and economic efficiency seemed to be increased while the amino acid catabolism was reduced in fish fed the P40/CHO20 diet. Higher FF increased growth and economic efficiency, and reduced glycolysis and lipogenesis pathways. In conclusion, a diet with P40/CHO20 ratio fed twice a day appears to be the most adequate strategy regarding feed utilization and economic efficiency for gilthead seabream juveniles in order to achieve optimum sustainable aquaculture.

1. Introduction

Increasing dietary incorporation of non-protein energy sources, such as lipids and carbohydrates (CHO), is one strategy to promote protein-sparing for growth, reducing environmental pollution associated with nitrogen wastes, and reducing feed costs (Metón et al., 1999; Fernández et al., 2007; Enes et al., 2011; Craig and Helfrich, 2017). Carbohydrates are the most economic energy source; however, fish, particularly carnivorous, do not tolerate high dietary CHO levels (Oliva-Teles et al., 2015). For instance, gilthead seabream (*Sparus aurata*), a carnivorous fish species, does not seem to tolerate more than 20% dietary CHO without negative effects on growth and feed utilization (Fernández et al., 2007; Couto et al., 2008; Enes et al., 2008, 2011; Bou et al., 2014; Magalhães et al., 2021). Moreover, higher dietary CHO levels affect intermediary metabolism and digestive and absorptive capacities (Fernández et al., 2007; Couto et al., 2008; García-Meilán et al., 2020).

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Table 1
Origen and main composition of the ingredients used in the experimental diets described in Table 2.

Ingredient	Origen	Main composition
Fishmeal	Sorgal. S.A., Ovar, Portugal	Crude protein: 73.5% DM Gross lipids: 17.0% DM
Fish oil	Sorgal. S.A., Ovar, Portugal	Gross lipids: 100% DM
Soybean meal	Sorgal. S.A., Ovar, Portugal	Crude protein: 54.3% DM Gross lipids: 1.8% DM
Corn gluten	Sorgal. S.A., Ovar, Portugal	Crude protein: 70.0% DM Gross lipids: 3.3% DM
Wheat gluten	Sorgal. S.A., Ovar, Portugal	Crude protein: 84.2% DM Gross lipids: 1.0% DM
Wheat meal	Sorgal. S.A., Ovar, Portugal	Crude protein: 13.8% DM Gross lipids: 1.1% DM
Monocalcium phosphate	Sorgal. S.A., Ovar, Portugal	(not applicable)
Lysine	Sorgal. S.A., Ovar, Portugal	(not applicable)
Taurine	Sorgal. S.A. Ovar, Portugal	(not applicable)
Vitamin mix	Premix. Lda., Viana do Castelo, Portugal	18,000 IU/kg diet, retinol acetate; 2000 IU/kg diet, cholecalciferol; 35 mg/kg diet, alpha tocopherol acetate; 10 mg/kg diet, sodium menadione bisulphate; 15 mg/kg diet, thiamin-HCl; 25 mg/kg diet, riboflavin; 50 mg/kg diet, calcium pantothenate; 200 mg/kg diet, nicotinic acid; 5 mg/kg diet, pyridoxine HCl; 10 mg/kg diet, folic acid; 0.02 mg/kg diet, cyanocobalamin; 1.5 mg/kg diet, biotin; 50 mg/kg diet, ascorbic acid; 400 mg/kg diet, inositol
Mineral mix	Premix. Lda., Viana do Castelo, Portugal	17%, calcium; 13%, phosphorus; 6%, potassium; 7%, cloride; 4%, sodium chloride
Binder	Liptosa, Madrid, Spain	(not applicable)
Choline chloride (50%)	Premix. Lda., Viana do Castelo, Portugal	(not applicable)

DM: Dry matter.

Table 2
Ingredients, proximate composition, and price of the experimental diets.

	Diets	
	P50/CHO10	P40/CHO20
Ingredients (% DM)		
Fishmeal	15.6	12.5
Fish oil	14.0	14.7
Soybean meal	25.0	20.0
Corn gluten	20.0	15.0
Wheat gluten	11.4	6.4
Wheat meal	9.4	26.2
Monocalcium phosphate	0.7	1.0
Lysine	0.1	0.5
Taurine	0.2	0.2
Vitamin mix	1.0	1.0
Mineral mix	1.0	1.0
Binder	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})[1]	20.8	19.8
Estimated diet price (€ kg^{-1})	1.57	1.38

CHO: Carbohydrates; DM: Dry matter; P: Protein.
[1] 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 CHO).

Several studies have been performed to establish the most adequate dietary protein/energy (P/E) ratio for gilthead seabream (Vergara et al., 1996; Sanz et al., 2000; Lupatsch et al., 2001, 2003; Fountoulaki et al., 2005; García-Meilán et al., 2013). However, the P/E ratio seems to be strongly influenced by fish size (Lupatsch et al., 2001, 2003). For instance, Vergara et al. (1996) suggested that the minimum dietary protein level producing maximum growth of gilthead seabream fry was 55% when the P/E ratio was 27.4. However, in juveniles, the recommended P/E ratio was between 23 and 33, when the initial body weight was 100 and 10 g, respectively (Lupatsch et al., 2003). Furthermore, García-Meilán et al. (2013) also concluded that gilthead seabream juveniles (with about 70 g) fed between 44% and 47% of protein presented only minimal adaptive changes and grew equally well.

In a recent study, major differences in growth performance and intermediary metabolism of gilthead seabream fed with diets containing different proportion of protein (P) and CHO (*i.e.*, P40/CHO20 or P50/CHO10) fed to satiety twice a day were not found (Basto-Silva et al., 2021). However, it is known that feeding frequency (FF) influences feed utilization and fish growth performance (Basçinar et al., 2001; Dwyer et al., 2002; Seo and Lee, 2008; Küçük et al., 2014; Sun et al., 2016; Eriegha and Ekokotu, 2017; Oh et al., 2018; Silva et al., 2020), and may also affect dietary CHO utilization. For instance, in white sturgeon (*Acipenser transmontanus*), hybrid tilapia (*Oreochromis niloticus x O. aureus*), and rainbow trout (*Oncorhynchus mykiss*), FF manipulation enhanced the use of dietary CHO, improving feed utilization and growth (Tung and Shiau, 1991; Hung and Storebakken, 1994; Lin et al., 1997). In contrast, in gibel carp (*Carassius auratus gibelio*) and common carp (*Cyprinus carpio*), no major effects were observed on growth performance, feed utilization, and CHO metabolism due to different dietary P/CHO ratio and FF conditions (Zhao et al., 2016; Cheng et al., 2019).

Most studies evaluating the effects of FF in gilthead seabream provided the animals with the same amount of feed per day, independently of the number of meals, thus not allowing the animals to self-regulate feed intake (Guinea and Fernandez, 1997; Yúfera et al., 2014; Gilannejad et al., 2019; Busti et al., 2020; Gilannejad et al., 2021). This does not allow a clear evaluation of the effects of FF on growth performance, feed utilization, or metabolic responses. For instance, in a study with gilthead seabream fed *ad libitum* at different FF, Yilmaz and Eroldogan (2011) observed that a higher FF improved growth, and affected whole-body composition, but did not affect feed utilization.

Feeds represent about 50–70% of the operational production costs in aquaculture (Rana et al., 2009), and dietary composition and FF highly affect the economic efficiency of fish production (Lozano et al., 2007; Aderolu et al., 2010; Martínez-Llorens et al., 2012; Güroy et al., 2017; Moutinho et al., 2017; Arru et al., 2019). Thus, optimizing feed composition and management may have a high impact on aquaculture profitability. For instance, reducing dietary protein content from 48% to 44% increased growth and economic profit of meagre juveniles (*Argyrosomus regius*) (Güroy et al., 2017), while African catfish (*Clarias gariepinus*) fed 3 times per day presented improved growth and economic profit in comparison with fish fed 1 or 2 times per day (Aderolu et al., 2010).

Thus, the present study aimed to assess the effects of FF (1, 2, or 3 meals per day) combined with different dietary P/CHO ratios (50/10 or 40/20) on growth, feed utilization, economic efficiency, body and liver composition, plasma metabolites indicators of nutrient metabolism, and gene expression of intermediary metabolism-related enzymes in gilthead seabream juveniles.

Table 3
Genes and primers used for qPCR.

Gene	ID primer	Sequence (5′- 3′)	[1]Accession n°	Tm (°C)	Efficiency (%)
Translation elongation factor 1α	*ef1α*	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	AF184170	60	98.0
Ribosomal protein S18	*rps18*	F: GGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACCA	AM490061.1	60	96.5
Growth hormone	*gh*	F: GCCCCATCGACAAGCACG R: GAGTCTACATTTTGCCACCGTCAG	AY038038	60	107.7
Growth hormone receptor-i	*ghr-i*	F: ACCTGTCAGCCACCACATGA R: TCGTGCAGATCTGGGTCGTA	AF438176	60	90.0
Growth hormone receptor-ii	*ghr-ii*	F: GAGTGAACCCGGCCTGACAG R: GCGGTGGTATCTGATTCATGGT	AY573601	60	99.8
Insulin-like growth factor-1	*igf-1*	F: ACAGAATGTAGGGACGGAGCGAATGGAC R: TTCGGACCATTGTTAGCCTCCTCTCTG	EF688016	60	81.6
Target of rapamycin	*mtor*	F: CAGACTGACGAGGATGCTGA R: AGTTGAGCAGCGGGTCaTAG	Azizi et al. (2016)	60	100.9
Glutamate dehydrogenase	*gdh*	F: GGTATCCACGGTCGTATCTCAGCC R: GAGACCCACATTACCAAAGCCCTG	JX073708	60	93.3
Glucokinase	*gk*	F: GACGCTATCAAGAGACGA*GGGAC R: CCACGGTCCTCATCTCCTCCAT	AF053330	60	98.1
Glucose 6-phosphatase	*g6pase*	F: CTGCTGTGGACGATGGAGAAAG R: TGTTGAGGGGCGAGTGAAGAC	AF151718	60	89.1
3-hydroxyacyl-CoA dehydrogenase	*hoad*	F: GAACCTCAGCAACAAGCCAAGAG R: CTAAGAGGCGGTTGACAATGAATCC	JQ308829	60	100.3
Fatty acid synthase	*fas*	F: TGGCAGCATACACACAGACC R: CACACAGGGCTTCAGTTTCA	AM952430	60	104.0

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

Table 4
Growth performance and feed utilization efficiency of gilthead seabream fed the experimental diets at different feeding frequencies.

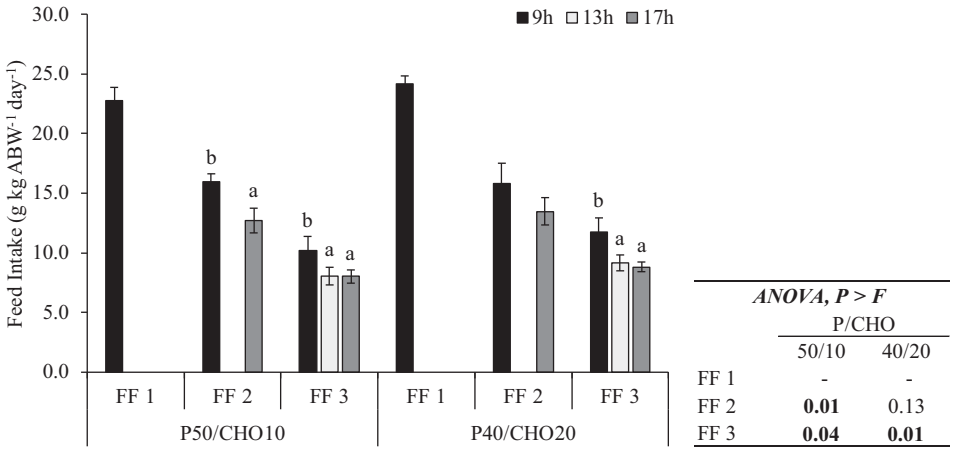
Ratio P/CHO	FF	IBW (g)	FBW (g)	DGI[2]	FI[3]	FE[4]	PER[5]	Mortality[6]	NI[7]	NR[8]
	1	9.1	41.6	6.1	22.8	0.95	1.8	1.7	87.7	30.7
50/10	2	9.1	50.9	7.2	28.7	0.81	1.6	1.7	101.5	26.5
	3	9.1	45.8	6.7	26.2	0.85	1.6	0.0	96.2	27.8
	1	9.1	39.6	5.8	24.1	0.85	2.0	3.3	78.6	33.3
40/20	2	9.1	48.4	7.0	29.3	0.78	1.8	0.0	85.9	30.2
	3	9.1	46.3	6.7	29.5	0.75	1.8	1.7	88.9	31.3
Pooled SEM		0.0	1.1	0.1	0.7	0.0	0.0	0.5	1.9	0.6
Main effect means[1]										
Ratio P/CHO	50/10	9.1	46.1	6.7	25.9 A	0.87 B	1.7 A	1.1	95.1 B	28.3 A
	40/20	9.1	44.8	6.5	27.7 B	0.79 A	1.9 B	1.7	84.5 A	31.6 B
	1	9.1	40.6 a	6.0 a	23.5 a	0.90 b	1.9 b	2.5	83.2 a	32.0 b
FF	2	9.1	49.7 b	7.1 b	29.0 b	0.80 a	1.7 a	0.9	93.7 b	28.4 a
	3	9.1	46.1 b	6.7 b	27.9 b	0.80 a	1.7 a	0.9	92.6 b	29.6 a
ANOVA, P > F										
Ratio P/CHO		1.00	0.34	0.16	**0.01**	**0.00**	**0.00**	0.63	**0.00**	**0.00**
FF		1.00	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	0.40	**0.00**	**0.00**
Interaction		1.00	0.64	0.63	0.18	0.10	0.11	0.40	0.08	0.78

Values presented as means (n = 3) and pooled standard error of the mean (pSEM). Different upper-case letters denote for significant differences between dietary P/CHO ratio and different lower-case letters denote for significant differences between feeding frequencies.
CHO: Carbohydrates; FBW: Final body weight; FF: Feeding frequency; I: Interaction; IBW: Initial body weight; P: Protein.
[1] Within each main effect, means with different letters are significantly different ($P < 0.05$).
[2] Daily growth index, DGI: ((FBW1/3− IBW1/3)/time in days) × 100.
[3] Feed intake, FI (g kg ABW^{-1} day^{-1}): FI (kg fish^{-1})/ABW/time in days, where average body weight, ABW = (IBW + FBW)/2.
[4] Feed efficiency, FE: wet weight gain/dry FI.
[5] Protein efficiency ratio, PER: wet weight gain/crude protein intake.
[6] Mortality (%): number of dead fish × 100/number of initial fish.
[7] Nitrogen intake, NI (g kg weight gain^{-1}): protein intake (g)/6.25 × 1000/weight gain.
[8] Nitrogen retention (%NI): NR (g kg^{-1} day^{-1})/NI (g kg^{-1} day^{-1}) × 100; where nitrogen retention, NR (g kg^{-1} day^{-1}) = (FBW × % final whole-body protein - IBW × % initial whole-body protein)/6.25 × 1000 / ABW × time in days.

2. Materials and methods

2.1. Diets composition

Two isoenergetic (20 kJ g^{-1}) and isolipidic (17% crude lipids) practical diets with different P/CHO ratios were formulated to include 50% protein (P) and 10% starch (CHO) or 40% P and 20% CHO. 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 °C and then stored in plastic containers at 4 °C until use. The origin and composition of the ingredients used in the experimental diets are presented in Table 1, and



ANOVA, P > F	P/CHO	
	50/10	40/20
FF 1	-	-
FF 2	**0.01**	0.13
FF 3	**0.04**	**0.01**

Fig. 1. Feed intake (g kg ABW^{-1} day^{-1}) at each mealtime. Values presented as means (n = 3) and standard error. Different letters denote significant differences between mealtime within each FF factor ($P \leq 0.05$). ABW: Average body weight; CHO: Carbohydrates; FF: Feeding frequency; P: Protein.

Table 5
Results of economic parameters at the end of the trial for gilthead seabream fed the experimental diets at different feeding frequencies.

Ratio P/CHO	FF	ECR[2]	EPI[3]
50/10	1	1.69	0.140
	2	1.95	0.150
	3	1.85	0.140
40/20	1	1.63	0.130
	2	1.78	0.150
	3	1.84	0.150
Pooled SEM		0.03	0.002
Main effect means[1]			
Ratio P/CHO	50/10	1.83 B	0.143
	40/20	1.75 A	0.143
FF	1	1.66 a	0.135 a
	2	1.87 b	0.150 b
	3	1.85 b	0.145 ab
ANOVA, P > F			
Ratio P/CHO		**0.01**	0.82
FF		**0.00**	**0.00**
Interaction		0.08	0.79

Values presented as means (n = 3) and pooled standard error of the mean (pSEM). Different upper-case letters denote for significant differences between dietary P/CHO ratio and different lower-case letters denote for significant differences between feeding frequencies.
CHO: Carbohydrates; FF: Feeding frequency; I: Interaction; P: Protein.
[1] Within each main effect, means with different letters are significantly different ($P < 0.05$).
[2] Economic conversion ratio, ECR (€ kg^{-1}): FCR × diet price (€ kg $diet^{-1}$), where feed conversion ratio, FCR = dry feed intake/wet weight gain.
[3] Economic profit index, EPI (€ $fish^{-1}$): [final weight (kg $fish^{-1}$) × fish sale price (€ kg $fish^{-1}$)] – [ECR (€ kg $fish^{-1}$) × weight gain (kg)]. The gilthead seabream sale price was fixed as 4.62€ (per kg), as reported in December 2018, at Warehouse Spain (FIS.com), for an aquaculture fish with 300–400 g.

diet composition and proximate analysis are presented in Table 2.

2.2. Fish and experimental conditions

The trial was performed at the Marine Zoology Station, University of Porto, Portugal, with gilthead seabream (*S. aurata*) juveniles 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 and 17% lipids; 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 °C, and each tank 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}, and dissolved oxygen was kept near saturation (6.0 ± 0.5 mg l^{-1}). Eighteen groups of 20 fish with an individual body weight of 9.10 ± 0.01 g were stocked in each tank, and the diets and feed frequency (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 on each meal was recorded, for the determination of feed intake (FI) per meal.

The experiment was performed by accredited scientists (following FELASA category C recommendations) and approved by the General Directorate of Food and Veterinary from Portugal (Certification number ORBEA-CIIMAR 30–2019), according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

2.3. Sampling

At the end of the trial, after 1 day of feed deprivation, fish in each tank were slightly anesthetized with 0.3 ml l^{-1} ethylene glycol monophenyl ether and bulk weighed. Thirteen fish from the initial stock population and 3 fish per tank at the end of the trial were euthanized by decapitation, and whole-fish, liver, and viscera weights were recorded for the determination of hepatosomatic (HSI) and visceral somatic (VSI) indices. Fish were then pooled by tank and stored at −20 °C until whole-body composition analysis.

The remaining fish continued to be fed for 2 more days to minimize manipulation stress and then, 5 h after the morning meal, blood from 6 fish per tank (3 pools of 2 fish) was collected from the caudal vein with heparinized syringes and immediately centrifuged at 3000 ×*g* for 10 min. Plasma aliquots were frozen at −80 °C until plasma metabolites were analyzed. After blood collection, fish were euthanized by decapitation and dissected on chilled trays. The liver of 3 fish was collected for histology and composition analysis. The histology samples were immediately fixed in Bouin (code 57211, Thermo Scientific - Richard-Allan Scientific, Kalamazoo, USA) for 24 h and subsequently transferred to ethanol (70%) until further processing. The samples for composition analysis were immediately frozen at −80 °C until used. The liver of the other 3 fish was stored in RNAlater (25 mM sodium citrate, 10 mM EDTA, and 70 g ammonium sulphate for a total of 100 ml at pH 5.2), left at 4 °C overnight, and subsequently stored at −80 °C until gene expression analysis.

Table 6
Whole-body and liver composition (wet weight basis), hepatosomatic (HSI) and visceral somatic indices (VSI) of gilthead seabream fed the experimental diets at different feeding frequencies.

Ratio P/CHO	FF	Whole-body				HSI[2]	VSI[3]	Liver	
		Protein	Lipid	Ash	Dry matter			Lipid	Glycogen
50/10	1	16.7	13.0	3.8	32.9	1.3	8.1	9.9	5.2
	2	16.7	13.7	3.9	33.5	1.1	8.1	9.5	5.4
	3	16.6	13.7	3.6	33.2	1.4	7.8	10.8	5.5
40/20	1	16.2	13.3	4.0	33.3	1.4	8.2	11.9	5.2
	2	16.2	13.9	4.0	34.0	1.3	8.6	11.3	7.7
	3	17.0	14.0	4.0	34.4	1.4	8.5	13.5	6.4
Pooled SEM		0.1	0.2	0.0	0.2	0.0	0.1	0.4	0.3
Main effect means[1]									
Ratio P/CHO	50/10	16.7	13.5	3.8 A	33.2	1.3	8.0	10.1 A	5.4 A
	40/20	16.5	13.7	4.0 B	33.9	1.4	8.4	12.2 B	6.4 B
FF	1	16.5	13.2	3.9	33.1	1.4 ab	8.2	10.9	5.2
	2	16.5	13.8	4.0	33.8	1.2 a	8.4	10.4	6.6
	3	16.8	13.9	3.8	33.8	1.4 b	8.2	12.2	6.0
ANOVA, P > F									
Ratio P/CHO		0.30	0.51	**0.01**	0.06	0.13	0.12	**0.00**	**0.03**
FF		0.18	0.34	0.39	0.18	**0.03**	0.85	0.09	0.08
Interaction		0.11	0.99	0.14	0.59	0.52	0.63	0.86	0.13

Values presented as means (%), body (n = 3), liver lipid and glycogen, VSI, and HSI (n = 9) and pooled standard error of the mean (pSEM). Different upper-case letters denote for significant differences between dietary P/CHO ratio and different lower-case letters denote for significant differences between feeding frequencies.
CHO: Carbohydrates; FF: Feeding frequency; I: Interaction; P: Protein.
[1] Within each main effect, means with different letters are significantly different ($P < 0.05$).
[2] Hepatosomatic index, HSI: (liver weight/body weight) × 100.
[3] Visceral somatic index, VSI: (viscera weight/body weight) × 100.

2.4. Proximate analysis

Dry matter, protein, lipid, and ash analysis of diets and whole-body were done following the Association of Official Analytical Chemists methods (AOAC, 2000). Dietary starch was determined as described by Beutler (1984). Liver glycogen and lipid contents were determined as described by Plummer (1987) and Folch et al. (1957), respectively. For each experimental condition, 3 groups of 3 pooled fish ($n = 3$) were used for whole-body composition analysis, and 9 fish ($n = 9$) were used to evaluate liver lipid and glycogen contents, VSI, and HSI.

2.5. Plasma metabolites

Plasma glucose, cholesterol, triglycerides, total protein, and total lipids 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). Nine fish (n = 9) were used for each experimental condition.

2.6. Histological processing and morphological evaluation

The liver was processed and sectioned using standard histological techniques and stained with hematoxylin and eosin (H&E). The samples were evaluated giving attention to lipid droplets as described in Basto-Silva et al. (2021). Shortly, in order to avoid any uncertainty between lipid droplets detection and glycogen, the images were first converted to greyscale, and all structures that could be confused by the software as lipid vacuoles (such as blood capillaries and adipose tissue) were manually removed. Since technique used for samples processing totally removes the lipid content from hepatocytes, the lipid vacuoles appear optically empty while glycogen granules are stained, thus not marked as a dark pixel during the threshold filter analysis in the Image J software, version 1.46 (National Institutes of Health, Maryland, USA). Digital images were acquired with Zen software (Blue edition; Zeiss, Jena, Germany). An $n = 9$ was used for each experimental condition.

2.7. Gene expression

Liver RNA extraction was done as described in Basto-Silva et al. (2021). RNA samples were used 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, from a starting amount of 3300 ng of total RNA. Samples were stored at −20 °C until used. Quantitative real-time PCR (qPCR) was performed as described in Basto-Silva et al. (2021) with the forward and reverse primers taken from the GenBank database (https://www.ncbi.nlm.nih.gov/) and presented in Table 3. The qPCR reactions followed Salmerón et al. (2013) procedure. *Translation elongation factor* (*ef1a*) and *ribosomal protein S18* (*rps18*) were selected as reference genes. The efficiency curves of the expressed genes ranged between 82 and 108%, and not between 95 and 105% as recommended, thus for the normalized gene expression was used the Pfaffl method (Pfaffl, 2001). For each experimental condition, 9 fish (n = 9) were used.

2.8. Economic analysis

The economic conversion ratio (ECR) and economic profit index (EPI) were evaluated as described in Martínez-Llorens et al. (2007). A higher ECR meaning an increase in the costs or a decrease in revenues, and a higher EPI indicating more financial benefits. The currency type for economic evaluations was the euro (€). The price of each diet was determined by multiplying the respective contribution of each feed ingredient by their respective cost per kg and summing the values obtained for all the ingredients in each of the formulated diets. The price (per kg) of each ingredient was provided by the ingredient's suppliers. Gilthead seabream sale price was fixed as 4.62€ (per kg), as reported in December 2018, at Warehouse Spain (FIS.com), for an aquaculture fish with 300–400 g.

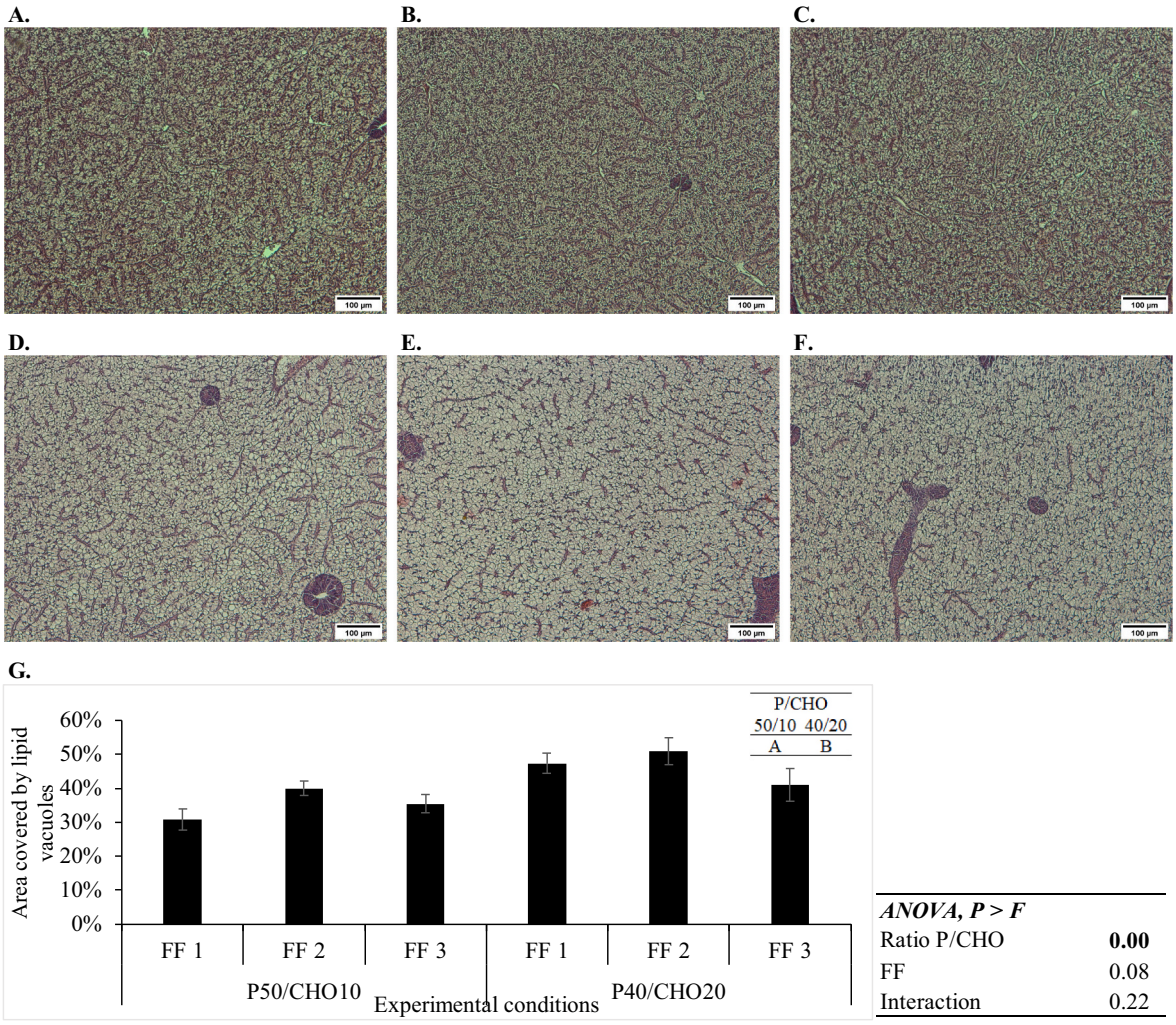


Fig. 2. Representative hematoxylin and eosin-stained histological sections of liver from fish fed diet P50/CHO10 one (A), two (B) and three meals per day (C); fish fed diet P40/CHO20 one (D), two (E) and three meals per day (F); and area covered by lipid vacuoles (%) in the liver (G). [1]Within each main effect, means with different letters are significantly different ($P < 0.05$). Images captured at 10× magnification. Values presented as means ($n = 9$) and standard error. Different uppercase letters denote for significant differences between P/CHO ratio. CHO: Carbohydrate; FF: Feeding frequency; I: Interaction; P: Protein.

2.9. Statistical analysis

Data are presented as mean ± standard error. 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. All data were analyzed by two-way ANOVA, with dietary P/CHO ratio and FF as main factors, except for FI at each mealtime, which was analyzed by one-way ANOVA. In the case of interaction between factors, a one-way ANOVA was performed for each factor. Significant differences among groups were determined by Tukey's multiple range test. All analyses were performed using SPSS 26 software package for Windows (IBM® SPSS® Statistics, New York, USA).

3. Results

Fish promptly accepted the experimental diets, and during the trial mortality was very low and unaffected by diet composition or FF (Table 4). Growth performance was unaffected by dietary P/CHO ratio but it was higher in fish fed 2 and 3 meals per day than 1 meal per day. FI was higher in fish fed with diet P40/CHO20 and 2 and 3 meals per day, independently of diet composition. Fish fed more than 1 meal per day consumed a higher amount of feed in the morning meal (Fig. 1). Feed efficiency (FE) was higher in fish fed with diet P50/CHO10 and in fish fed 1 meal per day, independently of diet composition. Protein efficiency ratio (PER) was higher in fish fed P40/CHO20 diet and in fish fed 1 meal per day, independently of diet composition. Nitrogen intake (NI) was higher in fish fed P50/CHO10 diet and 2 or 3 meals per day, independently of diet composition. Nitrogen retention (NR) as % of NI was higher in fish fed P40/CHO20 diet and in fish fed 1 meal per day, independently of diet composition.

The ECR was lower in fish fed with diet P40/CHO20 and 1 meal per day, independently of diet composition (Table 5). The EPI was only affected by FF, being lower in fish fed 1 meal per day, in comparison with those fed 2 meals per day.

There were no differences between the groups in whole-body protein, lipid, and dry matter content, while ash content was lower in fish fed with diet P50/CHO10 (Table 6). The HSI was higher in fish fed 3 meals per day than 2 meals per day while the VSI was not affected by diet composition nor FF. As shown in Fig. 2, fish fed the P40/CHO20 diet had a higher liver area covered by lipid vacuoles.

Interaction between dietary P/CHO ratio and FF was observed in plasmatic glucose, cholesterol, and total lipids. In fish fed the P50/CHO10 diet, FF did not affect plasma glucose level while in fish fed with diet P40/CHO20 plasma glucose was higher in fish fed 3 meals per day (Table 7). Plasma glucose was also higher in fish fed with diet P40/CHO20 3 meals per day than in those fed P50/CHO10 diet at the same

Table 7
Plasma glucose, cholesterol, triglycerides (mg dl^{-1}), total protein, and total lipids (g dl^{-1}) of gilthead seabream fed the experimental diets at different feeding frequencies.

Ratio P/CHO	FF	Glucose	Cholesterol	Triglycerides	Total proteins	Total lipids
	1	62.9	247.6 b	215.5	3.6	1.8 b
50/10	2	58.7	195.8 Aa	162.5	3.4	1.4 Aa
	3	56.9 A	176.7 Aa	153.7	3.3	1.5 ab
	1	58.6 a	221.6	206.6	3.4	1.9 b
40/20	2	56.8 a	253.1 B	199.3	3.5	1.9 Bb
	3	65.2 Bb	246.1 B	156.9	3.1	1.4 a
Pooled SEM		1.0	6.4	6.2	0.0	0.0
Main effect means[1]						
Ratio P/CHO	50/10	59.5	206.7	177.2	3.4	1.6
	40/20	60.2	240.3	187.6	3.3	1.7
	1	60.8	234.6	211.1 b	3.5 b	1.9
FF	2	57.8	224.5	180.9 ab	3.5 b	1.7
	3	61.1	211.4	155.3 a	3.2 a	1.5
ANOVA, $P > F$[2]						
Ratio P/CHO		0.72	**0.00**	0.36	0.16	**0.01**
FF		0.30	0.22	**0.00**	**0.01**	**0.00**
Interaction		**0.02**	**0.00**	0.22	0.05	**0.00**

Values presented as means (n = 9) and pooled standard error of the mean (pSEM). Different upper-case letters denote for significant differences between dietary P/CHO ratio and different lower-case letters denote for significant differences between feeding frequencies.
CHO: Carbohydrates; FF: Feeding frequency; I: Interaction; P: Protein.
[1] Within each main effect, means with different letters are significantly different ($P < 0.05$).
[2] In the case of significant interaction, individual treatment means within a P/CHO ratio or FF protocols were indicated with different upper-case or lower-case letters.

FF. In fish fed with diet P40/CHO20, FF did not affect the plasma cholesterol level, while this metabolite was higher in fish fed with diet P50/CHO10 1 meal per day than when fed 2 or 3 meals per day. Further, plasma cholesterol was also higher in fish fed with diet P40/CHO20 at 2 and 3 meals per day than in fish fed the P50/CHO10 diet at the same FF. Total lipids in plasma were higher in fish fed the P40/CHO20 diet at 2 meals per day than in fish fed the P50/CHO10 diet at the same FF. With the P50/CHO10 diet, fish fed 1 meal per day had higher plasmatic total lipids than fish fed 2 meals per day, while with the P40/CHO20 diet fish fed 1 and 2 meals per day had higher circulating total lipids than fish fed 3 meals per day. Plasma triglycerides and total proteins were only affected by FF, being lower in fish fed 3 meals per day compared with fish fed 1 or 1 and 2 meals per day, respectively.

Except for *glutamate dehydrogenase* (*gdh*) and *glucokinase* (*gk*), diet composition *per se* did not affect the expression of the other studied genes (Fig. 3). *gdh* expression was higher in fish fed with diet P50/CHO10 while *gk* expression was higher in fish fed P40/CHO20 diet and, independently of diet composition, in fish fed 1 meal per day.

Growth hormone (*gh*) and *target of rapamycin* (*mtor*) gene expression were not affected by dietary composition nor FF (Fig. 3). Fish fed 1 meal per day presented higher *growth hormone receptor* (*ghr*)-*i* expression. An interaction between dietary P/CHO ratio and FF was observed in *ghr-ii* and *insulin-like growth factor-1* (*igf-1*). The highest expression of *ghr-ii* was observed in fish fed diet P50/CHO10 at 2 meals per day while the lowest expression was observed in fish fed diet P50/CHO10 at 3 meals per day compared to fish fed diet P40/CHO20 at the same FF. Independently of the diet used, fish fed more meals per day had higher *ghr-ii* gene expression. In fish fed 2 meals per day, *igf-1* expression was higher with diet P50/CHO10 than with diet P40/CHO20. *Fatty acid synthase* (*fas*) expression was higher in fish fed 1 meal per day, independently of diet composition. *Glucose-6-phosphatase* (*g6pase*) and *3-hydroxyacyl-CoA dehydrogenase* (*hoad*) were neither affected by diet composition nor FF.

4. Discussion

Dietary nutrient manipulation, namely P/CHO ratio, and FF optimization are two important factors to take into account to optimize fish growth and feed utilization, which are major goals in aquaculture production. Several studies were already performed on those topics in gilthead seabream (Lupatsch et al., 2003; Fernández et al., 2007; Couto et al., 2008; Enes et al., 2011; Yilmaz and Eroldogan, 2011; García-Meilán et al., 2013; Bou et al., 2014; Yúfera et al., 2014; Castro et al., 2016; Busti et al., 2020; García-Meilán et al., 2020; Basto-Silva et al., 2021; Magalhães et al., 2021) but the simultaneous evaluation of both factors on fish growth and metabolism are yet scarcely studied (Zhao et al., 2016; Cheng et al., 2019), and none in gilthead seabream.

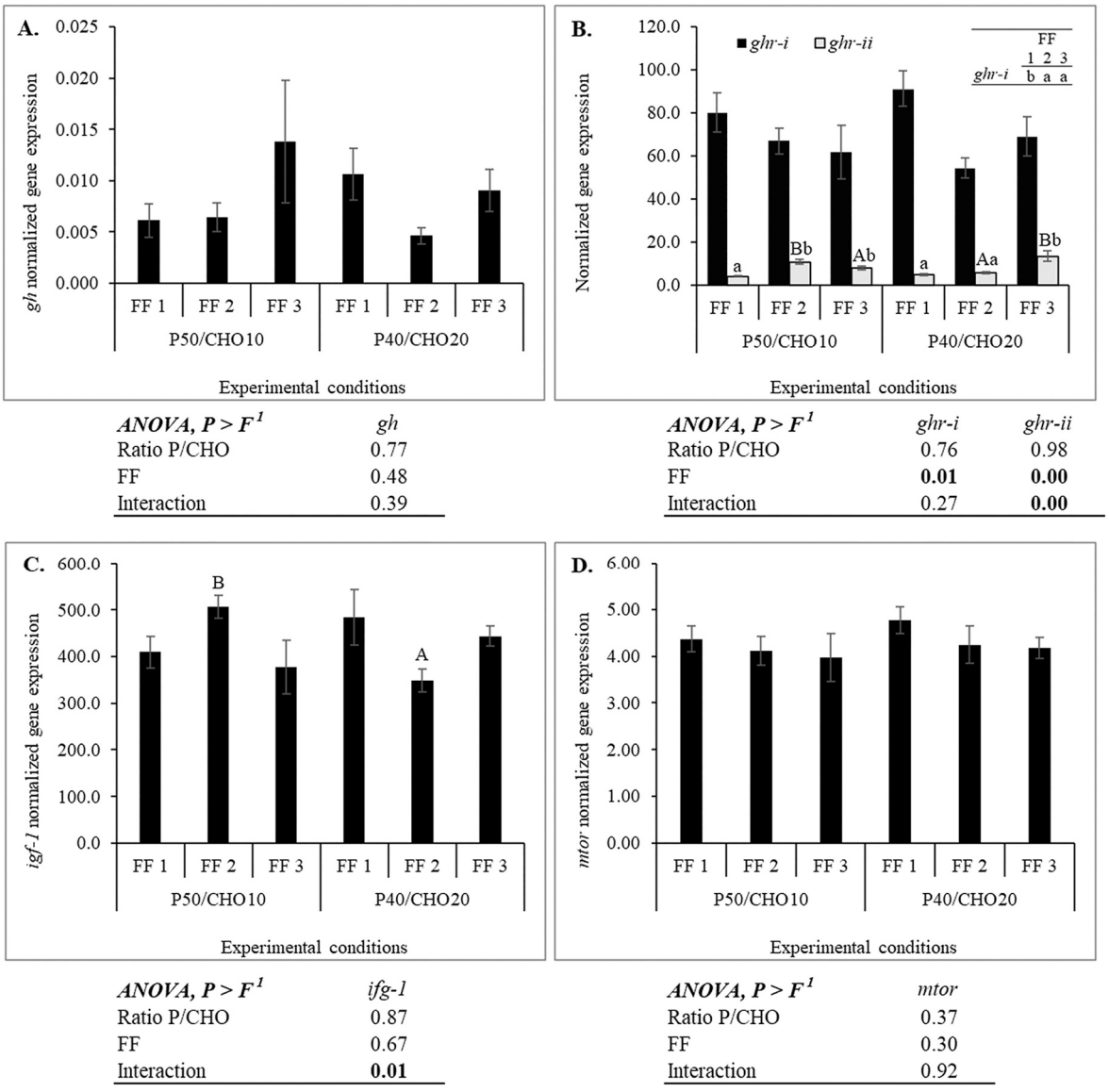
In the present study, interactions were not observed between dietary P/CHO ratio and FF on gilthead seabream growth and feed utilization. Similar results were also previously observed in gibel carp (Zhao et al., 2016). Present data also showed that dietary P/CHO ratio did not affect growth performance, but a lower dietary P/CHO ratio increased FI, PER, and NR (%NI), and reduced FE and NI. On the other hand, feeding more than one meal per day led to higher growth, FI, and NI, but decreased FE, PER, and NR (%NI). In contrast, in gibel carp, a higher dietary P/CHO ratio increased growth, and FE was also improved in fish fed more meals per day (Zhao et al., 2016).

The FI increase and FE reduction in fish fed P40/CHO20 diet compared with fish fed P50/CHO10 diet, might be explained by fish adjusting FI to meet their protein needs when fed low protein diets. Also in gilthead seabream, Santinha et al. (1996) observed that when the amount of dietary protein was below requirements, fish exhibited a higher FI.

In the present study, growth was not depressed with the lower dietary protein diet, while PER and NR (%NI) were increased in fish fed with the P40/CHO20 diet. This further suggests that CHO efficiently spared protein use for energy purposes (Fernández et al., 2007; Enes et al., 2011; Castro et al., 2016; Basto-Silva et al., 2021; Magalhães et al., 2021).

Present results also showed that, independently of diet composition, feeding 1 meal per day was inadequate for gilthead seabream, of the size range tested, to fulfill the nutritional/energy needs to maximize growth performance, since juveniles fish present high metabolic rate and fast gastric evacuation rate. This was also probably related with stomach size limitations (Ruohonen and Grove, 1996; Peterson and Small, 2006), thus leading to lower growth performance than that of fish fed 2 or 3 meals per day. In accordance, the higher FI in fish fed more meals per day can explain the observed higher FBW. Nonetheless, when fed more meals per day fish might present a faster transit rate which might impact digestion, moreover fish might be eating before gastric evacuation of previous feed ingested is completed (Andrade et al., 1996). While increasing the number of meals from 1 to 2 per day led to higher FI, increasing to 3 meals per day did not further increase FI. This further suggests that when a physical limitation (stomach fullness) is not imposed, gilthead seabream can regulate FI to meet nutrient/energy needs.

During feeding activities fish use energy, thus fish fed more meals per day might expend more energy (Guinea and Fernandez, 1997). However, it cannot be disregarded that part of the increase in growth of fish fed at higher FF can be related to a decrease in competition behavior between the animals and therefore a decrease of energy spent in aggressive behaviors, such as fin biting and feed seizing, compromising the energy available for growth of fish fed 1 meal per day. Indeed, it is known that gilthead seabream exhibit social hierarchy, especially when fish are reared under low densities (Montero et al., 2009). It was also to be expected that increasing FF could lead to improved CHO utilization, by decreasing the plasma glucose load and, thus sparing protein for



ANOVA, $P > F^1$	gh
Ratio P/CHO	0.77
FF	0.48
Interaction	0.39

ANOVA, $P > F^1$	ghr-i	ghr-ii
Ratio P/CHO	0.76	0.98
FF	**0.01**	**0.00**
Interaction	0.27	**0.00**

ANOVA, $P > F^1$	ifg-1
Ratio P/CHO	0.87
FF	0.67
Interaction	**0.01**

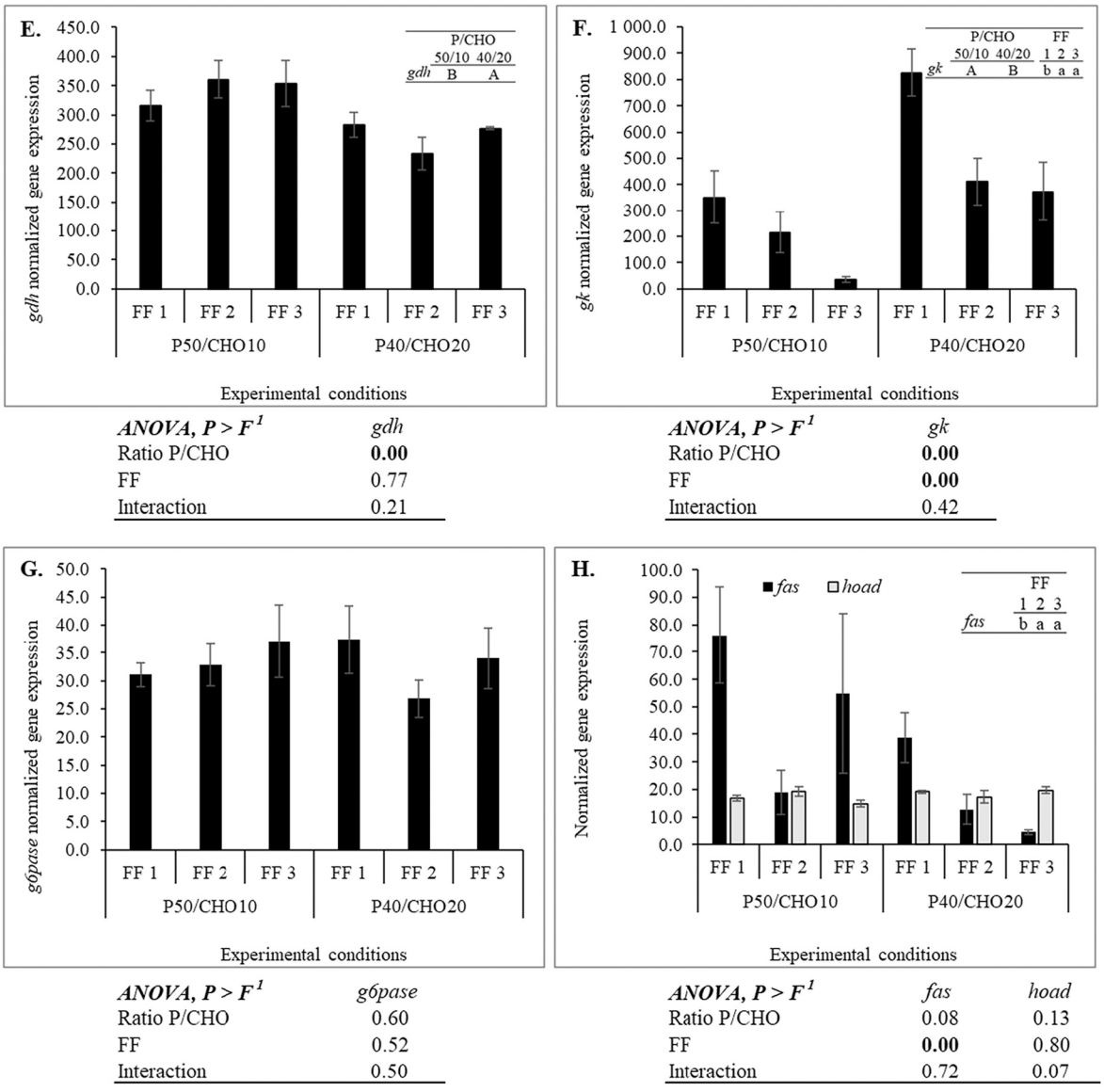
ANOVA, $P > F^1$	mtor
Ratio P/CHO	0.37
FF	0.30
Interaction	0.92

Fig. 3. Normalized expression of genes related to growth (A, B, C), amino acid catabolism (D, E), glycolysis (F), gluconeogenesis (G), and fatty acid metabolism (H) in the liver of gilthead seabream fed the experimental diets at different feeding frequencies. [1]In the case of significant interaction, individual treatment means within a P/CHO ratio or FF protocols were indicated with different upper-case or lower-case letters in the graph area. Upper-case letters denote for significant differences between dietary P/CHO ratio and lower-case letters denote for significant differences between FF. All values are expressed as arbitrary unit x 10^3 and presented as means (n = 9) and standard error. CHO: Carbohydrates; *fas*: *fatty acid synthase*; FF: Feeding frequency; *g6pase*: *glucose-6-phosphatase*; *gdh*: *glutamate dehydrogenase*; *gh*: *growth hormone*; *ghr-i, −ii*: *growth hormone receptor-i*; *−ii*; *gk*: *glucokinase*; *hoad*: *3-hydroxyacyl-CoA dehydrogenase*; I: Interaction; *igf-1*: *insulin-like growth factor-1*; *mtor*: *target of rapamycin*; P: Protein.

growth, as reported for instance for hybrid tilapia and rainbow trout (Tung and Shiau, 1991; Hung and Storebakken, 1994). However, no improvement in FE or interaction between FF and the dietary P/CHO ratio was observed to allow such a conclusion.

Despite the increase in growth and FI in fish fed 2 and 3 meals per day, the FE, PER, and NR (%NI) were lower than in fish fed 1 meal per day. This slight decrease in feed utilization in fish fed more than 1 meal per day might be associated with a faster transit time and thus less effective digestion, as also suggested for other species, such as Asian seabass (*Lates calcarifer*), dark-banded rockfish (*Sebastes inermis*), flounder fish (*Platichthys flesus luscus*), and Korean rockfish (*Sebastes schlegeli*) (Biswas et al., 2010; Küçük et al., 2014; Md Mizanur and Bai, 2014; Oh et al., 2018).

As expected, FI was higher in the morning meal as fish were starved due to the long interval between this meal and the previous one. Besides a FI regulation to meet energy needs, as discussed above, the lower FI in the subsequent meals might be also related to gut filling since the amount of feed in the gut limits the FI of the following meal (Peterson and Small, 2006; Küçük et al., 2014). However, a further reduction of FI in the third meal might also be expected, but no differences were noticed in FI between the second and third meals. This might be related to feeding preferences of gilthead seabream, as it was previously reported that when fed on-demand, gilthead seabream preferentially feeds in the afternoon and evening (Sánchez-Muros et al., 2003). Regarding the ECR, fish fed with diet P40/CHO20 present a lower cost than diet P50/CHO10. Nonetheless, the EPI was not affected by the dietary P/CHO ratio and the cost-effectiveness of diets was improved in fish fed 2 meals per day. This suggests that fish fed 2 meals per day, despite consuming more feed, will give the aquaculture farmer more economic return as it also induces higher fish growth. A higher economic profit and growth



ANOVA, $P > F^1$	gdh
Ratio P/CHO	**0.00**
FF	0.77
Interaction	0.21

ANOVA, $P > F^1$	gk
Ratio P/CHO	**0.00**
FF	**0.00**
Interaction	0.42

ANOVA, $P > F^1$	g6pase
Ratio P/CHO	0.60
FF	0.52
Interaction	0.50

ANOVA, $P > F^1$	fas	hoad
Ratio P/CHO	0.08	0.13
FF	**0.00**	0.80
Interaction	0.72	0.07

Fig. 3. (*continued*).

was also reported for African catfish fingerlings and juveniles fed more meals per day (3 or 4 compared with 1 or 2) (Aderolu et al., 2010).

Although fish fed diet P40/CHO20 presented higher liver lipid content and area covered by lipid vacuoles, differences were not observed in *fas* expression, which indicates that *de novo* lipid production was not increased. This suggests that glucose used for energy purposes also spared some of the dietary lipids that might have been directly deposited in the liver. Similarly, Nile tilapia (*O. niloticus*) fed lower dietary P/CHO ratios presented higher whole-body, liver, and white muscle lipid contents, and plasma triglycerides levels, despite the *acetyl-coenzyme A carboxylase α* and *fas* expression were not affected when compared with the higher P/CHO ratio (Chen et al., 2020). However, we cannot disregard that the activity of fas might be increased if measured. Indeed, as in the present study and also in this species, an absence of *fas* gene expression difference was previously reported by Castro et al. (2016), although fish fed a P50/CHO20 diet presented higher hepatic fas activity than fish fed a diet with 66% of protein and no CHO content (P66/CHO0 diet). This suggests that gene expression and enzymatic responses could have different behaviors, pointing to the necessity of, in future studies, monitoring changes at the different levels of biological organization, namely at the biochemical and molecular levels.

Liver glycogen content was also higher in fish fed with diet P40/CHO20 than with diet P50/CHO10, suggesting an increase of the glycogenesis pathway. Similar results were also observed by Enes et al. (2008), Castro et al. (2016) and Magalhães et al. (2021) for gilthead seabream fed 20% of dietary starch in comparison with fish fed lower starch levels (10%, 5%, or 0%).

Regarding plasmatic glucose, it was expected that a higher glucose level would be found in fish fed with diet P40/CHO20 compared with fish fed diet P50/CHO10, as reported by Basto-Silva et al. (2021) for the same fish species. However, this was only true for fish fed 3 meals per day, not being observed any further differences in plasma glucose levels between diets and FF.

In mammals, increasing FF was reported to decrease plasmatic glucose levels as a result of improved glucose tolerance (Bertelsen et al., 1993; Carlson et al., 2007). Contrary to mammals, most studies in fish showed that increasing FF did not affect plasmatic glucose level (Hung and Storebakken, 1994; Zolfaghari et al., 2011; Enes et al., 2015; Guo et al., 2018; Oh et al., 2018; Pedrosa et al., 2019; Busti et al., 2020). Nonetheless, mullet (*Mugil liza*) juveniles fed 5 meals per day presented higher plasma glucose levels than fish fed 1 or 3 meals per day, which could be attributed to the increased intake and absorption of nutrients in

that group (Silva et al., 2020).

In the present study, the increase of plasmatic triglycerides and total lipids, together with the increase of *fas* expression in fish fed 1 meal per day in comparison with those fed 3 meals per day, suggests that part of the final products of glycolysis were diverted for lipid synthesis in that group. However, this was not reflected in increased liver or the whole-body lipid content. Differently, in another study also in this species, no changes in the plasmatic triglycerides or cholesterol were observed by changing the FF protocol (Busti et al., 2020).

However, it is important to note that in diets including 10% CHO, increasing from 1 to 2 meals per day decreased the plasmatic lipid content but no further decrease was gained when increasing to 3 meals per day, whereas in diets with 20% CHO, it was needed 3 meals per day to drop the plasmatic lipid load. This observation together with the results of HSI, VSI, and whole-body lipid composition hint that a lower protein diet and with higher CHO content, provided at 2 meals per day, can indeed be the best option for the aquaculture producer. Since excess body fat is a factor to take into consideration both by aquaculture producers as buyers this should be carefully considered if the goal is to promote fat gain or have lean fish.

Regarding growth-related genes, previous studies found a positive relationship between gilthead seabream growth and hepatic expression of *ghr-i*, *ghr-ii*, *igf-1*, and *igf-2* (Pérez-Sánchez et al., 1995; Saera-Vila et al., 2007). However, this response seems to be affected by other factors, like dietary composition (Gómez-Requeni et al., 2004; Benedito-Palos et al., 2007, 2016; Basto-Silva et al., 2021), feeding rate (Pérez-Sánchez et al., 1995), age of the fish, as well as by sampling time (Gómez-Requeni et al., 2004; Benedito-Palos et al., 2007), and the target-tissue (Benedito-Palos et al., 2007; Saera-Vila et al., 2007; Benedito-Palos et al., 2016). In the present study, fish fed with diet P50/CHO10 at 2 meals per day showed a tendency for higher growth than those fed diet P40/CHO20 and, simultaneously, also showed a consistent increase of hepatic *ghr-ii* and *igf-1* expression. While, concerning the FF effects, it was the fish fed 1 meal per day that presented a decrease of growth, and a consistent lower expression of *ghr-ii*, independently of the dietary P/CHO ratios used.

In fish fed diet P40/CHO20 the reduction of *gdh* and increase of *gk* expression reflect the protein-sparing effect and the use of CHO for energy purposes, indicating a reduction of amino acid catabolism and an increase of glycolysis. Similar results were also previously reported for gilthead seabream fed diets with low P/CHO ratios (Couto et al., 2008; Enes et al., 2008; Basto-Silva et al., 2021; Magalhães et al., 2021).

Independently of diet composition, *gk* expression was higher in fish fed 1 meal per day than 2 or 3 meals per day, possibly due to the higher glucose load in that group, although this was not reflected in the plasma glucose level. This is consistent with the enhancement of the glycolysis pathway also observed in white seabream (Enes et al., 2015), which the authors attributed to a higher glucose load in fish fed 2 meals per day than in fish fed 3 or 4 meals per day.

The gluconeogenesis pathway did not seem to be influenced by the dietary P/CHO ratio or FF, as suggested by the unchanged *g6pase* gene expression. This agrees with our previous results for the same species fed diets with the same P/CHO ratios at 2 meals per day (Basto-Silva et al., 2021), and indicates that endogenous glucose synthesis was not particularly depressed by increasing the dietary starch content, as previously suggested by Enes et al. (2008).

Overall, glycogenesis, glycolysis, and economic efficiency seemed to be increased by using a diet with a lower P/CHO ratio (P40/CHO20 *versus* P50/CHO10), while the amino acid catabolism was reduced, reflecting the protein-sparing effect of dietary CHO.

Compared to feeding 1 meal per day, for gilthead seabream of this size, feeding 2–3 meals per day increased growth and economic efficiency, and reduced glycolysis and lipogenesis pathways.

Thus, a diet with P40CHO20 fed twice per day should be considered in order to improve aquaculture sustainability and profitability.

CRediT author statement

Catarina Basto-Silva performed the experiment and analyses, analyzed data, and participated in the conceptualization and the experimental design. Paula Enes and Inês Guerreiro supervised the *in vivo* experiment. Encarnación Capilla and Inês Guerreiro supervised the gene expression analysis. Aires Oliva-Teles participated in the experimental design, idea conception, and was part of the supervision team. Inês Guerreiro conceived the work, participated in the experimental design, and was part of the supervision team. The first manuscript draft was written by Catarina Basto-Silva and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

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.

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CHAPTER 5| EFFECT OF DIETARY PLANT-FEEDSTUFFS AND PROTEIN/CARBOHYDRATE RATIO ON GILTHEAD SEABREAM (*Sparus aurata*) GUT HEALTH AND FUNCTIONALITY

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Effect of Dietary Plant Feedstuffs and Protein/Carbohydrate Ratio on Gilthead Seabream (*Sparus aurata*) Gut Health and Functionality

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Abstract: This study investigated, for the first time, the integrated effects of dietary protein source and protein/carbohydrate (P/CH) ratio on gilthead seabream gut histomorphology, microbiota composition, digestive enzymes activity, and immunological and oxidative stress-related gene expressions. Four isolipidic diets: two fishmeal-based (FM) and two plant feedstuff (PF)-based diets, with P/CH ratios of 50/10 or 40/20 each (FM-P50/CH10; FM-P40/CH20; PF-P50/CH10; PF-P40/CH20), were tested. PF-based diets lead to more histomorphological alterations than FM-based diets. P/CH ratio had no relevant effect on gut histomorphology. Gut mucosa of fish fed PF-based diets presented a higher number of operational taxonomic units, and richness and diversity indices, while the P/CH ratio did not affect those parameters. The α-amylase activity was lower in fish fed with PF-based diets and in fish fed the P40/CH20 diets. Regarding the immune-related genes, only *cyclooxygenase-2* was affected, being higher in fish fed the P50/CH10 diets than the P40/CH20 diets. Fish fed the FM-based diets presented higher expression of *glutathione reductase* and *glutathione peroxidase*, while fish fed the P50/CH10 diet had higher expression of *superoxide dismutase*. In conclusion, PF-based diets can compromise gut absorptive and digestive metabolism, but decreasing the dietary P/CH ratio had little effect on the parameters measured.

Keywords: alternative ingredients; digestive enzymes; gut digesta; gut histomorphology; gut mucosa

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1. Introduction

Fishmeal (FM) was traditionally used as the main and most adequate protein source for carnivorous fish due to its high quality, high digestibility, and good palatability [1–3]. Presently, its use is in a clear downward trend [4]. This reduction is largely due to supply and price variation, coupled with the continuously increasing demand from the aquafeed industry [4]. Hence, the use of plant feedstuffs (PF) and the inclusion of carbohydrate (CH) sources in fish feeds have been good alternatives to, respectively, decreasing dietary FM inclusion as a protein source and spare protein use for growth [5–10]. Gilthead seabream (*Sparus aurata*), one of the species with higher production in Europe, seems able to cope with a total replacement of dietary FM by PF [9]. This species requires about 45% of dietary protein [11]. However, if digestible CHs are provided in a suitable quantity, dietary protein might be spared for growth instead of being used as an energy source and, therefore, reduce nitrogen wastes and dietary costs [6,7,12]. Nonetheless, the maximum dietary CH inclusion that does not cause negative effects in gilthead seabream is limited to 20% [7]. Higher dietary CH inclusion may compromise growth and the digestive and absorptive capacities [6,12]. Several studies with gilthead seabream were already conducted to separately

evaluate the effects of dietary inclusion of PF and the protein-sparing by CHs. Overall, results showed that PF-based diets often promoted gut morphological changes, modifications on microbiota composition, decreases in gut enzymatic activity, and increases in oxidative stress of fish [9,13–21]. The inclusion of 20% or more of dietary CHs also affected fish growth performance, digestive enzyme activities, and antioxidant status [7,22–24]. However, the interactive effects on gut functionality and the health of gilthead seabream fed diets with lower P/CH ratios and the replacement of FM by PF as a major dietary protein source has not received much attention, and the available information is somehow dispersed. For instance, Castro et al. [25] did not observe major changes in gut histomorphology, microbiota, α-amylase, and lipase activities of gilthead seabream fed diets with highly different P/CH ratios (50/17 and 66/0). Similarly, in the same species, Couto et al. [26] and Fountoulaki et al. [27] also did not find an effect of the dietary P/CH ratio on the proteolytic and amylolytic activities, nor did Castro et al. [23] on the gut oxidative status, or antioxidant enzymes activities. All these studies evaluating different dietary P/CH ratios were made with FM as the main dietary protein source. To our knowledge, only one study is available that evaluated dietary P/CH ratios using PF as the main protein source [12]. In this study, the authors reported that fish fed a P40/CH39 diet had higher lipase and trypsin activities and lower α-amylase activity than those fed a P46/CH19 diet.

Recently, we assessed the effects of FM- or PF-based diets with different P/CH ratios (50/10 and 40/20) in gilthead seabream growth, feed utilization, appetite regulation, and intermediary metabolism [28]. Results showed that diets only slightly modified fish appetite and metabolic parameters, although growth was higher in fish fed the FM-P50/CH10 diet than those fed the FM-P40/CH20 diet. Further, reducing the dietary P/CH ratio led to a decrease in the feed efficiency and an increase in the protein efficiency ratio.

The present study is a follow-up to our previous study [28]. While the previous study aimed to evaluate the effect of dietary protein sources (FM vs. PF) and P/CH ratio on gilthead seabream appetite regulation and intermediary metabolism, the present study aims to evaluate, for the first time, the effects of these factors (dietary protein source and P/CH ratio) on gilthead seabream gut function and health, by assessing gut histomorphology, gut microbiota composition, digestive enzymes activity, and gut immunological and oxidative stress genes expression.

2. Materials and Methods

2.1. Diets

Four isolipidic diets (18% crude lipids) were formulated to contain 100% FM or 20% FM + 80% PF as protein sources, and protein to carbohydrate (P/CH) ratios of P50/CH10 or P40/CH20 (diets FM-P50/CH10, FM-P40/CH20, PF-P50/CH10, and PF-P40/CH20). Details of diets, ingredient composition, and a proximate analysis are presented in the supplementary material (Table S1).

2.2. Experimental Conditions and Sampling

Fish-rearing conditions are described in detail in Basto-Silva et al. [28]. Briefly, 180 gilthead seabream (140 ± 0.1 g, initial body weight) were randomly distributed to twelve 300-L water capacity tanks in a temperature-controlled recirculation life-support system. The diets were randomly distributed to triplicate groups, and fish were fed with the corresponding diet by hand until apparent visual satiation—two meals per day, for 41 days, 6 days a week. The length of the trial was chosen based on previous studies conducted on fish, also including gilthead seabream, which show that this duration was enough to induce dietary effects at intestinal level [17,29].

At the end of the 41 days, 6 fish per tank were sampled 5 h after the first meal of the day and euthanized with a sharp blow to the head (Figure 1). Three fish were sampled for midgut, pyloric caeca (PC), and stomach, all with digestive content, for digestive enzymes evaluation. From the same fish, midgut and PC were also collected for histomorphology evaluation. The remaining 3 fish were sampled to collect midgut to perform

gene expression analysis. Two of these three fish were also sampled for allochthonous (digesta) and autochthonous (mucosa) microbiota characterization. Digesta samples were collected by squeezing the entire gut, and mucosa samples were obtained by scrapping the internal surface of gut. Midgut was considered as the portion which began after the PC and finished before the hindgut, which is the final section of the gut [30], and the portions collected were the ones from the beginning of the midgut. Samples for enzymes activity and microbiota characterization were immediately frozen in liquid nitrogen and stored at −80 °C until analyses. Histology and gene expression samples were freed from the adjacent adipose and connective tissue, rinsed in phosphate-buffered saline (PBS), and the excess PBS was removed using a paper towel before being stored. Histology samples were fixed in phosphate-buffered formalin (4%, pH 7.4) for 24 h and then transferred to ethanol (70%) until further processing. Samples for gene expression were stored in RNA later, left at 4 °C overnight, and afterwards stored at −80 °C until analysis.

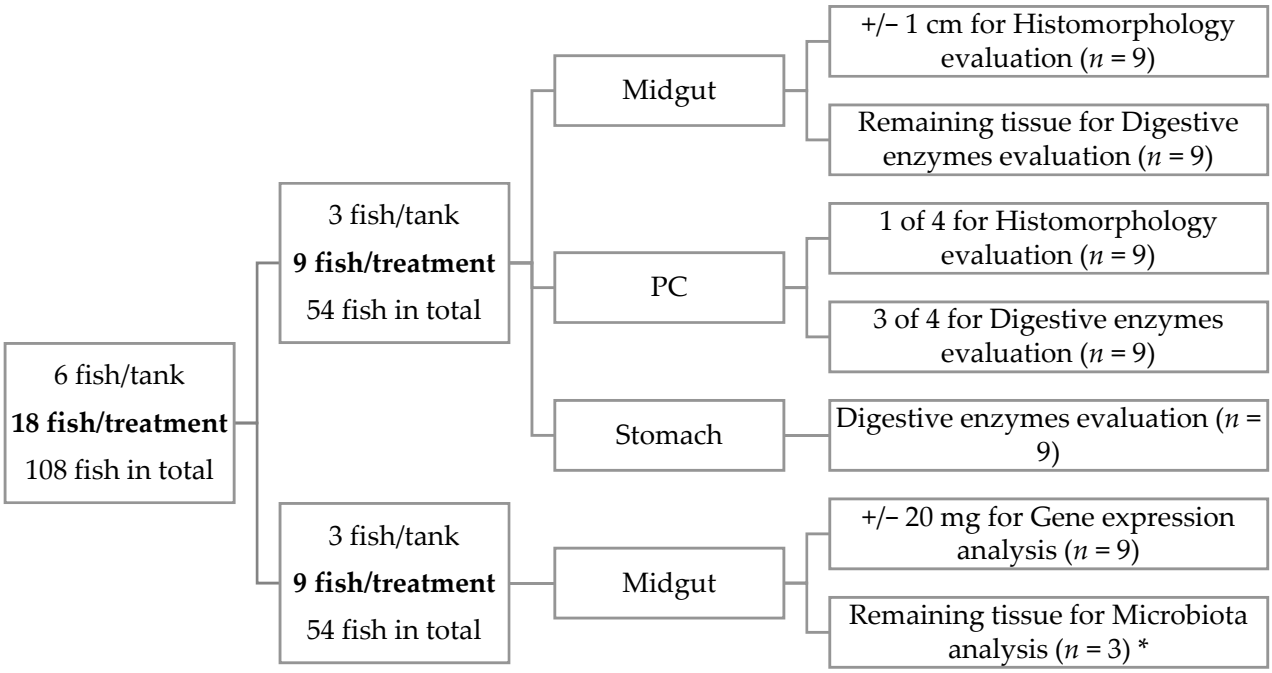

Figure 1. Schematic representation of sampling methodology applied in the present work. * In microbiota, only 2 of 3 fish per tank were used, and the samples were pooled to reduce individual variation, accounting for *n* = 3 per treatment.

2.3. Histological Evaluation

PC and midgut samples were processed and sectioned using standard histological techniques, stained with hematoxylin and eosin, and evaluated through a blinded semi-quantitative method, as described in Castro et al. [25], with slight modifications, namely, considering the nucleus position and hyper-vacuolization within the enterocytes. A score of 1 was given to the tissue with the least changes, and subsequent scores (up to 5) accounted for increasing histomorphological alterations, as described by Penn et al. [31]. Digital images were acquired with Zen software (Blue Edition; Zeiss, Jena, Germany), and using a light microscope Axio Imager.A2 (Zeiss, Oberkochen, Germany).

2.4. Microbial Diversity Analysis

Digesta and mucosa samples of the 2 fish per tank were pooled to reduce individual variation, accounting for *n* = 3 per treatment, each representing the microbial community of 6 fish. DNA extractions, polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE), band excisions, and re-amplifications were performed as described by Castro et al. [25], with each PCR product being loaded on a polyacrylamide

gel at 8%, made of a denaturing gradient of 30 to 60% 7 M urea/40% formamide. Amplicons were sequenced to identify microbiota operational taxonomic units (OTUs), and a phylogenetic analysis was performed to identify the closest known species as described in Castro et al. [25].

2.5. Digestive Enzyme Activities and Zymograms

All samples were individually homogenized with a Ystral homogenizer—Laboratory Series X10 (Ballrechten-Dottingen, Germany) in 4 parts of ice-cold 50-mM Tris-HCl buffer pH 7.5, containing 0.1 mM EDTA (reference code E5134, Sigma-Aldrich, Sintra, Portugal), and 0.1% (*v*/*v*) Triton X-100 (reference code T8787, Sigma-Aldrich, Sintra, Portugal). Homogenates were centrifuged (30,000× *g*, 30 min, 4 °C) and supernatants were recovered and stored at −80 °C until use.

Pepsin activity was measured in the stomach, as described in Alarcón et al. [32], total protease activity was measured in PC and midgut, as described in Moyano et al. [33], and lipase (EC 3.1.1.3) and α-amylase (EC 3.2.1.1) activities were measured in PC and midgut using commercial kits from Spinreact (Girona, Spain), with code #1001275 and #41201, respectively.

Pepsin and proteolytic activities were expressed as units (U) per mg of soluble protein, and α-amylase and lipase as mU per mg of soluble protein, with one U of enzyme activity defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol/min of the substrate at the assay temperature.

Protein concentration of the samples was measured according to Bradford [34], using a Sigma-Aldrich (Sintra, Portugal) protein assay kit (reference code B6916) and albumin bovine serum (BSA; reference code A4503, Sigma-Aldrich, Sintra, Portugal) as standard.

All enzyme activities were measured in a Multiskan GO microplate reader (model 51119200; Thermo Scientific, Nanjing, China).

Alkaline protease zymograms were obtained after resolving, by SDS-PAGE, the homogenates, as described in Castro et al. [35]. The commercial Precision Plus Protein™ All Blue Prestained Standard (reference code 1610373, Bio-Rad Laboratories Lda., Amadora, Portugal) was used to estimate the proteins' molecular weight. The specific trypsin-like and chymotrypsin-like activities were identified based on García-Meilán et al. [24], where 6 bands with protease activity were identified in gilthead seabream. Coomassie-stained gels were imaged with a ChemiDoc XRS+ (Bio-Rad Laboratories Lda., Amadora, Portugal), and qualitatively evaluated by the presence or absence of bands.

2.6. RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR (qPCR)

The total RNA extraction from intestinal samples, the RNA concentration, the purity and integrity evaluation, the cDNA synthesis, and the quantitative real-time PCR (qPCR) were performed as described in Basto-Silva et al. [28]. The forward and reverse primers used (Table 1) were searched in the GenBank database [36], and their efficiency curves were evaluated according to the assay conditions. Most of the primers' amplification efficiencies were between 90% and 110%, which are the recommended efficiency values [37]. However, as not all used primers conform to this criteria, we used the Pfaffl method [38] to ensure the robustness of the data. The Bio-Rad CFX Manager 3.1 (California, CA, USA) was the software used to measure the expression levels. *Elongation factor 1α* (*ef1α*) and *ribosomal protein S18* (*rps18*) were used as reference genes.

Table 1. Genes and primers used for qPCR.

ID Primer	Sequence (5′–3′)	[1] Accession n°	Tm (°C)	Efficiency (%)
ef1α	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	AF184170	60	87.2
rps18	F: GGGTGTTGGCAGACGTTAC R: CTTCTGCCTGTTGAGGAACCA	AM490061.1	60	88.0
hsp70	F: AATGTTCTGCGCATCATCAA R: GCCTCCACCAAGATCAAAGA	EU805481	57	90.1
cat	F: TTCCCGTCCTTCATTCACTC R: CTCCAGAAGTCCCACACCAT	JQ308823	60	98.5
cox2	F: GAGTACTGGAAGCCGAGCAC R: GATATCACTGCCGCCTGAGT	AM296029	60	94.6
gpx1	F: GAAGGTGGATGTGAATGGAAAAGATG R: CTGACGGGACTCCAAATGATGG	DQ524992	60	91.2
gr	F: TGTTCAGCCACCCACCCATCGG R: GCGTGATACATCGGAGTGAATGAAGTCTTG	AJ937873	60	97.0
igM	F: CAGCCTCGAGAAGTGGAAAC R: GAGGTTGACCAGGTTGGTGT	AM493677	60	87.0
Il1β	F: GGGCTGAACAACAGCACTCTC R: TTAACACTCTCCACCCTCCA	AJ277166	60	99.0
sod	F: CCTGACCTGACCTACGACTATGG R: AGTGCCTCCTGATATTTCTCCTCTG	JQ308833	60	91.6
tnf α	F: TCGTTCAGAGTCTCCTGCAG R: CATGGACTCTGAGTAGCGCGA	AJ413189	60	96.0

cat: catalase; cox2: cyclooxygenase 2; ef1α: translation elongation factor 1α; F: forward; gpx1: glutathione peroxidase; gr: glutathione reductase; hsp70: 70 kilodalton heat shock proteins; igM: immunoglobulin M heavy chain; il1β: interleukin 1β; R: reverse; rps18: ribosomal protein S18; sod: superoxide dismutase; Tm: melting temperature; tnf α: tumor necrosis factor α. [1] from the GenBank database [36].

2.7. Statistical Analysis

Statistical analyses were completed using SPSS 25 software package for Windows (IBM® SPSS® Statistics, New York, NY, USA). Homogeneity of variances and data normality were tested by the Levene and Shapiro–Wilk tests, respectively. When normality was not verified, data were transformed before ANOVA. However, all data are presented as the mean and standard error of the mean (SEM), without any transformation. Differences were considered statistically significant at $p < 0.05$.

Since histological data was not normal nor homogenous even after transformation, statistical analysis of the histomorphology evaluation was completed by the non-parametric Kruskal–Wallis test, followed by all-pairwise comparisons. Furthermore, the significance values were adjusted by the Bonferroni correction for multiple tests.

The remaining data were evaluated by two-way ANOVA tests, with the protein source and P/CH ratios as factors. In the case of interaction between factors, one-way ANOVA was performed for the P/CH ratio within each protein source, and for the protein source within each P/CH ratio.

Statistical analysis related to the DGGE was performed as described in Castro et al. [25].

3. Results

During the trial, all experimental diets were well-accepted by the fish, and the fish survival rate was 100%. Results of the rearing trial were not the aim of this study and are presented elsewhere [28].

Regarding the PC histomorphology, fish fed the PF-P50/CH10 diet presented a higher total mean score (2.23) than those in the remaining experimental conditions, where the total mean score ranged between 1.78 and 1.88 (Table 2). Lamina propria width was higher in fish fed the PF-P50/CH10 diet than in those fed the FM-based diets (Figure 2I). Fish fed the PF-P50/CH10 diet also presented higher submucosa widths than those remaining in the experimental conditions (Figure 2II). Lamina propria cellularity was higher in fish fed

the FM-P50/CH10 and PF-P50/CH10 diets than the FM-P40/CH20 diet. The enterocytes vacuolization was higher in fish fed the PF-based diets.

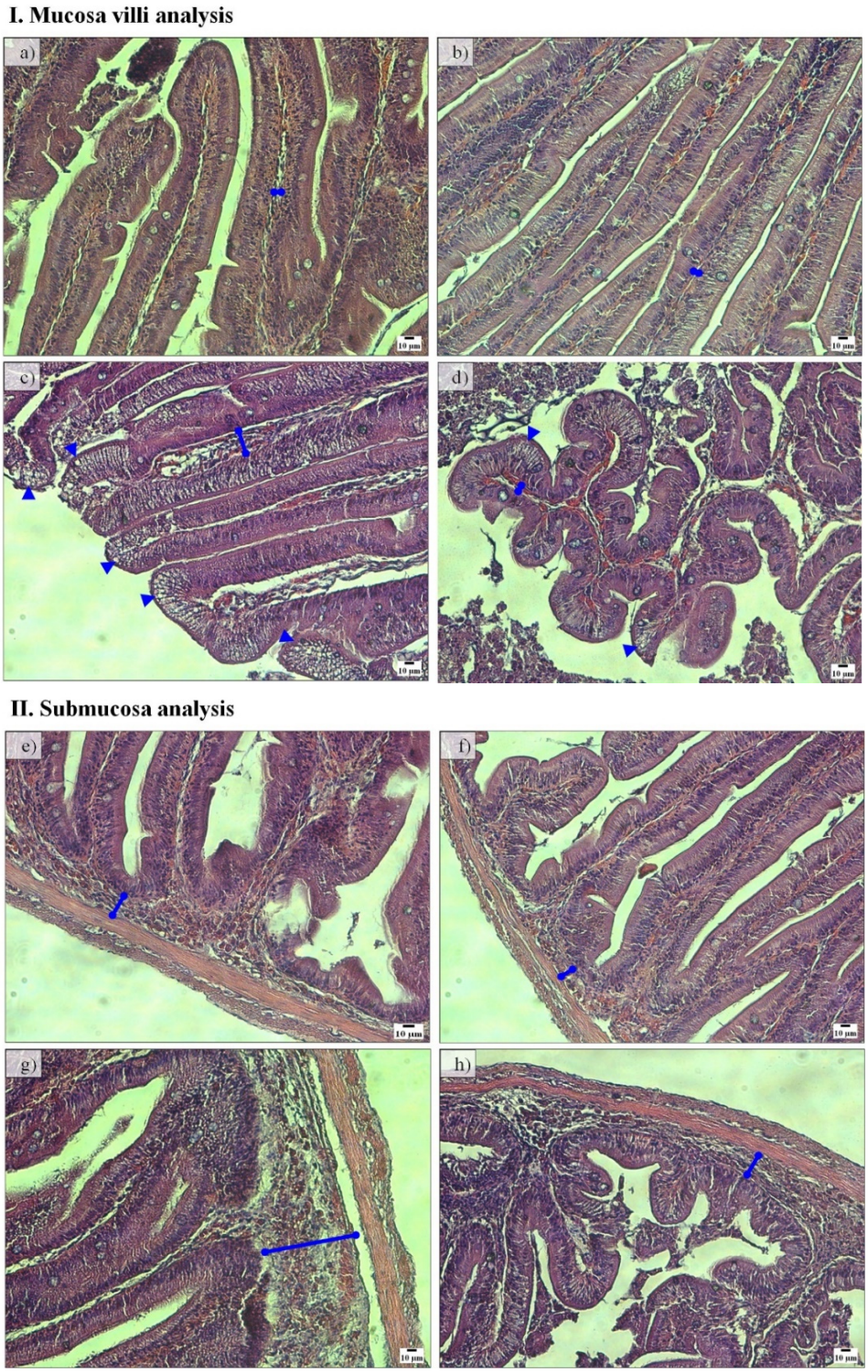

Figure 2. Representative histological sections of pyloric caeca mucosa villi (**I**) and submucosa (**II**) stained with hematoxylin and eosin of fish fed FM-P50/CH10 (**a**,**e**), FM-P40/CH20 (**b**,**f**), PF-P50/CH10 (**c**,**g**), and PF-P40/CH20 (**d**,**h**). ►enterocytes vacuolization. (**I**): Lamina propria width was higher in fish fed the PF-P50/CH10 diet (**c**) than fish fed the FM-based diets (**a**,**b**). Enterocytes vacuolization was higher in fish fed the PF-based diets (**c**,**d**) than those in the remaining conditions (**a**,**b**). (**II**): Submucosa width was higher in fish fed diet PF-P50/CH10 (**g**), than those in the remaining conditions (**e**–**h**).

Table 2. Details of the score-based evaluation of the pyloric caeca histology of gilthead seabream fed the experimental diets.

Protein Source	FM		PF		SEM	*p*-Value
P/CH Ratio	50/10	40/20	50/10	40/20		
Gut fold height	1.44	1.33	1.72	1.50	0.14	0.97
Lamina propria—width	1.61 ^{a}	1.61 ^{a}	2.22 ^{b}	1.94 ^{a,b}	0.09	0.04
Lamina propria—cellularity	2.22 ^{b}	1.56 ^{a}	2.61 ^{b}	2.00 ^{a,b}	0.12	0.03
Submucosa—width	1.44 ^{a}	1.39 ^{a}	2.00 ^{b}	1.50 ^{a}	0.08	0.04
Submucosa—cellularity	1.94	2.00	2.11	1.61	0.10	0.28
Intraepithelial leucocytes infiltration	2.78	2.83	2.67	2.06	0.13	0.11
Eosinophilic granulocytes presence	2.11	1.94	2.44	1.89	0.13	0.33
Enterocytes nucleus alignment	2.33	2.28	2.44	2.61	0.09	0.66
Enterocytes vacuolization	1.00 ^{a}	1.11 ^{a}	1.83 ^{b}	1.72 ^{b}	0.10	0.00
Mean score	1.88 ^{a}	1.78 ^{a}	2.23 ^{b}	1.87 ^{a}	0.06	0.03

Values presented as means (n = 9) and standard error of the mean (SEM). Different lower-case letters stand for statistical differences across dietary groups as determined by the Kruskal–Wallis all-pairwise comparisons. Furthermore, the significance values have been adjusted by the Bonferroni correction for multiple tests. CH: carbohydrate; FM: fishmeal; PF: plant feedstuffs; P: protein.

Regarding midgut histomorphology, fish fed the PF-based diets presented a higher total mean score (2.77) and gut fold height than fish fed FM-based diets, which have a total mean score of 2.28 (Figure 3 and Table 3). No further differences between groups were detected.

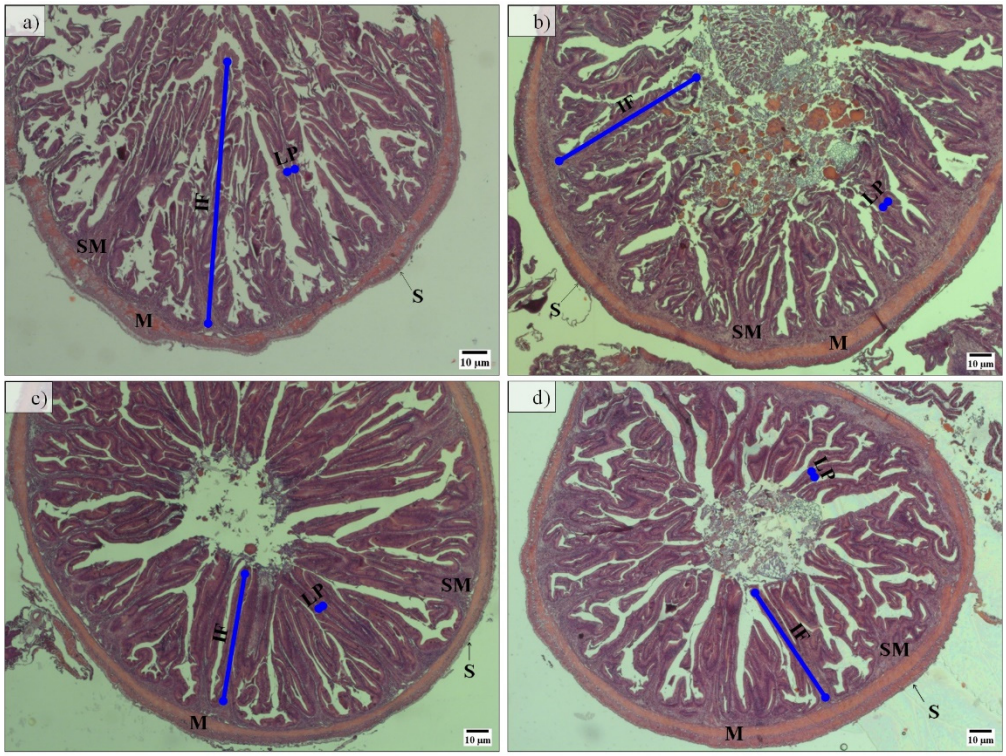

Figure 3. Representative hematoxylin and eosin-stained histological sections of midgut from fish fed FM-P50/CH10 (**a**), FM-P40/CH20 (**b**), PF-P50/CH10 (**c**), and PF-P40/CH20 (**d**). IF, intestine fold; LP, lamina propria; M, muscularis layer; S, serosa layer; SM, submucosa layer. Intestine fold height showed higher histomorphology deformations in fish fed the PF-based diets (**c**,**d**) than in fish fed the FM-based diets (**b**), except for fish fed the FM-P50/CH10 diet (**a**), which was not significantly different from fish fed the PF-P40/CH20 (**d**).

Table 3. Details of the score-based evaluation of the midgut histology of gilthead seabream fed the experimental diets.

Protein Source	FM		PF		SEM	*p*-Value
P/CH Ratio	50/10	40/20	50/10	40/20		
Gut fold height	1.50 ^{a,b}	1.22 ^{a}	2.33 ^{c}	2.00 ^{b,c}	0.14	0.02
Lamina propria—width	2.67	2.00	2.89	2.44	0.14	0.15
Lamina propria—cellularity	3.00	2.67	3.11	2.78	0.14	0.62
Submucosa—width	2.88	2.11	3.13	3.29	0.18	0.16
Submucosa—cellularity	2.75	2.44	3.25	3.29	0.15	0.08
Intraepithelial leucocytes infiltration	2.78	2.72	3.56	3.22	0.23	0.44
Eosinophilic granulocytes presence	3.11	2.78	3.13	3.56	0.14	0.39
Enterocytes nucleus alignment	2.44	2.11	2.56	2.89	0.14	0.29
Enterocytes vacuolization	1.00	1.00	1.22	1.22	0.05	0.22
Mean score	2.44 ^{a,b}	2.12 ^{a}	2.79 ^{b}	2.74 ^{b}	0.09	0.01

Values presented as means (n = 9) and standard error of the mean (SEM). Different lower-case letters stand for statistical differences across dietary groups as determined by the Kruskal–Wallis all-pairwise comparisons. Furthermore, the significance values have been adjusted by the Bonferroni correction for multiple tests. CH: carbohydrate; FM: fishmeal; PF: plant feedstuffs; P: protein.

DGGE fingerprints of the hypervariable V3 region of the 16S rRNA genes present in digesta and mucosa gut samples revealed that, independently of the dietary treatment, gut bacterial communities maintained a similarity, near 40% within both gut samples (Figure 4). Moreover, two clusters were observed in both gut microbiota regions, corresponding to samples recovered from fish fed the FM- and the PF-based diets, except for the FM-P50/CH10 diet in the digesta, which did not cluster with the remaining FM-based diets, and the PF-P50/CH10 diet in the mucosa, which did not cluster with the remaining PF-based diets. Despite this clear cluster separation, in digesta samples, the dietary composition did not affect the average number of OTUs, richness, and diversity indices (Table 4). Only the similarity index was higher in fish fed PF-P50/CH10 than in fish fed the FM-P50/CH10 diet. In mucosa samples, PF-based diets led to a higher number of gut OTUs, richness, and diversity indices than FM-based diets, while the similarity index was not different between groups. Sequence analysis from DGGE-selected bands showed that the dominant allochthonous and autochthonous bacteria detected were either corresponding to uncultured bacteria not yet assigned to a specific taxon or were closely related to genera belonging to the phylum Firmicutes and Proteobacteria, namely, Lactobacillus, Pseudomonas, Klebsiella, and Vibrio (Table 5 and Figure 4). Except for band 15, which was only found in digesta, all other bands were detected in digesta and mucosa samples.

Concerning digestive enzymes, α-amylase activity was lower in fish fed the PF-based diets, for both PC and midgut, and in fish fed the P40/CH20 diet only in the PC (Table 6). Proteolytic activity was higher in the PC of fish fed the P50/CH10 diet, but only within the PF-based diet-fed fish. Pepsin and lipase activities were not affected by dietary composition.

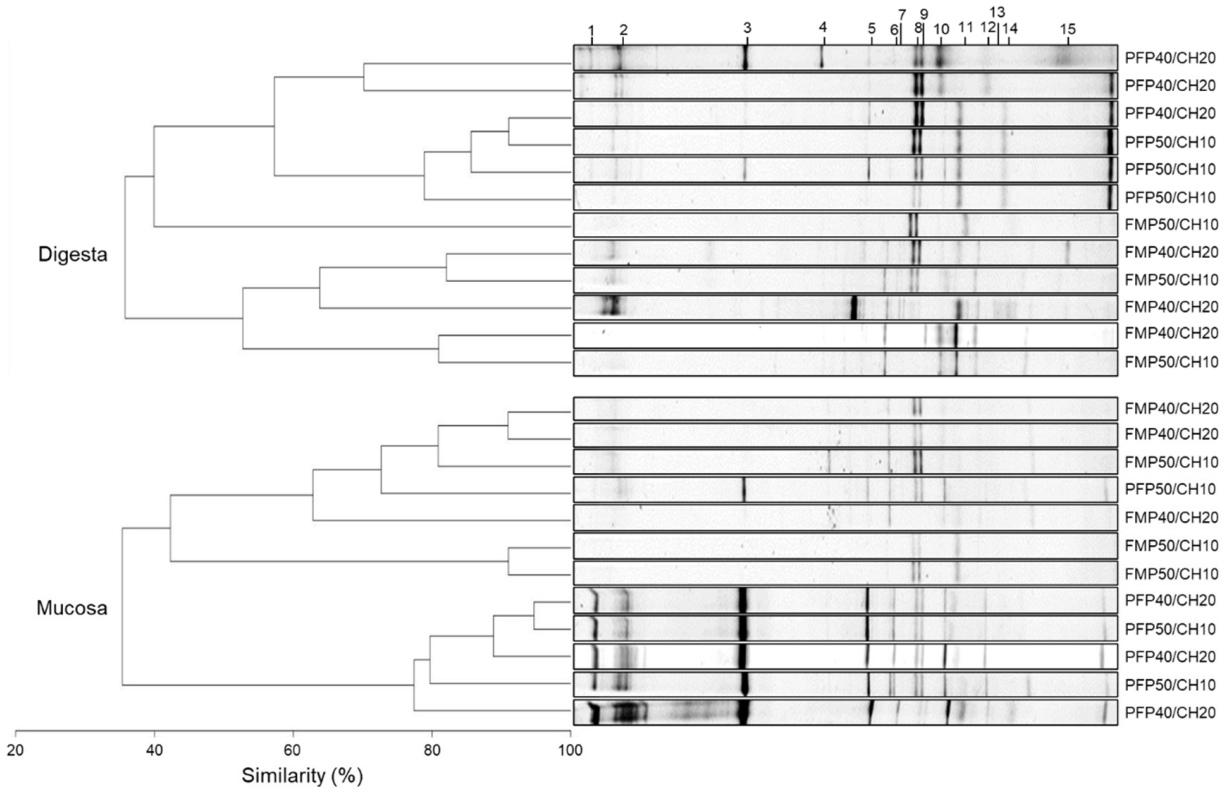

Figure 4. Dendrogram and PCR-DGGE fingerprints of the microbiota found in digesta and mucosa samples recovered from the gut of gilthead seabream fed the experimental diets. Numbers (1–15) on top of the figure correspond to the gel bands sequenced to identify the corresponding bacterial species, described on Table 5.

Table 4. Ecological parameters obtained from PCR- DGGE fingerprints of gut microbiota of gilthead seabream fed the experimental diets.

PS	FM		PF		SEM	Two-Way ANOVA		
P/CH Ratio	50/10	40/20	50/10	40/20		PS	P/CH Ratio	I
Digesta								
OTUs	8.7	13.7	10.0	11.3	0.9	0.76	0.08	0.29
Richness [1]	0.88	1.38	1.02	1.14	0.09	0.75	0.10	0.28
Diversity [2]	2.08	2.56	2.24	2.37	0.09	0.94	0.11	0.33
SIMPER Similarity (%) [3]	34.1 [A]	57.0	80.4 [B]	65.9	6.0	0.01	0.59	0.04
Mucosa								
OTUs	6.0	8.3	14.0	11.7	1.1	0.00	1.00	0.11
Richness [1]	0.60	0.87	1.41	1.15	0.11	0.00	0.97	0.08
Diversity [2]	1.67	2.11	2.59	2.39	0.12	0.01	0.48	0.09
SIMPER Similarity (%) [3]	65.3	71.2	72.8	83.8	4.3	0.29	0.37	0.78

Values presented as means (n = 3 per treatment pooled from 6 fish), and standard error of the mean (SEM). Different upper-case letters denote significant differences between dietary protein sources. In the case of interaction between factors, one-way ANOVA was performed for the P/CH ratio within each protein source, and for the protein source within each P/CH ratio. The significant interactions between the factors are presented in the upper part of the table. CH: carbohydrate; FM: fishmeal; I: interaction; OTUs: average number of operational taxonomic units; PF: plant feedstuffs; P: protein; PS: protein source. [1] Margalef species richness: $d = (S - 1)/\log(N)$. [2] Shannon's diversity index: $H' = -\sum(pi(\ln pi))$. [3] SIMPER: similarity percentage within group replicates.

Table 5. Identified bacterial species from the DNA sequencing of the allochthonous and autochthonous gut bacteria communities of gilthead seabream fed the experimental diets.

Band	Closest Known Species (BLAST)	Phylum	Similarity (%)	Accession Number of Nearest Neighbor
1	Uncultured bacterium from Turkey fecal microbial community	-	99	EU873831.1
2	Uncultured *Pseudomonas* sp.	Proteobacteria	100	LC032367.1
3	*Lactobacillus aviarius* subsp. *aviarius*	Firmicutes	96	LC071825.1
4	Uncultured marine bacterium	-	96	HM437606.1
5	Uncultured *Lactobacillus* sp.	Firmicutes	97	LT571746.1
6	Uncultured *Pseudomonas* sp.	Proteobacteria	99	GU250534.1
7	Uncultured bacterium from gut microbiota of Atlantic salmon (*Salmo salar* L.)	-	100	EU009390.1
8	*Klebsiella pneumoniae*	Proteobacteria	100	CP031798.1
9	Uncultured *Klebsiella* sp.	Proteobacteria	97	MH767054.1
10	Uncultured bacterium from gut bacterial communities of *Mythimna separata*	-	80	JQ013040.1
11	Uncultured *Vibrio* sp.	Proteobacteria	97	HM214586.1
12	Uncultured bacterium from environmental samples	-	95	FJ785825.1
13	Uncultured bacterium from environmental samples	-	100	LT720113.1
14	Uncultured bacterium from intestine of Atlantic cod (*Gadus morhua*)	-	98	HM115943.1
15	Uncultured bacterium from environmental samples	-	100	KC527347.1

Table 6. Specific activity of pepsin (U mg protein^{-1}) in the stomach, and α-amylase, lipase (mU mg protein^{-1}), and proteolytic activity (U mg protein^{-1}) in the pyloric caeca, and midgut of gilthead seabream fed the experimental diet.

PS	FM		PF		SEM	Two-Way ANOVA		
P/CH Ratio	50/10	40/20	50/10	40/20		PS	P/CH Ratio	I
				Stomach				
Pepsin	34.7	23.7	22.4	18.6	3.7	0.26	0.34	0.64
				Pyloric caeca				
α-Amylase	45.2	27.1	19.0	6.3	4.0	0.00	0.01	0.42
Lipase	0.56	0.45	0.61	0.42	0.05	0.91	0.17	0.71
Proteolytic activity	17.4	16.5	45.4 [b]	11.4 [a]	4.7	0.67	0.09	0.03
				Midgut				
α-Amylase	207.3	191.0	57.6	52.2	24.6	0.00	0.69	0.39
Lipase	3.58	4.19	2.86	3.52	0.39	0.39	0.43	0.97
Proteolytic activity	254.8	284.2	234.4	239.7	30.3	0.28	0.19	0.53

Values presented as means (n = 9), and standard error of the mean (SEM). Different lower-case letters denote significant differences between dietary P/CH ratios. 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. The significant interactions between the factors are presented in the upper part of the table. CH: carbohydrate; FM: fishmeal; I: interaction; PF: plant feedstuffs; P: protein; PS: protein source.

Alkaline protease zymograms, from both PC and midgut, revealed the presence of six bands with proteolytic activity against casein, three identified as trypsin-like proteases (90, 60, and 55 KDa), and the other three as chymotrypsin-like proteases (50, 30, and 25 KDa). All treatments presented the same number of proteolytic bands (Figure 5).

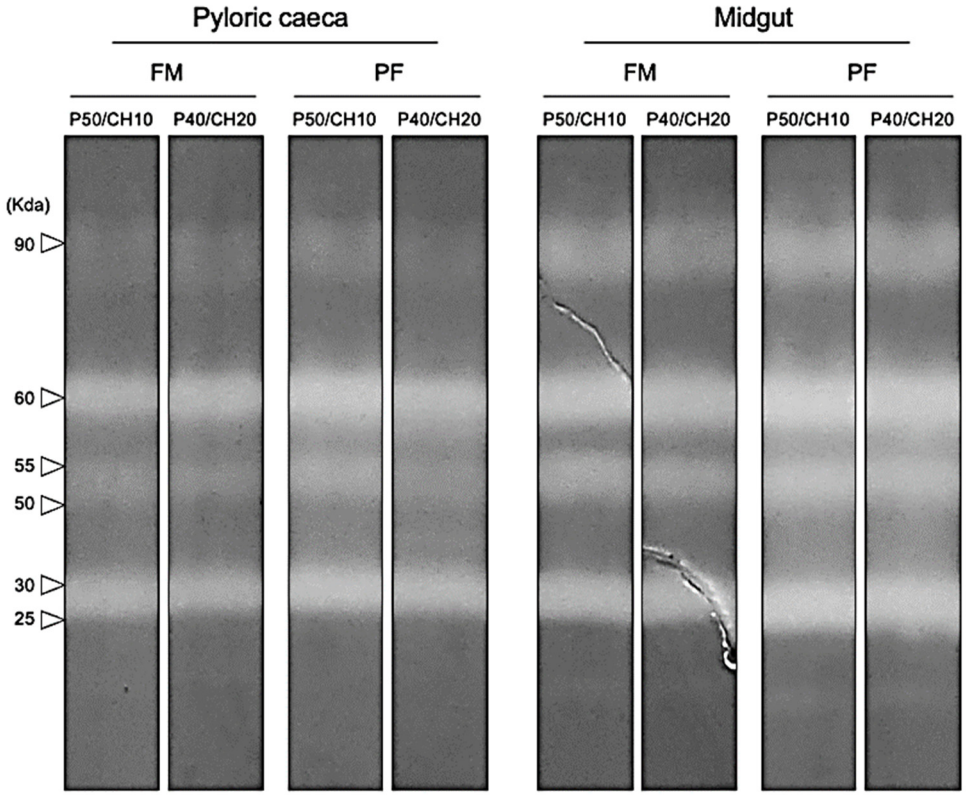

Figure 5. Representative model zymogram of alkaline proteases in pyloric caeca and midgut extracts. The molecular weight of each band with proteolytic activity is indicated. All samples were analyzed individually.

Concerning immune-related gene expressions, only *cyclooxygenase-2* (*cox2*) presented significant changes, being higher in fish fed the P50/CH10 diet (Table 7). Gene expression of *immunoglobulin M heavy chain* (*igM*), *interleukin-1β* (*il1β*), and *tumor necrosis factor-α* (*tnf-α*) was not affected by dietary composition.

Table 7. Normalized gene expression [1] of immunology and oxidative stress-related genes in midgut of gilthead seabream fed the experimental diets.

PS	FM		PF		SEM	Two-Way ANOVA		
P/CH Ratio	50/10	40/20	50/10	40/20		PS	P/CH Ratio	I
				Immunology				
cox2	0.20	0.13	0.19	0.13	0.01	0.75	0.01	0.64
igM	19.5	16.0	11.1	18.0	1.5	0.28	0.58	0.09
il1β	0.15	0.11	0.14	0.10	0.02	0.78	0.44	0.48
tnf-α	0.13	0.09	0.10	0.11	0.01	0.61	0.37	0.22
				Oxidative Stress				
hsp70	195.1	178.1	168.2	171.5	10.8	0.78	0.50	0.42
cat	61.5	46.1	47.1	44.0	4.1	0.33	0.26	0.77
gr	8.9	4.6	3.8	4.7	0.6	0.01	0.29	0.06
gpx1	13.3	9.3	8.7	8.4	0.6	0.02	0.05	0.09
sod	69.3	32.4	42.7	20.1	6.9	0.14	0.01	0.97

[1] All values expressed as arbitrary unit × 10^2. Values presented as means (n = 9), and standard error of the mean (SEM). cat: catalase; CH: Carbohydrate; cox2: cyclooxygenase 2; FM: fishmeal; gpx1: glutathione peroxidase; gr: glutathione reductase; hsp70: 70 kilodalton heat shock proteins; igM: immunoglobulin M heavy chain; I: interaction; il1β: interleukin 1β; PF: plant feedstuffs; P: protein; PS: protein source; sod: superoxide dismutase; tnf-α: tumor necrosis factor α.

Regarding the oxidative stress-related genes, PF-based diets led to a lower expression of *glutathione reductase* (*gr*) and *glutathione peroxidase* (*gpx1*), while *superoxide dismutase* (*sod*) expression was lower in fish fed the P40/CH20 diet. The gene expression of *70 kilodalton heat shock proteins* (*hsp70*) and *catalase* (*cat*) was not affected by dietary composition.

4. Discussion

The presence of antinutritional factors on PF, namely, in soybean products, was reported as leading to gut inflammation in gilthead seabream [15,18,20,21,39]. Among the observed gut morphological alterations caused by soybean meal were a decrease in gut fold height, an enlargement of submucosa and lamina propria, an increased number of inflammatory cells on tissues, and modifications on enterocytes vacuolization [15,18,20,21]. Although we have assessed the midgut and PC, and previous studies analyzed the distal gut, the present results agree with the reported observations in this species, since fish fed the PF-P50/CH10 diet, which has a higher soybean meal content (25% compared with 19% for PF-P40/CH20, and no soybean meal content for FM-based diets), also presented more histological alterations when compared with fish fed the other diets. The histological modifications observed in the midgut and PC were mainly in gut fold height, width and cellularity of lamina propria, width of the submucosa, and/or in enterocytes vacuolization. Similarly, gilthead seabream juveniles fed 30% soybean meal presented a moderately and diffusely expanded distal gut lamina propria [14], while juveniles fed soy saponins and phytosterols presented histomorphological alterations of the intestinal mucosal structure [17]. Nonetheless, during the on-growing period (fish of similar sizes to those of the present study) gilthead seabream showed a high tolerance to soy saponins and phytosterols [29]. This indicates that fish responses can be different, depending on the life stage, dietary ingredients/antinutrients combinations, and intestine portions.

Moreover, in the present study, PC seemed to be more sensitive to dietary composition changes than midgut, where fewer histomorphological alterations were observed. This agreed with the study of Couto et al. [29], which observed that dietary soy saponins and phytosterols affected PC histomorphology but not the distal gut of on-growing gilthead seabream.

However, it is important to add that the observed histomorphology modifications were not enough to consistently affect gilthead seabream growth [28]. Nonetheless, a longer experimental trial could have exposed those differences.

The composition of gut microbiota also affects gut functionality since, for instance, bacteria might have a role in nutrients' digestion and immune functions, being affected by diet composition [39]. In the present study, protein source was the single factor affecting gut microbiota. The only detectable effect on digesta microbiota was an increase of the similarity index in fish fed the PF-P50/CH10 diet, indicating that this diet might modulate gut bacteria populations towards a higher similarity between samples. The absence of any other major effect on digesta microbiota in fish fed different dietary compositions was previously observed in gilthead seabream [25]. This lack of effect could be expected, since digesta microbiota comprises transient (allochthonous) microorganisms, which are often surrounded by the resident microbiota to the gut wall and, thus, do not last a long time in the gut [40].

The higher number of OTUs, richness, and diversity indices observed in the mucosa microbiota of fish fed the PF-based diets agree with what was previously reported for this species, at the juvenile stage, fed soybean meal-based diets compared with FM-based diets [16], and for other species also fed PF-based diets, such as Senegalese sole (*Solea senegalensis*) and Atlantic salmon [41–43]. These results could be explained by the presence of non-digestible carbohydrates on PF, which provide the required substrate for gut bacteria proliferation [44,45]. It should be noticed that higher richness and diversity indices, as in fish fed the PF-based diets, can be undesirable since they can be associated with the presence of pathogenic bacteria in gut microbiota [18,46]. On the other hand, a diverse gut microbiome, with the increase of microorganisms from the Firmicutes phylum, can

stimulate a fish's innate immunity and reduce the gut surface area for the establishment of pathogenic bacteria, improving the fish's health [47–49]. Although, in the present study, none of the immune-related genes measured were affected by the use of PF, the dominant allochthonous and autochthonous bacteria detected were indeed the most closely related to the Firmicutes and Proteobacteria phyla, as already described in gilthead seabream fed different dietary compositions [18,47]. However, in future studies, a higher-resolution method, such as next-generation sequencing and FISH, could improve the characterization of the bacterial communities under different dietary feeding regimes, providing not only the full identification of the species and/or subspecies of the bacteria, but also allowing for their quantification. This more in-depth characterization and quantification of the bacterial species and/or subspecies will possibly allow for a clearer connection between microbiota and gut functionality.

Both *Pseudomonas* sp. and *Lactobacillus* sp. can produce α-amylase [50]; however, as their presence was detected in fish fed all experimental diets, no link can be made between the presence of α-amylase-producing bacteria, the dietary ratios, and α-amylase activity measured. Indeed, the lack of differences in the gut microbiota of fish fed different dietary P/CH ratios could be partially explained by the use of pregelatinized maize starch as the main carbohydrate source. Gilthead seabream presents almost 100% starch digestibility of diets including 10 to 30% of this ingredient [26]; thus, pregelatinized maize starch does not seem to provide a substantial substrate for microbial fermentation and development. A similar lack of changes in gut microbiota was reported for gilthead seabream and other fish species fed also with highly digestible starch [26,51,52].

For diets' digestion, several enzymes are needed, with each enzyme presenting a specific role. α-amylase, proteases, and lipase are, respectively, responsible for the enzymatic hydrolysis of starch, proteins, and lipids [51–53]. Despite that we did not observe any major effect on the feed intake of fish fed the different diets [28], in the present study, α-amylase activity in PC and midgut and proteolytic activity in PC were affected by the dietary composition. The α-amylase activity was lower in the PC and midgut of fish fed the P40/CH20 diet, and in the PC, it was also lower in fish fed the PF-based diets than those fed the FM-based diets. The influence of dietary P/CH ratio can be related to the adsorption of α-amylase by starch, as suggested by Spannhof and Plantikow [54], who observed that α-amylase secreted by fish during the digestive process was adsorbed by the starch present in the diets [55,56]. This lower α-amylase activity observed in fish fed the P40/CH20 diet can partially explain the lower feed efficiency observed in our previous study in fish fed the P40/CH20 diet, in comparison with those fed the P50/CH10 diet [28]. The effects of dietary protein sources may be related to the ingredients used, namely, wheat gluten, which is a source of α-amylase inhibitors [55,56].

According to Hidalgo et al. [57] and Fernández et al. [58], α-amylase activity is more dependent on fish nutritional habits than the proteolytic activity, and this is further supported by the lack of effects on the proteolytic activity reported in gilthead seabream fed diets with different P/CH ratios [25–27]. However, studies in other fish species showed that higher dietary protein levels increased proteolytic activity [59–62]. In the present study, higher proteolytic activity in fish fed the diet with a higher protein content was also observed in the PC, but only in fish fed the PF-based diets. Moreover, no differences were found regarding the alkaline protease pattern, as observed in the zymograms of the different dietary treatments, suggesting the proteases present are the same independently of the diet offered. Differently, García-Meilán et al. [24] observed that, in gilthead seabream fed FM-based diets, PC proteolytic activity was higher in fish fed lower dietary protein-content diets (P35 and P38), while in the midgut, the proteolytic activity increased progressively as dietary protein increased, stabilizing at 41% to 47% of protein. Thus, more studies should be conducted to clarify the effects of dietary protein level and source on proteolytic activity in the gut.

In the present study, fish fed the PF-based diets presented lower *gr* and *gpx1* gene expression than those fed FM-based diets, which may indicate that the former were more

vulnerable to oxidative stress [63]. This evidence seems to be in agreement with the presence of soybean meal antinutritional factors, such as the β-conglycinin, which has been identified as one of the major feed allergens [64,65]. This allergen has an N-glycan structure, essential for the formation of di-tyrosine bridges, which trigger the process responsible for oxidative stress, increasing the malondialdehyde content, and causing oxidative damages [64].

Regarding dietary P/CH ratio effects on oxidative stress, the decrease of *sod* gene expression in fish fed the P40/CH20 diet may indicate that those fish were also more susceptible to oxidative stress. Nevertheless, Castro et al. [23] observed in gilthead seabream that the intestinal sod activity was not affected by the use of different dietary P/CH ratios. Indeed, a disconnection between the gene expression and enzymatic activity results was previously reported by other studies [22,66]. Thus, we might not disregard that the response at the biochemical level might be different of the one obtained at molecular level. Hence, future studies should also include enzymatic activities which, together with the gene expression analyses, will allow for a more complete conclusion.

Sitjà-Bobadilla et al. [13] and Kokou et al. [19,20] reported, in gilthead seabream fed PF-based diets, a synchronism between the immune and stress responses and the gut histomorphological alterations. A similar relationship was observed in the present study, although no effects were observed in the immune-related genes analyzed, except for *cox2* expression, which was higher in fish fed the high-protein diets. Cox2 is linked mainly to inflammation [67,68], so it might be expected that an increase of *cox2* gene expression would be accompanied by higher histomorphological scores in this group, which did not happen. The absence of effects on immune-related responses seems to agree with the lack of mortality or diseases observed in our previous study [28].

5. Conclusions

The present study aimed to provide an integrated view of the effects on gut health and functionality of gilthead seabream when fed diets with FM or PF as the main dietary protein sources and different P/CH ratios. However, no major statistical interactions between those two factors were observed, and in general, only independent effects were reported, which did not allow us to conclude on the cumulative effect of both factors. Dietary P/CH ratio has little effects on gut health or functionality; only a decrease of α-amylase activity and gut *cox2* and *sod* gene expression were observed.

PF-based diets are more prone to compromise CH digestibility, induce gut histomorphological changes and modifications of gut mucosa microbiota profile, and decrease expression of oxidative stress-related genes. Overall, the present data demonstrates the need of fine-tunning fish feed formulations with PF to properly preserve fish intestinal physiology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7020059/s1, Table S1: Details of diets, ingredient composition, and proximate analysis published in Basto-Silva, et al. [28].

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Institutional Review Board Statement: The experiment was performed by accredited scientists (following FELASA category C recommendations) and approved by the General Directorate of Food and Veterinary from Portugal (Certification number ORBEA-CIIMAR 30-2019), according to the European Union directive 2010/63/EU on the protection of animals for scientific purposes.

Data Availability Statement: The data used to generate the results in this manuscript can be made available if requested to the corresponding author.

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CHAPTER 6| EFFECTS OF FEEDING FREQUENCY AND DIETARY PROTEIN/CARBOHYDRATE RATIOS ON GILTHEAD SEABREAM (*Sparus aurata*) INTESTINAL FUNCTIONALITY AND HEALTH

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Research Article

Effects of Feeding Frequency and Dietary Protein/Carbohydrate Ratios on Gilthead Seabream (*Sparus aurata*) Intestinal Functionality and Health

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The present study evaluated the effects of feeding frequency (FF) and dietary protein/carbohydrate (P/CH) ratios on intestinal histomorphology, microbiota profile, and digestive and oxidative stress-related enzyme activities of gilthead seabream (*Sparus aurata*). To this purpose, two practical diets were formulated: one with 50% P and 10% CH (P50/CH10) and other with 40% P and 20% CH (P40/CH20). Triplicate groups of fish with 9.1 ± 0.01 g were fed these diets for 60 days until visual satiation at a FF of 1, 2, or 3 meals per day. Distal intestine histomorphology was not affected by diet composition or FF. However, the pyloric caeca (PC) of fish fed 1 meal per day presented more gut fold height alterations than the other groups, except in fish fed diet P50/CH10 3 meals per day, where no changes was observed. Fish fed diet P40/CH20 3 meals per day also presented higher PC submucosa cellularity than the other groups. Fish fed diet P40/CH20 presented a higher number of operational taxonomic units, microbial richness, and diversity indices than fish fed diet P50/CH10. Amylase was the only measured digestive enzyme affected by the experimental conditions, presenting higher activity in fish fed diet P50/CH10 once per day. Glucose-6-phosphate dehydrogenase activity was lower in fish fed 2 meals per day than only 1. While catalase activity was lower in fish fed 2 than 3 meals per day. Glutathione reductase activity was the only measured parameter affected both by dietary P/CH ratio and FF, being inferior in fish fed once per day the P50/CH10 diet than the P40/CH20 diet and, also in the P50/CH10 diet, to fish fed 1 than those fed 3 meals per day. Overall, no major interactions was observed between dietary P/CH ratio and FF; however, a P40/CH20 diet fed 2 meals per day might be recommended for gilthead seabream juveniles.

1. Introduction

The intestine, as the complex multifunctional organ, that is, assumes great importance in the overall performance of fish [1]. It was already established that one of the most important factors to maintain intestinal health is the use of balanced diets which fulfil the basic nutritional species requirements [2]. Carnivorous fish, such as gilthead seabream (*Sparus aurata*), evolved to digest highly digestible and nutritionally dense diets, rich in proteins (P) and low in carbohydrates (CH) [1]. Accordingly, dietary protein requirements of gilthead seabream are between 45 and 55%, depending on the

life stage, while only up to 20% CH can be used in the diets without causing major negative effects [3–6]. Dietary macronutrients can have an impact on intestinal health and functionality depending on levels and ratios between nutrients [7]. Therefore, it is important to understand how dietary nutrient ratios affect intestinal functionality and health. For instance, in gilthead seabream, although differences in intestinal histomorphology and microbiota diversity were not observed in fish fed different dietary P/CH ratios, differences were reported in digestive enzymes activity and oxidative-related parameters [8–12].

Feeding frequency (FF) optimization also helps to improve fish growth, health, and welfare [13]. The FF may modulate intestinal feed transit, digestion rate, and nutrient utilization efficiency, thus impacting growth, gut functionality, and health. In juvenile gilthead seabream, it was observed that although daily FF did not change the feed transit speed and the time that feed was in the intestine, it affected pepsin and trypsin activity [14, 15]. Furthermore, also in gilthead seabream, an increase in daily α-amylase and lipase activities was observed when FF increased from 1 to 2-3 meals per day, although these differences tended to disappear when activities were reported per meal [16].

The effects of FF on intestinal function and health have not yet been well-explored in gilthead seabream, and only scarce and diverse results are available for other fish species. For instance, in Nile tilapia (*Oreochromis niloticus*) and arapaima juveniles (*Arapaima gigas*), changes in FF did not affect the activities of digestive enzymes [17, 18], while in Lebranche mullet (*Mugil liza*) and white seabream (*Diplodus sargus*) juveniles, FF affected some digestive enzyme activities [19, 20]. Dolly Varden char (*Salvelinus malma*) juveniles fed increasing FF (up to 6 meals per day) presented higher serum malondialdehyde (MDA, usually used as a marker of lipid peroxidation) content [21], while blunt snout bream (*Megalobrama amblycephala*) juveniles fed 3 or 4 meals per day presented lower liver MDA content in comparison with those fed with lower (1 or 2) or higher (5 or 6) meals per day [22]. Regarding the effects of FF on intestinal histomorphology, in lumpfish (*Cyclopterus lumpus*), the severity of the inflammation increased in fish fed daily compared to fish fed only 3 or 4 days a week [23]. Nile tilapia fed on alternate days presented higher intestinal microbial biodiversity than fish fed every third day or kept unfed [24].

While results regarding FF effects on intestine functionality and health are disperse and seem contradictory and dependent on fish species, our recent observation that 2 or 3 meals a day improved growth of gilthead seabream juveniles fed P50/CH10 or P40/CH20 diets, when compared with only 1 meal a day [25], led us to inquire if FF manipulation might improve intestine functionality and health. In fact, it is known that FF affects CH utilization improving feed utilization and growth [26–28]. However, studies on intestine functionality and health, which might explain those improvements are lacking. Actually, there are only two studies in fish assessing simultaneously the effects of P/CH ratios and FF on parameters related with intestinal functionality, namely, in digestive enzymes, and none is in gilthead seabream [29, 30]. Thus, the current study aimed to evaluate the effects of FF (1, 2, or 3 meals per day) and dietary P/CH ratio (P50/CH10 or P40/CH20) on gilthead seabream intestinal histomorphology, microbiota diversity, and digestive and oxidative stress status.

2. Materials and Methods

2.1. Experimental Conditions and Sampling. Two plant-feedstuff- (PF-, 77%) based, isolipidic (17% crude lipids), and isoenergetic (20 kJ g^{-1}) diets with different P/CH ratios were formulated. One diet included 50% P and 10% CH, while the other diet included 40% P and 20% CH. The main source of CH used was wheat meal, while fish oil was the main lipid source. The composition of the experimental diets and proximate analysis is presented in Table 1.

The experimental trial was performed at the Marine Zoology Station of the University of Porto (Portugal) in a recirculating water system equipped with 18 fiberglass tanks (100 L water capacity), thermo-regulated to 24 ± 1°C, with a salinity of 36.0 ± 1.0 g L^{-1}, dissolved oxygen of 6.0 ± 0.5 mg L^{-1}, and where each tank was supplied with a continuous flow of filtered seawater (6.0 L min^{-1}). Gilthead seabream (*Sparus aurata*) juveniles were acquired from Sonríonansa Pesués (Cantabria, Spain). After a quarantine period of 19 days, 360 fish with a mean individual initial body weight of 9.10 ± 0.01 g were randomly distributed by 18 tanks (20 fish per tank). The diets and different FF were randomly assigned to triplicate groups. Fish were fed by hand for 60 days, 6 days a week, until visual satiation, at a FF of 1 meal (09 : 00), 2 meals (09 : 00 and 17 : 00), or 3 meals (09 : 00, 13 : 00, and 17 : 00) per day.

At the end of the trial, 8 fish from each tank were sampled 5 h after the morning meal, euthanized with a sharp blow to the head, and dissected on ice-cold trays. Three fish were sampled for collection of the distal intestine (DI, distinguished from the mid intestine by an enlarged diameter and darker mucosa) and pyloric caeca (PC) for histology evaluation. Samples were rinsed in phosphate-buffered saline, blotted dry with a paper towel, fixed in Bouin (code 57211, Thermo Scientific-Richard-Allan Scientific, Kalamazoo, USA) for 24 h, and then transferred to ethanol (70%) until further processing. The whole intestine with PC and intestinal content from 3 other fish was collected, immediately frozen in liquid nitrogen, and stored at -80°C until the analysis of digestive enzyme activity and lipid peroxidation (LPO). The remaining 2 fish were sampled under aseptic conditions to collect mucosa for microbiota characterization. Autochthonous microbiota samples were obtained by scraping the internal intestinal mucosa surface, immediately frozen in liquid nitrogen, and stored at -80°C until microbiota characterization.

2.2. Histological Processing and Morphological Evaluation. The DI and PC samples were processed and sectioned using standard histological techniques and stained with hematoxylin and eosin. Samples were evaluated as indicated by Krogdahl et al. [31], through a blinded semiquantitative analysis

Table 1: Composition of the experimental diets and proximate analysis.

	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})[a]	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. [a]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).

focusing on changes in (1) widening and shortening of the mucosal fold heights, (2) increased cellularity of the connective tissue and widening of lamina propria and submucosa, (3) infiltration of mixed leucocyte population (namely, intraepithelial lymphocytes and eosinophilic granular cells) in both the above-mentioned layers, (4) nucleus position and hypervacuolization within the enterocytes, and (5) increased number of goblet cells per analyzed area. The number of goblet cells was counted in each selected area/section, previously measured, as in the following equation:

$$\begin{aligned}\textbf{\textit{Goblet cells (GC) frequency}} = [&(\textbf{\textit{n}}^{\circ}\text{ of }\textbf{\textit{GC on section 1}} \div \textbf{\textit{area from section 1}}) \\ &+ (\cdots) + (\textbf{\textit{n}}^{\circ}\text{ of }\textbf{\textit{GC on section 4}} \\ &\div \textbf{\textit{area from section 4}})] \div 4.\end{aligned} \quad (1)$$

The 4 most intact villus sections were evaluated on each cut. The score 1 was given to the tissue with the least changes, and subsequent scores (up to 5) accounted for increasing histomorphology alterations, as described by Penn et al. [32]. The presence of goblet cells equal to the average was assigned with score 1. Scores 2, 3, 4, and 5 were assigned to sections where the presence of goblet cells was, respectively, 25%, 50%, 75%, or 100% above average. Digital image obtention and measurement of the selected areas were done with the Zen software (Blue edition; Zeiss, Jena, Germany). Three individual histological cuts were evaluated from each of nine fish ($n = 9$) within each experimental condition.

2.3. Microbial Diversity Analysis. Intestinal mucosa samples of the two fish per tank were pooled to reduce individual variation. DNA extraction, PCR amplification, polymorphism analyses of 16S rRNA genes by denaturing gradient gel electrophoresis (DGGE), bands excision, and reamplification were performed as described by Castro et al. [11] with slight modifications. Namely, samples were homogenized in a Precellys Evolution tissue homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France). Each PCR product was loaded on an 8% polyacrylamide gel with a denaturing gradient of 30 to 60% of 7 M urea/40% formamide. Amplicons were sequenced to identify microbiota operational taxonomic units (OTUs). Phylogenetic analysis to identify the closest known species was done as described in Castrol et al. [11]. Only sequences higher than 100 bp reads and a query coverage of 85-100% were considered for a valid identification.

2.4. Enzymatic Activities and Lipid Peroxidation (LPO). Fish intestines were homogenized (Ystral homogenize -Laboratory Series X10, Ballrechten-Dottingen, Germany) in 4 parts of ice-cold 50 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM EDTA (ref. E5134, Sigma-Aldrich, Sintra, Portugal) and 0.1% (*v*/*v*) Triton X-100 (ref. T8787, Sigma-Aldrich, Sintra, Portugal). After centrifugation of homogenates (30 000*g*, 30 min, 4°C), the supernatants were recovered and stored at -80°C until use. All enzyme activities were measured at 37°C in a Multiskan GO microplate reader (model 51119200; Thermo Scientific, Nanjing, China) according to the specific assay conditions.

α-amylase (EC 3.2.1.1), lipase (EC 3.1.1.3), and total alkaline proteases activities were measured as described by Couto et al. [33]. Superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), glutathione peroxidase (GPX; EC 1.11.1.9), and glutathione reductase (GR, EC 1.6.4.2) activities were evaluated as described by Guerreiro et al. [34]. The optimal substrate and protein concentrations

Table 2: Details of the score-based evaluation of distal intestine histomorphology of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			P40/CH20			SEM	p value
FF	1	2	3	1	2	3		
Intestine fold height	2.44	2.00	2.22	2.78	2.78	3.22	0.15	0.16
LP-width	1.89	2.33	1.89	1.89	2.00	1.89	0.06	0.14
LP-cellularity	1.89	2.11	1.67	1.44	1.67	1.44	0.09	0.25
SM-width	2.00	2.00	1.78	1.78	1.78	2.00	0.06	0.59
SM-cellularity	1.11	1.56	1.44	1.33	1.11	1.11	0.07	0.25
GCs	1.44	1.00	2.00	1.22	2.11	1.89	0.17	0.14
IELs	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
EGCs	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
Ent.- nucleus alignment	1.89	2.33	2.33	2.22	2.00	2.22	0.08	0.44
Ent.-vacuolization	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
Mean score	1.57	1.63	1.63	1.57	1.64	1.68	0.03	0.72

Values presented as means ($n = 9$) and pooled SEM. The results were analyzed using Kruskal-Wallis followed by all pairwise comparisons, and the significance values were adjusted using Bonferroni correction for multiple tests. No significant differences was found. CH: carbohydrate; EGC: eosinophilic granulocytes presence; Ent.: enterocytes; FF: feeding frequency; GC: goblet cell presence; IEL: intraepithelial leucocyte infiltration; LP: Lamina propria; P: protein; SEM: standard error of the mean; SM: submucosa.

Table 3: Details of the score-based evaluation of pyloric caeca histomorphology of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			P40/CH20			SEM	p value
FF	1	2	3	1	2	3		
Intestine fold height	1.78^{b}	1.11^{a}	1.44^{ab}	1.89^{b}	1.13^{a}	1.11^{a}	0.09	0.01
LP-width	2.00	2.00	2.00	2.11	2.25	2.00	0.03	0.15
LP-cellularity	1.89	1.89	2.00	2.11	1.88	1.89	0.07	0.91
SM-width	1.78	2.00	1.78	1.67	2.13	2.22	0.09	0.39
SM-cellularity	1.00^{a}	1.11^{a}	1.22^{a}	1.11^{a}	1.25^{a}	1.67^{b}	0.06	0.01
GCs	1.11	1.44	1.67	1.33	1.63	1.33	0.12	0.77
IELs	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
EGCs	1.00	1.00	1.11	1.00	1.00	1.00	0.02	0.43
Ent.- nucleus alignment	2.44	2.67	2.67	2.33	2.50	2.78	0.07	0.42
Ent.-vacuolization	1.67	1.22	1.67	1.67	1.63	1.33	0.08	0.35
Mean score	1.57	1.54	1.66	1.62	1.64	1.63	0.03	0.74

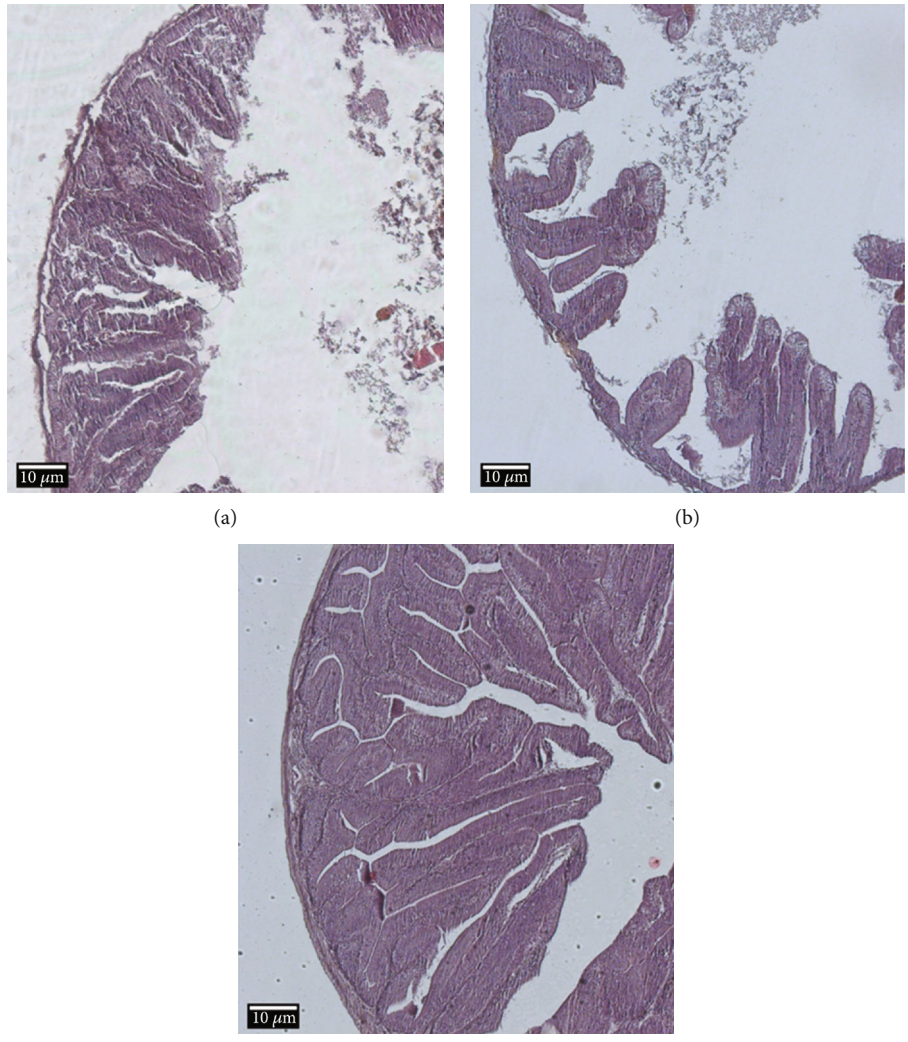
Values presented as means ($n = 9$) and pooled SEM. Different lowercase letters indicate statistical differences between experimental conditions groups as analysed by the Kruskal-Wallis followed by all pairwise comparisons. The significance values were adjusted by Bonferroni correction for multiple tests. CH: carbohydrate; EGC: eosinophilic granulocytes presence; Ent.: enterocytes; FF: feeding frequency; GC: goblet cell presence; IEL: intraepithelial leucocyte infiltration; LP: Lamina propria; P: protein; SEM: standard error of the mean; SM: submucosa.

for the measurement of the maximal activity for each oxidative stress enzyme were established by preliminary tests. The molar extinction coefficients used for H_2O_2 and NADPH were 0.039 and 6.22 mM^{-1} cm^{-1}, respectively.

CAT and SOD were expressed as units (U) per mg of soluble protein, and all other enzymes were expressed as mU/mg protein. Except for SOD, whose activity unit was defined as the amount of enzyme needed to produce 50% inhibition of the ferricytochrome C reduction rate, and one unit (U) of enzyme activity was defined as the amount of enzyme needed to catalyse the hydrolysis of 1 μmol/min of substrate at assay temperature (37°C). Protein concentration was measured according to Bradford [35] using Bio-Rad Protein Assay Dye Reagent (ref. 5.000.006, Amadora, Portugal), with albumin bovine serum (ref. A4503, Sigma-Aldrich, Sintra, Portugal) as standard.

Malondialdehyde (MDA) concentration was measured as described in Couto et al. [33]. Results were expressed as nmol MDA per g of tissue, calculated from a calibration curve.

2.5. Statistical Analysis. All data are presented as the mean and standard error of the mean (SEM). Statistical analysis was done using SPSS 25 software package for Windows (IBM® SPSS® Statistics, New York, USA). Data were tested for normality by the Shapiro-Wilk test and homogeneity of variances by the Levene test. When normality was not verified, data were transformed before ANOVA.



(a) (b)

(c)

Figure 1: Detail of the alterations observed on intestine fold height in the pyloric caeca of gilthead seabream fed P50/CH10 diet or P40/CH20 diet, 1 meal per day (a and b), comparing with those fed P50/CH10 diet, 2 meals per day (c). Images with haematoxylin-eosin staining captured at 10x magnification.

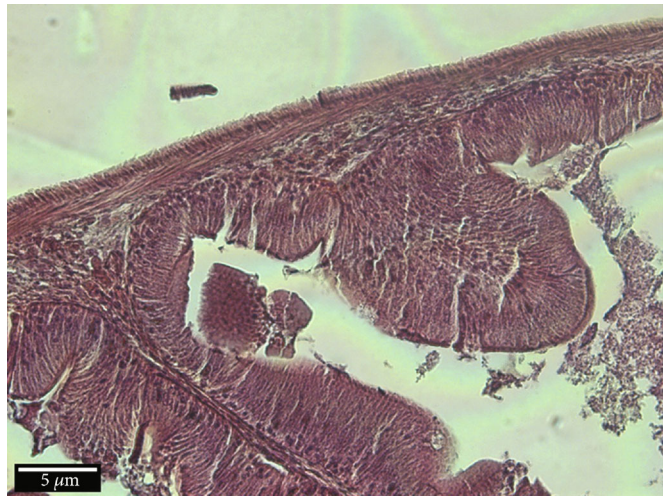
The enzyme activity data and LPO were analyzed by two-way ANOVA, with the dietary P/CH ratio and FF as factors. In the case of interaction between factors, one-way ANOVA was performed for the P/CH ratio within each FF, and FF within each P/CH ratio. Significant differences among groups were determined by the Tukey's multiple range test. Differences were considered statistically significant when $p < 0.05$. Since data for histomorphology evaluation were not normal nor homogenous, a nonparametric Kruskal-Wallis test followed by all pairwise comparisons was performed, and the significance values were adjusted by using the Bonferroni correction for multiple tests.

Statistical analysis related with the DGGE was performed as described in Castro et al. [11]. Intestine microbiota data were then subjected to two-way ANOVA with P/CH ratio and FF as factors, as described for the other parameters.

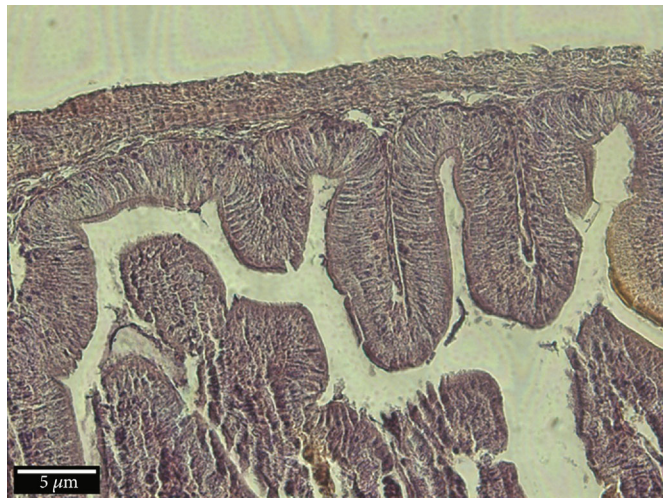
3. Results

The results of the growth trial were not the goal of the present study being presented in Basto-Silva et al. [25]. Shortly, feed intake was increased in fish fed the P40/CH20 diet and 2 or 3 meals per day, while fish fed 1 meal per day presented higher protein efficiency ratio (PER), feed efficiency (FE), and nitrogen retention (NR), but lower final fish weight than the other groups. Furthermore, the P40/CH20 diet led to an increase in PER and NR and a decrease in FE compared to fish fed the P50/CH10 diet.

3.1. Intestinal Histomorphology. Experimental diets and FF did not affect the histomorphology of the DI (Table 2). However, the PC of fish fed 1 meal per day presented a higher fold height compared to the remaining experimental conditions, except for fish fed the P50/CH10 diet 3 meals per day



(a)



(b)

Figure 2: Detail of the alterations observed on submucosa cellularity in the pyloric caeca of gilthead seabream fed P40/CH20 diet, 3 meals per day (a), comparing with those fed P50/CH10 diet, 1 meal per day (b). Images with haematoxylin-eosin staining captured at 40x magnification.

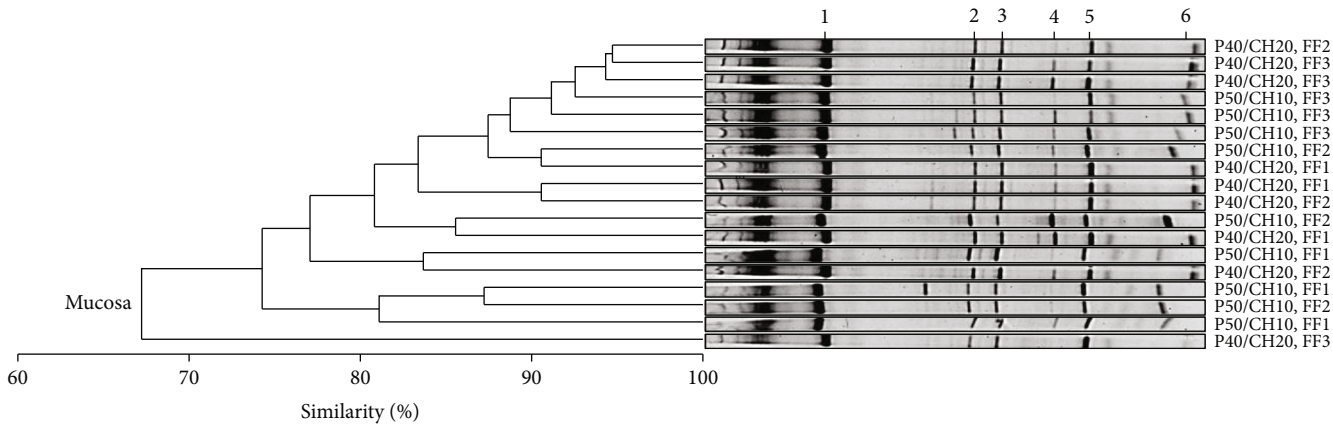


Figure 3: Dendrogram and PCR-DGGE fingerprint of the intestines' autochthonous microbiota of gilthead seabream fed the experimental diets at different feeding frequencies. The bacterial species identified and described in Table 4 correspond to the sequenced gel bands represented in this figure by numbers (1-6).

Table 4: Ecological parameters obtained from PCR-DGGE fingerprints of the intestines' autochthonous microbiota of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			SEM	P40/CH20			SEM
FF	1	2	3		1	2	3	
OTUs	9.67	11.33	13.33	0.63	13.00	13.67	12.67	0.45
Richness[1]	0.56	0.65	0.77	0.04	0.75	0.80	0.73	0.03
Diversity[2]	2.10	2.28	2.43	0.06	2.43	2.45	2.37	0.04
SIMPER similarity (%)[3]	78.83	80.89	90.02	1.99	81.02	79.05	77.71	2.74

Two-way ANOVA	P/CH ratio	FF	I	P/CH ratio		FF		
				P50/CH10	P40/CH20	1	2	3
OTUs	0.02	0.13	0.07	A	B	—	—	—
Richness[1]	0.02	0.13	0.06	A	B	—	—	—
Diversity[2]	0.02	0.20	0.06	A	B	—	—	—
SIMPER similarity (%)[3]	0.30	0.39	0.29	—	—	—	—	—

Values presented as means and pooled SEM ($n = 3$ per treatment pooled from 6 fish). The results were analyzed by using two-way ANOVA, followed by the Tukey's test. Different uppercase letters indicate significantly different P/CH ratios. CH: carbohydrates; FF: feeding frequency; I: interaction; OTUs: average number of operational taxonomic units; P: protein; SEM: standard error of the mean. [1]Margalef species richness: d = (S-1)/log(N). [2]Shannon's diversity index: $H^{'} = -\sum(\text{pi}(\ln\text{pi}))$. [3]SIMPER: similarity percentage within group replicates.

Table 5: Closest known species identified from the DNA sequencing of the autochthonous intestinal bacteria communities of gilthead seabream fed the experimental diets at different feeding frequencies.

Band	Closest known species (BLAST)	Phylum	Similarity (%)	Accession number of nearest neighbor
1	*Lactobacillus aviarius* subsp. *aviarius*	Firmicutes	100	LC071825.1
2	*Lactobacillus acidophilus*	Firmicutes	100	MT645504.1
3	Uncultured bacterium from environmental samples	n/a	98	EU009390.1
4	Uncultured bacterium from environmental samples	n/a	86	LC031369.1
5	*Pseudomonas* sp.	Proteobacteria	100	MK033128.1
6	Uncultured bacterium from environmental samples	n/a	100	KY857639.1

where no changes were observed (Table 3, Figure 1). Furthermore, fish fed the P40/CH20 diet 3 meals per day showed higher cellularity of the submucosa (SM) compared to the remaining experimental conditions (Figure 2).

3.2. Microbiota Diversity. The Bray-Curtis dendrogram and PCR-DGGE fingerprint analysis showed that the intestine bacterial communities maintained a similarity of up to 60% (Figure 3). However, no clustering was detected between samples from different experimental diets or FF. The average number of OTUs, microbial richness, and diversity indices were higher in fish fed the P40/CH20 diet, while the similarity index was not affected by the dietary composition or FF (Table 4). Sequence analysis of DGGE selected bands showed that the dominant autochthonous bacteria detected corresponded to uncultured bacteria not yet assigned to a specific taxon or were most closely related to genera belonging to the phylum Firmicutes and Proteobacteria, namely, *Lactobacillus* sp. and *Pseudomonas* sp., respectively (Table 5).

3.3. Digestive and Oxidative Stress-Related Enzymes and Lipid Peroxidation. The α-amylase activity was increased in fish fed diet P50/CH10 and also in fish fed 1 meal per day (Table 6). Total alkaline protease and lipase activities were not affected by diet or FF.

G6PD and CAT activities were affected by FF, but not by the dietary P/CH ratio (Table 7). Fish fed 2 meals per day presented lower G6PD and CAT activities than fish fed 1 or 3 meals per day, respectively. GR activity was higher in fish fed 3 meals than 1 meal per day, but only in fish fed P50/CH10 diet. Furthermore, in fish fed diet P50/CH10 1 meal per day GR activity was also lower than in fish fed the P40/CH20 diet in the same FF. GPX, SOD, and LPO were not affected by diets or FF.

4. Discussion

Potential interactions between the dietary P/CH ratio and FF on growth, feed utilization, and metabolism of CH were recently evaluated in gilthead seabream [25], as well as in gibel carp (*Carassius auratus gibelio*) and common carp (*Cyprinus carpio*) [29, 30]. While Cheng et al. [30] also presented data on α-amylase activity and Zhao et al. [29] on trypsin activity, this is the first study to determine the combined effects of the dietary P/CH ratio and FF on several

Table 6: Specific activity of digestive enzymes, α-amylase, lipase, and total alkaline protease activity (mU/mg protein) of gilthead seabream fed experimental diets at different feeding frequencies.

(a)

P/CH ratio	P50/CH10			SEM	P40/CH20			SEM
FF	1	2	3		1	2	3	
α-Amylase	663.8	407.0	391.8	39.9	441.1	370.5	373.1	26.3
Lipase	15.1	12.0	13.8	0.8	13.6	11.8	11.6	0.6
Total alkaline protease	624.3	669.8	582.5	19.7	674.8	611.5	662.4	20.7

(b)

Two-way ANOVA	P/CH ratio	FF	I	P/CH ratio		FF		
				P50/CH10	P40/CH20	1	2	3
α-Amylase	0.03	0.00	0.10	B	A	b	a	a
Lipase	0.18	0.12	0.71	—	—	—	—	—
Total alkaline protease	0.40	0.73	0.12	—	—	—	—	—

Values presented as means ($n = 9$) and pooled SEM. The results were analyzed by two-way ANOVA, followed by the Tukey's test. Different uppercase letters indicate significantly different P/CH ratios, and lowercase letters indicate significantly different feeding frequencies. CH: carbohydrates; FF: feeding frequency; I: interaction; P: protein; SEM: standard error of the mean.

parameters of intestinal morphology, functionality, and health of fish.

In the current study, neither the dietary P/CH ratio nor FF affected DI histomorphology, but some minor alterations were observed in the PC in fold-height and submucosa cellularity. This may suggest that PC was more sensitive than DI to the dietary treatments and FF imposed. However, the minor alterations observed in PC most probably did not have biological significance, since the PC mean score was similar between groups, and no correlation was observed between the remaining functionality and health intestine parameters. Previously, Couto et al. [36] also observed in gilthead seabream that dietary soy purified antinutrients affected the PC but not DI histomorphology. Likewise, the absence of DI histomorphology alterations in gilthead seabream fed with different P/CH ratio diets was previously observed by other authors [11, 37].

Gut microbiota composition is strongly influenced by dietary composition and FF either in mammals or fish [38–42]. Although changing the dietary P/CH ratio alters the available nutrients for bacteria fermentation, the associated changes in gut microbiota composition remain unclear [38, 40, 42]. In the current study, fish fed the diet P40/CH20 had an increased average number of OTUs, richness, and diversity indices. The present experiment does not allow us to conclude if these differences are due to the different amounts of protein or CH in the diet. In European seabass (*Dicentrarchus labrax*), a dietary increase of CH lead to increased gut microbiota diversity [43] and this may suggest that the results observed in the current study might also be related with the increased CH content of diet P40/CH20. However, in gilthead seabream, fish fed 0% or 20% of CH diets did not present differences in gut microbiota composition [11]. However, in that study, only the allochthonous microbiota was analyzed, whereas in the present study, we analyzed the autochthonous microbiota. Differences between the two studies might also be related to the CH source used: wheat meal in the current study and gelatinized starch in the study by Castro et al. [11], thus providing different substrates for bacteria proliferation [38, 40]. Besides these differences, the different outcomes might be connected to the different fish sizes used in both studies (9 g in the current study against 71 g in the study by Castro et al. [11]), as it is known that fish developmental stages influence microbiota composition [44].

In the current study, no differences was observed in the autochthonous gut microbiota with FF. Differently, Sherif et al. [41] observed in Nile tilapia that exchanging the feeding regime on a alternately weekly basis affected the intestine microbiota, changing the abundance and proportions of *Lactobacillus*, *Aeromonas*, *Pseudomonas*, and *Edwardsiella* spp. However, in the present study, gut microbiota composition was evaluated by DGGE, a technique that has relatively low resolution. Therefore, for a comprehensive assessment of dietary and FF effects on fish, further studies should be done using methods with a higher-resolution, as, for instance, next-generation sequencing.

Similar to the present study, Cheng et al. [30] also assessed the combined effects of dietary P/CH ratios and FF on α-amylase activity in common carp. The authors tested diets with 3 P/CH ratios (P32/CH5, P30/CH10, and P28/CH20) fed 2 or 4 meals per day and, as in the present study, did not report any significant interaction between those two factors. However, as in the current study, fish fed the higher CH diet and the higher FF presented lower α-amylase activity. These results agree with previously reported results in gilthead seabream fed low P/CH ratio diets [12]. A possible explanation for these results is that in high CH diets, α-amylase molecules could be adsorbed by crude starch, thus inhibiting starch hydrolysis and, at the same time, accelerating intestinal transit speed, leading to a reduction in the time available for intestinal absorption

Table 7: Intestine specific activity of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), catalase (CAT), superoxide dismutase (SOD) (U/mg protein), and lipid peroxidation (LPO) (nmol malondialdehyde g^{-1} tissue) of gilthead seabream fed the experimental diets at different feeding frequencies.

P/CH ratio	P50/CH10			SEM	P40/CH20			SEM
FF	1	2	3		1	2	3	
G6PD	14.1	8.6	12.1	1.0	16.7	10.8	12.0	0.9
GPX	7.9	8.5	19.3	1.9	12.8	17.4	11.6	2.0
GR	20.1^{Aa}	25.8^{ab}	31.6^{b}	1.5	33.1^{B}	23.1	31.2	2.1
CAT	28.0	18.6	44.4	6.4	18.2	15.3	55.3	9.9
SOD	663.2	782.4	776.3	45.2	807.6	709.8	784.1	53.9
LPO	51.5	61.5	42.4	4.2	50.8	46.8	45.3	2.9

Two-way ANOVA	P/CH ratio	FF	I	P/CH ratio		FF		
				P50/CH10	P40/CH20	1	2	3
G6PD	0.20	0.00	0.61	—	—	b	a	Ab
GPX	0.34	0.18	0.12	—	—	—	—	—
GR	0.21	0.02	0.02					
CAT	0.94	0.03	0.87	—	—	Ab	a	b
SOD	0.83	0.72	0.61	—	—	—	—	—
LPO	0.46	0.21	0.31	—	—	—	—	—

Values presented as means ($n = 9$) and pooled SEM. The results were analyzed by two-way ANOVA, followed by Tukey's test. Two-way ANOVA: if the interaction was significant, one-way ANOVA was performed for P/CH ratio within feed frequency and for feed frequency within P/CH ratio. In this case, significant differences were indicated in the upper part of the table. Different uppercase letters indicate significantly different P/CH ratios, and lowercase letters indicate significantly different feeding frequencies. CH: carbohydrates; FF: feeding frequency; I: interaction; P: protein; SEM: standard error of the mean.

[45]. Another explanation is that when fish are fed fewer meals per day, the higher feed load by meal promotes higher pancreatic secretion of α-amylase [19].

It could be expected that the change in the level of dietary protein affected proteolytic activity, as previously observed by García-Meilán et al. [9, 12] also in gilthead seabream. Nevertheless, no differences in total alkaline protease activity was observed in the current study. Similar to the present results, a lack of effects on proteolytic activity was also reported in gilthead seabream fed different dietary P/CH ratios [8]. The authors tested different levels of P, lipids, and CH in the diet and concluded that intestinal total proteolytic activity was only influenced by changes in dietary lipid, suggesting that proteolytic activity is more sensitive to changes in dietary fat than variations in dietary P or CH.

When the production of reactive oxygen species (ROS) is higher than the respective removal, LPO occurs. CAT reduces H_2O_2 to O_2 and H_2O, being more active when the production of H_2O_2 is high, while G6PD is involved in NADPH regeneration which is a coenzyme required for the normal functioning of CAT, GPX, and GR [46, 47]. In the current study, although LPO levels were not affected by the FF, lower G6PD and CAT activities were observed in fish fed 2 meals per day, which might indicate a reduction of total ROS production. The available data suggests that an intermediary FF contributes to improving the antioxidant capacity of fish. Accordingly, in juvenile Dolly Varden char, total antioxidant capacity increased with FF up to 5 meals per day, decreasing at higher FF [21]. Also, in blunt snout bream fed between 1 and 6 meals per day, the lowest hepatic CAT and GPX activities were detected in fish fed 3 or 4 meals per day, while the total antioxidant capacity was higher in these groups [22]. Similarly, in juvenile tiger puffer (*Takifugu rubripes*), fish fed 4 or 6 meals per day exhibited lower antioxidant enzyme activities, namely, SOD, CAT, and GPX activities, than those fed only 2 meals per day or continuous feeding [48].

In the current study, the dietary P/CH ratio did not have any major effect on LPO or antioxidant enzyme activities. This is similar to what was previously reported for gilthead seabream and European seabass [10, 49].

GR which catalyzes the NADPH-dependent regeneration of reduced glutathione from oxidized glutathione generated by GPX was the only oxidative stress-related enzyme presenting an interaction between dietary P/CH ratio and FF. Despite no differences was observed regarding GPX activity, GR results might suggest that fish fed diet P40/CH20 at 1 meal per day might be under a higher overall ROS production than fish fed diet P50/CH10 at the same FF. Within fish fed the P50/CH10 diets, the same seems true for fish fed 3 meals instead of 1 meal per day. However, since no other interactive effect was observed in the remaining stress oxidative-related enzymes, or any other measured parameter, it is not possible to draw any conclusion regarding the interactive effect of using different FF and P/CH ratios.

In conclusion, the present results indicate that there are no major interactions between the dietary P/CH ratios and FF with respect to the intestinal functionality and health of gilthead seabream. Present results further support the conclusion of Basto-Silva et al. [25] where a diet with a lower P/CH ratio (P40/CH20 vs. P50/CH10) fed 2 meals per day appears to be the most adequate strategy for gilthead seabream juveniles.

Data Availability

The data used to generate the results in this manuscript can be made available if requested to the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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CHAPTER 7| GILTHEAD SEABREAM (*Sparus aurata*) IN VITRO ADIPOGENESIS AND ITS ENDOCRINE REGULATION BY LEPTIN, GHRELIN, AND INSULIN

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Gilthead seabream (*Sparus aurata*) *in vitro* adipogenesis and its endocrine regulation by leptin, ghrelin, and insulin

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ABSTRACT

Leptin, ghrelin, and insulin influence lipid metabolism and thus can directly affect adipose tissue characteristics, modulating the organoleptic quality of aquaculture fish. The present study explored gilthead seabream (*Sparus aurata*) cultured preadipocytes development, and the regulation of adipogenesis by those three hormones. Preadipocytes presented a fibroblast-like phenotype during the proliferation phase that changed to round-shaped with an enlarged cytoplasm filled with lipid droplets after complete differentiation, confirming the characteristics of mature adipocytes. *peroxisome proliferator-activated receptor-γ* (*pparγ*) expression was higher at the beginning of the culture, while *fatty acid synthase* and *3-hydroxyacyl-CoA dehydrogenase* gradually increased with cell maturation. The expression of *lipoprotein lipase-like*, *lysosomal acid lipase* (*lipa*), *fatty acid translocase/cluster of differentiation-36* (*cd36*), and *leptin receptor* (*lepr*) were not affected during cell culture development; and undetectable expression levels were observed for *leptin*. Concerning regulation, leptin inhibited lipid accumulation significantly reducing *pparγ* and *cd36* gene expression, both in early differentiating and mature adipocytes, while ghrelin decreased the expression of *pparγ* in the early differentiating phase but did not reduce intracellular lipid content significantly. Additional insulin past the onset of adipogenesis did not affect lipid accumulation either. In conclusion, at present culture conditions leptin has an anti-adipogenic function in differentiating preadipocytes of gilthead seabream and continues exerting this role in mature adipocytes, while ghrelin and insulin do not seem to influence adipogenesis progression. A better understanding of leptin, ghrelin, and insulin impact on the adipogenic process could help in the prevention of fat accumulation, improving aquaculture fish production and quality.

1. Introduction

In fish, the adipose tissue has an important role in whole-organism energy homeostasis, particularly in lipid metabolism, namely by regulating tissue lipogenesis, lipolysis, and *β*-oxidation (Salmerón, 2018). In mature adipocytes, lipogenesis converts fatty acids (FA) or other substrates (as glucose, amino acids or carbohydrates) from the diet into triglycerides (TG) for long-term storage until later use is required (Weil et al., 2013). During energy requirement periods, lipolysis and *β*-oxidation pathways are activated promoting the release of FA and glycerol into the blood from where they are captured by cells to provide energy for metabolic processes (Weil et al., 2013; Salmerón, 2018). The adipose tissue grows either by hypertrophy (increase in size by TG storage) and hyperplasia (*i.e.* adipogenesis), the later occurring by differentiation of precursor cells (Otto and Lane, 2005). The adipogenic process includes two main phases: (i) proliferation, where cells from the stromal vascular fraction divide and are committed to differentiate towards the adipocyte lineage, mainly through the coordination of Peroxisome proliferator-activated receptor-γ (*Pparγ*) and CCAAT/enhancer-binding protein-*α* (*C/ebpα*), and (ii) differentiation, in which

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those transcription factors promote the expression of characteristic proteins (as Lipoprotein lipase, *Lpl*, or Fatty acid translocase/cluster of differentiation 36, *Cd36*) involved in lipid uptake, transport, synthesis and storage of FA and subsequent adipokines secretion (Rosen and MacDougald, 2006; Salmerón, 2018).

Leptin and ghrelin are two hormones that mainly take part in appetite regulation, but also affect many other processes, such as lipid metabolism, in fish as in mammals (Kim et al., 2008; Liu et al., 2009; Salmerón et al., 2015). Leptin was already described in several fish species, for instance, orange-spotted grouper (*Epinephelus coioides*), pacu (*Piaractus mesopotamicus*) and rainbow trout (*Oncorhynchus mykiss*) (Murashita et al., 2008; Zhang et al., 2013; Volkoff et al., 2017), as being mainly produced in the liver, but also in other tissues, such as adipose tissue, stomach, and intestine (reviewed by Rønnestad et al., 2017). Leptin has been described as a satiety signal, anti-obesogenic hormone, and regulator of the liberation or storage of lipids from tissues (Copeland et al., 2011). In rainbow trout, *in vitro* leptin treatment stimulated lipolysis in adipocytes, supporting an anti-adipogenic role of this hormone (Salmerón et al., 2015). Similar results were observed by Lu et al. (2012) and Song et al. (2015) in grass carp (*Ctenopharyngodon idellus*) and yellow catfish (*Pelteobagrus fulvidraco*), respectively, where leptin treatment stimulated both, hepatic lipolysis and *β*-oxidation, while inhibiting lipogenesis. In both studies, leptin treatment promoted a release of glycerol, a reduction of hepatic lipid content, a decrease of *pparγ* gene and protein expression, and an upregulation of key *β*-oxidation-related genes, such as *pparα*, and *carnitine palmitoyl transferase-1* (*cpt-1*).

Ghrelin is mainly expressed in the stomach but also in the gastrointestinal tract, pancreas, heart, and hypothalamus, and seems to act mainly as a hunger signal, although differences exist between fish species (Jönsson, 2013; Perelló-Amorós et al., 2018; Bertucci et al., 2019). These authors suggested that ghrelin has species-specific functions in fish, not only in appetite regulation but also concerning other metabolic responses; however, available data regarding its effects on lipid metabolism are still scarce and contradictory. In rainbow trout adipocytes, ghrelin treatment seemed to activate lipid turnover, stimulating the synthesis of TG (i.e. lipogenesis), their mobilization and use (Salmerón et al., 2015), while in Mozambique tilapia (*Oreochromis mossambicus*) long-term ghrelin treatment with micro-osmotic pumps increased liver and muscle total fat content (Riley et al., 2005). Differently, in *in vivo* studies with rainbow trout and brown trout (*Salmo trutta*), ghrelin did not affect lipid metabolism or deposition (Jönsson et al., 2010; Tinoco et al. 2014; Chisada et al., 2014).

Insulin acts as a growth promoter and affects lipid metabolism by inducing adipocytes differentiation and increasing adipose fat stores in red sea bream (*Pagrus major*), Atlantic salmon (*Salmo salar*), and large yellow croaker (*Pseudosciaena crocea* R.) (Oku et al., 2006; Sánchez-Gurmaches et al., 2011; Wang et al., 2012). Insulin promoted rainbow trout preadipocyte differentiation and stimulated *lpl* gene expression in proliferating and freshly isolated adipocytes of the same species (Bouraoui et al., 2012; Cruz-Garcia et al., 2015). However, insulin did not seem to increase lipid accumulation during the differentiation phase on rainbow trout (Salmerón et al., 2015). On the other hand, insulin injection promoted *lpl* gene expression in gilthead seabream (*Sparus aurata*) adipose tissue, suggesting also an adipogenic role of insulin in this species (Albalat et al., 2007). Consistently, insulin induced lipid accumulation in primary cultured preadipocytes of gilthead seabream, as it does in rainbow trout, which suggests that insulin can trigger the process of differentiation of adipocytes also in sparids (Bouraoui et al., 2008; Salmerón et al., 2013).

Gilthead seabream represents about 7% of all marine fish produced in the world (FIGIS, 2019), and has an important economic value for Mediterranean aquaculture. Since hormonal factors, like ghrelin, leptin, and insulin, influence lipid metabolism in a species-specific manner, it is of utmost importance to have a better understanding of their effects on adipocyte cells of gilthead seabream, as this may influence adipose tissue characteristics and consequently hamper fish quality, by affecting both carcass and fillet yields, and organoleptic parameters. Moreover, understanding and increasing knowledge on fish adipose tissue biology has great scientific interest. Thus, the present study aims to contribute to the characterization of adipogenesis and the evaluation of leptin, ghrelin, and insulin effects in the adipogenic process using an *in vitro* primary cell culture model of gilthead seabream preadipocytes.

2. Material and methods

2.1. Fish maintenance and ethics statement

Gilthead seabream (*Sparus aurata*) juveniles of approximately 30 g body weight were obtained from Piscimar S.L. (Burriana, Castellón, Spain) and maintained at the animal facilities of the Faculty of Biology at the University of Barcelona (Spain). Fish were kept in 0.4 m^3 tanks in a temperature-controlled seawater recirculation system at 23 ± 1 °C, salinity of 36 ± 1 g L^{-1}, dissolved oxygen kept near saturation, and a 12 h light/12 h dark photoperiod. Fish were fed *ad libitum* twice daily with a commercial diet (OptiBream, Skretting, Burgos, Spain), and fasted 24 h before performing the cell cultures to avoid contamination from the gastrointestinal tract. Before adipose tissue extraction, fish were anesthetized (MS-222, 0.1 g L^{-1}) and subsequently sacrificed by cranial concussion. All animal handling procedures were done by accredited scientists (following FELASA category C recommendations) and approved by the Ethics and Animal Care Committee of the University of Barcelona (certification number CEEA OB34/17), following the European Union, Spanish, and Catalan government-established norms and procedures.

2.2. Gilthead seabream cultured preadipocytes: characterization and endocrine regulation

2.2.1. Establishment of the preadipocyte primary culture

The establishment of the preadipocyte primary cultures followed the procedure described by Salmerón et al. (2013). For each culture, 6 to 9 gilthead seabream juveniles were used, collecting a pool of 3 g of visceral adipose tissue. In fact, pooling adipose tissue samples from different animals allows to obtain sufficient and homogeneus preparations of precursor cells, not biased by a particular individual condition, to perform at once all the experimental treatments for them to be comparable. Briefly, the extracted tissue was first washed and minced with Krebs-HEPES buffer (pH 7.4) with 1% antibiotic/antimycotic solution and digested for 1 h with type II collagenase (130 UI mL^{-1}) in Krebs-HEPES buffer plus 1% BSA at 18 °C with gentle agitation. Next, the cell suspension was filtered through a 100 μm cell strainer, centrifuged (1500 rpm, 10 min) to get rid of mature adipocytes, and the obtained pelleted cells were counted using a Neubauer chamber. Cells were seeded in 1% gelatin-treated 6- or 12-well plates at a final density of 4.3 × 10^4 cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% of antibiotic/antimycotic solution and 60 mM NaCl (growth medium, GM), and incubated at 23 °C with 2.5% CO_2. The medium was changed every 2 days during the whole experiment.

2.2.2. Cell culture development characterization

The primary cultured preadipocytes were maintained during 16 days as described in Salmerón et al. (2013), first cultured in GM and then, on day 8, the medium was replaced by a differentiation medium (DM), composed by GM plus 10 μg mL^{-1} porcine insulin (corresponding to 1700 nM), 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) and 0.25 μM dexamethasone. To properly induce adipocyte maturation, 5 μL mL^{-1} of lipid mixture (4.5 mg mL^{-1} cholesterol, 10 mg mL^{-1} cod liver oil fatty acids (methyl esters), 25 mg mL^{-1} polyoxyethylene sorbitan monooleate and 2.0 mg mL^{-1} D-*α*-tocopherol actetate) (L5146, Sigma) were also added to the DM. Four days after induction of

differentiation the medium was changed to GM plus lipid mixture (5 μL mL^{-1}) and the cells were maintained on it until the end of the experiment. During the development of the cells, representative images were taken at different times with an Axiovert 40C inverted microscope (Carl Zeiss, Germany) coupled to a Canon EOS 1000D digital camera (Tokyo, Japan).

For gene expression characterization, preadipocyte samples were collected at days 4 and 8 (i.e. before the induction of differentiation), and at days 12 and 16 (i.e. mature adipocytes). After being washed with phosphate-buffered saline (PBS), cell samples of two duplicate wells of the 6 well-plates were collected with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) using a cell scraper, transferred to an RNase-free polypropylene tube, and kept at −80 °C until performing RNA extraction, as described in Section 2.4.1. Results are the average of 7 independent adipocyte cultures ($n = 7$).

2.2.3. Endocrine regulation of adipocytes differentiation

For the evaluation of the endocrine regulation of the adipogenic process, cells were stimulated at two moments. First, preadipocytes at day 8 were induced to differentiate with DM containing lipid mixture (5 μL mL^{-1}), leptin (100 nM), or ghrelin (10 nM). Second, adipocytes at day 12 were treated with GM plus lipid mixture (5 μL mL^{-1}), leptin (100 nM), ghrelin (10 nM), or insulin (1000 nM). Insulin was only tested at day 12, since this hormone is *per se* already included in the cocktail used for differentiation at a concentration of 1700 nM (i.e. DM), and thus an additional 1000 nM would not have make a difference according to previous data (Bouraoui et al., 2012). But in fact, it is wellkwown that insulin enhances fish adipocytes differentiation by itself, as reported by several authors (Oku et al., 2006; Sánchez-Gurmaches et al., 2011; Wang et al., 2012). The recombinant rainbow trout leptin (29% of sequence identity with gilthead seabream) used was a kind gift of Dr. Ivar Rønnestad (University of Bergen, Norway), who produced it following the procedure described in Murashita et al. (2008). The synthetic 20 amino-acid octanoylated rainbow trout ghrelin (80% of sequence identity with gilthead seabream) used was a kind gift of Dr. Elisabeth Jönsson (University of Gothenburg, Sweden), who obtained it from the Peptide Institute Inc., Osaka (Japan). The porcine insulin (67% and 88% of sequence identity of insulin chains A and B respectively, with gilthead seabream sequences) was obtained from Sigma. In all cases, identity of leptin, ghrelin and insulin was verified by Blast and BlastP searches, and the concentrations used were chosen based on previous literature (Salmerón et al., 2015). The DM or GM plus lipid mixture treatments at days 8 and 12, respectively, were used as positive controls since they represent the standard culturing procedure. Six hours after being subjected to the treatments, cells were washed with PBS and, for each condition, two duplicate wells of the 6 well-plates were collected with 1 mL of TRI Reagent Solution using a cell scraper, transferred to an RNase-free polypropylene tube, and kept at −80 °C until performing gene expression analyses. Cell samples were obtained from 7 independent experimental adipocyte cultures ($n = 7$).

Furthermore, in parallel 12-well plates, cells at day 8 were treated for 72 h with DM or DM plus leptin (100 nM), ghrelin (10 nM), insulin (1000 nM), or lipid mixture (5 μL mL^{-1}), as a positive control to evaluate lipid accumulation by Oil Red O (ORO) staining. To corroborate the pro-adipogenic effect of lipid mixture in the current experimental conditions, cells maintained only in DM were used as a negative control. Six independent adipocyte cultures ($n = 6$) were performed.

2.3. Oil red O staining

To evaluate leptin, ghrelin, and insulin effects in adipocyte differentiation, after each treatment cells were stained with ORO (O0625, Sigma) as described by Capilla et al. (2011). Cells were fixed with 10% formalin for 1 h and stained with 0.3% ORO diluted in 36% triethylphosphate for 2 h. After washing excessive dye with distilled water, representative images of the development of the cells were obtained using an Axiovert 40C inverted microscope coupled to a Canon EOS 1000D digital camera. Then, quantification of the lipid content was done by extraction of the lipids with 2-propanol for 30 min and reading the absorbance at 490 nm in duplicate 96-wells (Tecan Infinite M200, Switzerland). For total protein extraction, cells were then washed with distilled water, stained with Comassie brilliant blue G-250 dye for 1 h, and incubated at 60 °C with 85% propylene glycol (398039, Sigma) during 1 h. Quantification of total protein was obtained from the absorbance measured at 630 nm in duplicate 96-wells using the same microplate reader. Final TG quantification was calculated as the quotient of the absorbances measured at 490 nm and at 630 nm.

2.4. Gene expression

2.4.1. RNA extraction and cDNA synthesis

RNA extraction followed the TRI Reagent Solution manufacturer's instructions (Applied Biosystems, Alcobendas, Spain). Total RNA concentration and purity were determined in a NanoDrop 2000 (Thermo Scientific, Alcobendas, Spain). Four-hundred fifty ng of total RNA were used for cDNA synthesis using DNase I enzyme (Life Technologies, Alcobendas, Spain) to remove all genomic DNA, and Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) according to the manufacturer's recommendations. Samples were stored at −20 °C until used.

2.4.2. Quantitative real-time PCR (qPCR)

qPCR analyses followed the requirements of MIQE guidelines (Bustin et al., 2009) and were performed in a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain). All samples were analyzed in duplicate, by adding 2.5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM of forward and reverse primers (Table 1), 1 μL of each cDNA sample at the appropriate dilution, and autoclaved water until a final volume of 5 μL. The qPCR reactions included the activation step (1 cycle of 3 min at 95 °C; followed by 40 cycles of 10 s at 95 °C and 30 s at primer melting temperature); and the amplicon dissociation step (increasing temperature by 0.5 °C every 30 s from 55 to 95 °C). The appropriate cDNA dilution, primers efficiency, and absence of primer-dimers were determined by a dilution curve with a pool of samples. *Ribosomal protein l27* (*rpl27*) and *β-actin* were selected as reference genes since they did not show significant differences between groups ($P > 0.05$), and relative expression was calculated following the Pfaffl (2001) method.

2.5. Statistical analysis

All data are presented as mean ± standard error (SE). 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 log-transformed. Data on gene profile characterization were analyzed by one-way ANOVA, followed by Tukey's test to determine differences between means. Hormone (leptin, ghrelin, and insulin) effects were assessed by one-way ANOVA, followed by Dunnett's test. The lipid accumulation effects on gilthead seabream adipocyte cells were evaluated comparing each treatment with the negative control and, the gene expression data were evaluated using the lipid treatment as the positive control. A statistical significance of $P < 0.05$ was set for all the statistical tests performed. All statistical analyses were carried out using SPSS 25 software package for Windows (IBM® SPSS® Statistics, New York, USA).

3. Results

3.1. Characterization of preadipocyte cell culture development

On day 4 (Fig. 1A), preadipocyte cells showed a triangular fibroblastic shape that became increasingly elongated by day 8 (Fig. 1B).

Table 1
Genes and primers used for qPCR.

Gene	Sequence (5′–3′)	Accession n°	Tm (°C)	Efficiency (%)
Transcription factor				
pparγ	F: CGCCGTGGACCTGTCAGAGC R: GGAATGGATGGAGGAGGAGGAGATGG	AY590304	66	97.9
Lipogenesis markers				
fas	F: TGGCAGCATACACACAGACC R: CACACAGGGCTTCAGTTTCA	AM952430	60	97.0
lpl-lk	F: CAGAGATGGAGCCGTCACTCAC R: TCTGTCACCAGCAGGAACGAATG	JQ390609	60	93.0
Lipolysis marker				
lipa	F: TACTACATCGGACACTCTCAAGGAAC R: GTGGAGAACGCTATGAATGCTATCG	JQ308831	60	94.0
β-oxidation marker				
hoad	F: GAACCTCAGCAACAAGCCAAGAG R: CTAAGAGGCGGTTGACAATGAATCC	JQ308829	60	95.3
Fatty acid transporters				
cd36	F: GTCGTGGCTCAAGTCTTCCA R: TTTCCCGTGGCCTGTATTCC	Riera-Heredia et al. (2019)	60	94.0
fatp1	F: CAACAGAGGTGGAGGGCATT R: GGGGAGATACGCAGGAACAC	Riera-Heredia et al. (2019)	60	102.0
Appetite regulation-related				
leptin	F: TCTCTTCGCTGTCTGGATTCCTGGAT R: CTCCTTCTTGCTCTGTAGCTCTT	KP822924	60	–
lepr	F: GGCGGAACTGATTCTACTCTG R: AGTATCGGACCTCGTATCTCA	MG570178	60	111.0
Reference genes				
β-actin	F: TCCTGCGGAATCCATGAGA R: GACGTCGCACTTCATGATGCT	X89920	60	102.0
rpl27	F: AAGAGGAACACAACTCACTGCCCCAC R: GCTTGCCTTTGCCCAGAACTTTGTAG	AY188520	68	100.2
ef1α	F: CTTCAACGCTCAGGTCATCAT R: GCACAGCGAAACGACCAAGGGGA	AF184170	60	84.3

F: forward; R: reverse; Tm: melting temperature; *pparγ*: *peroxisome proliferator-activated receptor-γ*; *fas*: *fatty acid synthase*; *lpl-lk*: *lipoprotein lipase like*; *lipa*: *lysosomal acid lipase*; *hoad*: *3-hydroxyacyl-CoA dehydrogenase*; *cd36*: *fatty acid translocase/cluster of differentiation 36*; *fatp1*: *fatty acid transport protein 1*; *lepr*: *leptin receptor*; *β-actin*: *beta-actin*; *rpl27*: *ribosomal protein L27*; *ef1α*: *translation elongation factor 1 alpha.*

After DM addition, the differentiating adipocyte cells acquired a rounded shape (Fig. 1C) and, its continuous growth promoted the enlargement of the cytoplasm, where lipid droplets could be found, characteristic of a mature adipocyte (Fig. 1D).

Concerning transcriptional characterization, the expression of the key adipogenic factor *pparγ* was significantly higher at day 4 of culture development when compared with all other days (Table 2). Similarly, the gene expression of the *fatty acid transport protein 1* (*fatp1*) was higher at the beginning of the culture, at day 4 compared to day 8; whereas on the contrary, *fatty acid synthase* (*fas*) and *3-hydroxyacyl-CoA dehydrogenase* (*hoad*) gene expression significantly increased with adipocyte differentiation. The gene expression of *lipoprotein lipase-like* (*lpl-lk*), *lysosomal acid lipase* (*lipa*), *cd36*, and *leptin receptor* (*lepr*) were not affected during cell culture development. Undetectable levels of expression were observed for *leptin* throughout the whole adipogenic process.

3.2. Leptin, ghrelin, and insulin effects on adipocyte differentiation

Lipid accumulation in adipocyte cells measured using ORO staining was significantly inhibited by leptin treatment (Fig. 2C and F), while ghrelin and insulin treatments had no effect (Fig. 2D, E, and F) when compared to the negative control cells, induced to differentiate only with the DM, containing the usual hormonal cocktail but not lipid mixture (Fig. 2A and F). The addition of lipid mixture to the DM consistently promoted the highest lipid accumulation on adipocyte cells (Fig. 2B and F), confirming its effectiveness as a positive control.

During the initial preadipocyte differentiation (at day 8), leptin promoted a decrease in *pparγ* and *cd36* gene expression, while ghrelin also downregulated *pparγ* expression (Table 3). The mRNA levels of all other genes analyzed (namely *fas*, *lpl-lk*, *lipa*, *hoad*, *fatp1,* and *lepr*) were not affected by any of the hormonal treatments.

When the hormonal treatments were applied in more advanced stages of adipocyte differentiation (at day 12), leptin also caused a decrease of both *pparγ* and *cd36* transcript levels, while ghrelin and insulin did not further affect any of the genes analyzed (Table 4).

4. Discussion

In the present study, the morphological changes of adipocytes during culture development followed the same pattern previously reported by Salmerón et al. (2013) for primary cultured preadipocytes of the same species. Namely, with preadipocyte cells showing a fibroblast appearance during the proliferation phase, and mature adipocytes presenting a rounded shape and a larger cytoplasm with lipids accumulated after complete differentiation. Similar morphological evolution was also reported for cultured adipocytes of other fish species like Atlantic salmon (Vegusdal et al., 2003), rainbow trout (Bouraoui et al., 2008), large yellow croaker (Wang et al., 2012), and grass carp (Liu et al., 2015).

The transcriptional characterization during gilthead seabream *in vitro* adipogenesis initiated in Salmerón et al. (2016) has been extended in the present study. As previously reported, the key transcription factor of adipogenesis, *pparγ,* showed higher gene expression during the cell proliferation phase, evidencing its importance only up to the onset of adipocyte differentiation (Salmerón et al., 2016). However, in other fish species, such as Atlantic salmon, rainbow trout, and large yellow croaker, *pparγ* gene expression seemed to be longer promoted during

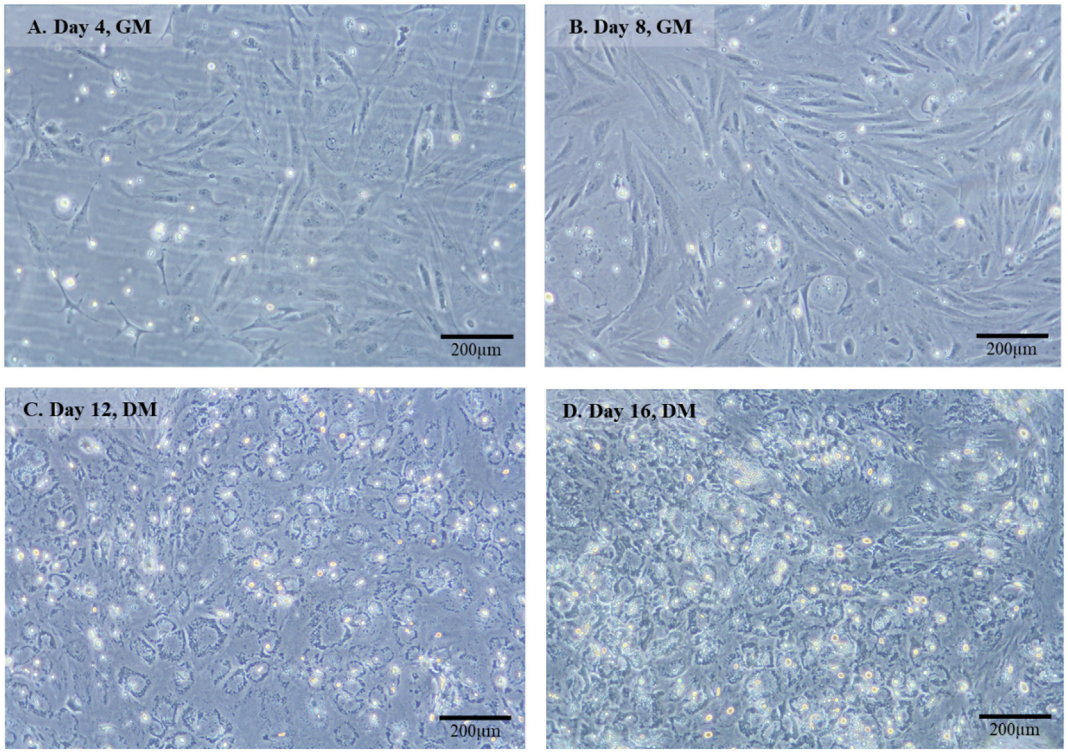

Fig. 1. Representative phase-contrast images of gilthead seabream preadipocyte cells growing in growth medium (GM), at day 4 (A) and day 8 (B); and adipocytes in differentiation medium (DM), at day 12 (C) and day 16 (D). Magnification 10×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the adipocyte differentiation process (Vegusdal et al., 2003; Bouraoui et al., 2008; Wang et al., 2012), while in red sea bream *pparγ* gene expression was not affected during adipogenesis (Oku and Umino, 2008). Nevertheless, also in rainbow trout, a recent detailed analysis during the early differentiation phase (days 7 to 11) showed a *pparγ* expression profile similar to the current one, with a transient upregulation and a subsequent abrupt decrease within 24 h after induction of differentiation by the addition of a DM (Riera-Heredia et al., 2019). Thus, *Pparγ* seems to have a critical role in early adipogenesis, but more studies should be done for a better understanding of its specific function during this process in fish.

Similar to what was previously observed in red sea bream and grass carp (Oku and Umino, 2008; Liu et al., 2015), in the present study *fas* gene expression increased during adipogenesis. This was expected, since in the adipocytes Fas participates in *de novo* lipogenesis for fat storage (Wang et al., 2012). However, in our previous study in gilthead seabream, *fas* gene expression gradually decreased during adipocyte differentiation (Salmerón et al., 2016), suggesting a negative feedback mechanism, due to the high availability of FA in the culture medium. Such negative feedback was also shown in Atlantic salmon preadipocytes treated with palmitic acid (Bou et al., 2016). In that study, it was observed a decrease of Acetyl-CoA carboxylase expression, and consequently in the malonyl-CoA production needed for palmitate synthesis through fas action. Although in the present study such negative feedback was not detected, at least regarding *fas* expression, in primary fetal rat calvarial cultured cells, palmitate treatment reduced the expression of *pparγ* (Yeh et al., 2014), which could explain the observed decrease in *pparγ* gene expression in the present study. In fact, the upregulation of *fas* expression may lead to increased production of palmitate, which in turn might cause a reduction in *pparγ* gene

Table 2
Normalized gene expression profile in gilthead seabream adipocytes during culture development.

	Days			
	4	8	12	16
pparγ	0.00432 ± 0.00055^{b}	0.00201 ± 0.00029^{a}	0.00154 ± 0.00017^{a}	0.00236 ± 0.00029^{a}
fas	0.039 ± 0.006^{a}	0.089 ± 0.033^{ab}	0.102 ± 0.028^{ab}	0.139 ± 0.040^{b}
lpl-lk	0.00088 ± 0.00042	0.00091 ± 0.00038	0.00129 ± 0.00044	0.00071 ± 0.00025
lipa	0.036 ± 0.010	0.030 ± 0.005	0.032 ± 0.007	0.036 ± 0.007
hoad	0.180 ± 0.041^{a}	0.220 ± 0.066^{a}	0.358 ± 0.071^{ab}	4.121 ± 3.527^{b}
cd36	0.269 ± 0.048	0.498 ± 0.076	0.606 ± 0.137	0.359 ± 0.063
fatp1	0.042 ± 0.007^{b}	0.020 ± 0.003^{a}	0.024 ± 0.006^{ab}	0.028 ± 0.004^{ab}
lepr	0.00047 ± 0.00020	0.00086 ± 0.00014	0.00059 ± 0.00014	0.00073 ± 0.00010

Preadipocyte cells (days 4 and 8) and mature adipocyte cells (days 12 and 16). At day 8, after preadipocyte cells collection, a differentiation medium (DM) was used to promote cell differentiation. Values are presented as means ($n = 7$) ± standard error (SE). Results were analyzed by one-way ANOVA, followed by Tukey's test. Values with different superscripts are significantly different ($P < 0.05$). Transcription factor: *pparγ*, *peroxisome proliferator-activated receptor-γ*; lipogenesis markers: *fas*, *fatty acid synthase*; and *lpl-lk*, *lipoprotein lipase like*; lipolysis marker: *lipa*, *lysosomal acid lipase*; *β*-oxidation marker: *hoad*, *3-hydroxyacyl-CoA dehydrogenase*; fatty acid transporters: *cd36*, *fatty acid translocase/cluster of differentiation 36*; and *fatp1*, *fatty acid transport protein 1*; appetite regulation-related gene: *lepr*, *leptin receptor*.

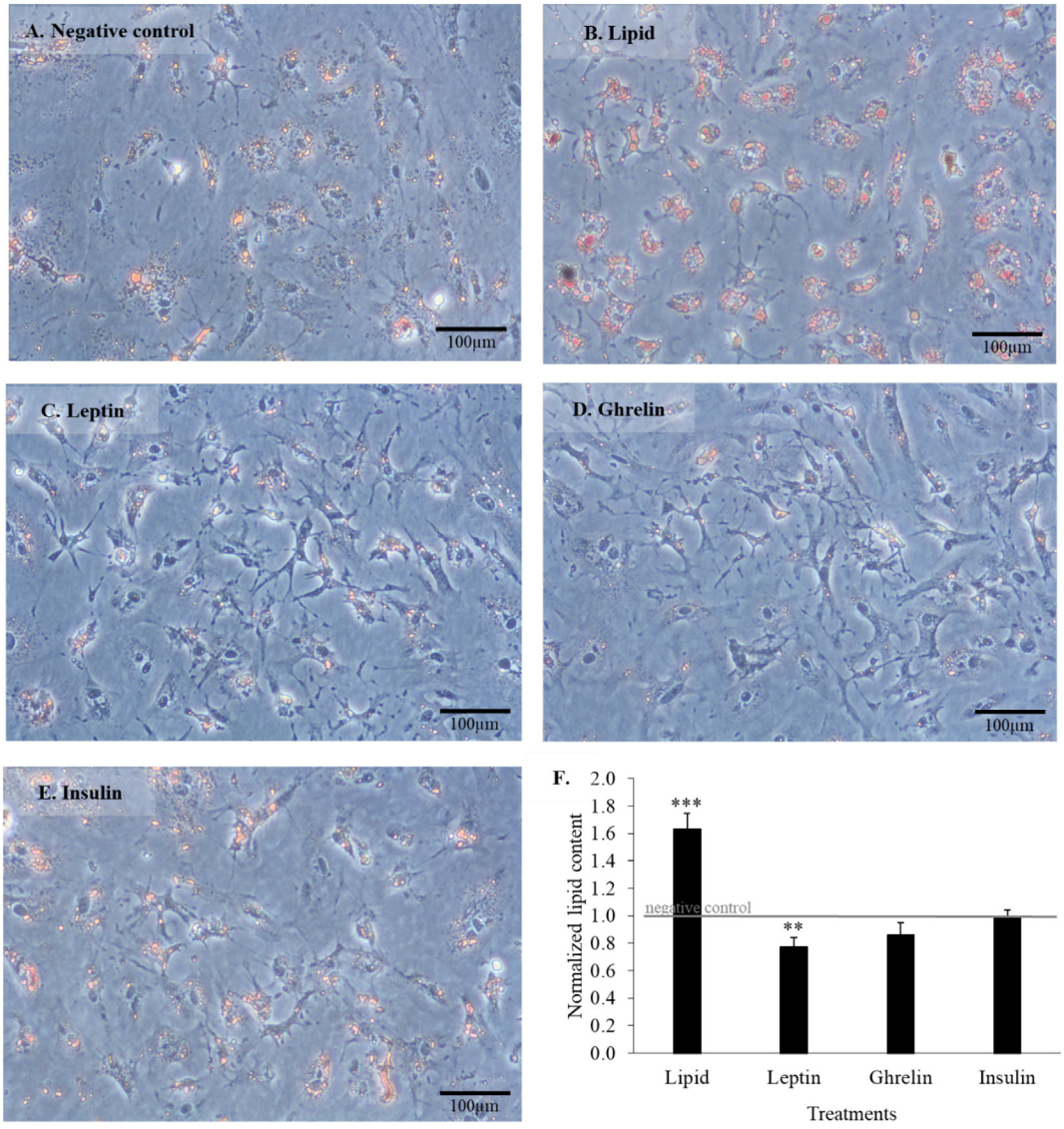

Fig. 2. Lipid, leptin, ghrelin, and insulin effects on lipid accumulation in gilthead seabream adipocyte cells. Representative phase-contrast images of gilthead seabream adipocyte cells treated at day 8 with only differentiation medium (DM) as negative control (A), 5 µL mL^{-1} lipid mixture (B) 100 nM leptin (C), 10 nM ghrelin (D), or 1000 nM insulin (E) for 72 h and stained with Oil red O. Magnification 20×. (F) Quantification of lipid content normalized by protein content and expressed as fold change respect to the negative control treatment (grey line). Data are presented as means ($n = 6$) and standard error (SE). Results were analyzed by one-way ANOVA, followed by Dunnett's test. Significant differences between the negative control and each one of the treatments tested are indicated by $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression.

The Lpl is a key enzyme involved in lipid deposition and metabolism and has been recognized as a lipogenesis marker, being an indicator of preadipocytes differentiation (Weil et al., 2013). In previous studies, *lpl* gene expression increased during adipocytes differentiation in Atlantic salmon (Todorčević et al., 2008), rainbow trout (Bouraoui et al., 2012), large yellow croaker (Wang et al., 2012), and grass carp (Liu et al., 2015). Previously, also in gilthead seabream, *lpl* expression gradually increased during adipocytes differentiation, although a major decrease was observed during the proliferation phase and upon adipogenic induction (Salmerón et al., 2016). In the present study, the gene expression of *lpl-lk* was also evaluated. Lpl-lk is an exclusive fish lineage isoform of Lpl, that was found in zebrafish (*Danio rerio*), tuna (*Thunnus orientalis*), and red sea bream, in addition to gilthead seabream (Benedito-Palos et al., 2013). The correlation between Lpl and Lpl-lk metabolic regulation seems to be tissue-specific. While in skeletal muscle *lpl* and *lpl-lk* had different expression responses (Benedito-Palos et al., 2013), in the liver both lipases were up-regulated in fasted fish in comparison to fed fish (Benedito-Palos et al., 2014). In the present study, *lpl-lk* mRNA levels were not affected by cell development, suggesting a different regulation for both isoforms in these conditions. Nonetheless, *lpl* and *lpl-lk* gene expression patterns and specific functions during adipocyte development in gilthead seabream still need to be better elucidated.

Lipa is essential for TG hydrolysis in lysosomes (Du et al., 2001); however, its effects in fish adipogenesis remain unclear. In *lipa*-deficient adult mice, a significant reduction of white and brown adipose tissues was observed, suggesting that this enzyme has important roles in adipocyte differentiation, lipid metabolism or fat mobilization (Du et al., 2001). However, data of the present study indicated that *lipa* gene expression did not change during adipocytes development, suggesting that this enzyme may not participate in the adipogenic pathway, at least in the cell culture times studied.

In Atlantic salmon, *acyl-coA dehydrogenase* expression, an enzyme involved in mitochondrial *β*-oxidation, decreased at later stages of adipocyte differentiation (Todorčević et al., 2008), leading the authors

Table 3
Normalized gene expression in gilthead seabream preadipocyte cells at day 8 after 6 h of lipid mixture (5 μL mL^{-1}), leptin (100 nM) and ghrelin (10 nM) treatments.

	Treatments		
	Lipid	Leptin	Ghrelin
pparγ	0.0087 ± 0.0023	0.0039 ± 0.0003*	0.0036 ± 0.0005*
fas	0.059 ± 0.004	0.045 ± 0.008	0.040 ± 0.010
lpl-lk	0.0132 ± 0.0101	0.0073 ± 0.0020	0.0068 ± 0.0035
lipa	0.074 ± 0.003	0.082 ± 0.014	0.056 ± 0.007
hoad	0.105 ± 0.015	0.096 ± 0.017	0.087 ± 0.011
cd36	0.368 ± 0.057	0.146 ± 0.020*	0.209 ± 0.076
fatp1	0.0022 ± 0.0005	0.0010 ± 0.0000	0.0013 ± 0.0002
lepr	0.00007 ± 0.00001	0.00016 ± 0.00006	0.00010 ± 0.00001

Values are presented as means (n = 7) ± standard error (SE). Results were analyzed by one-way ANOVA, followed by Dunnett's test. Significant differences between the lipid (= positive control) and each one of the treatments tested are indicated by $^*P \leq 0.05$. Transcription factor: *pparγ*, *peroxisome proliferator-activated receptor-γ*; lipogenesis markers: *fas*, *fatty acid synthase*; and *lpl-lk*, *lipoprotein lipase like*; lipolysis marker: *lipa*, *lysosomal acid lipase*; *β*-oxidation marker: *hoad*, *3-hydroxyacyl-CoA dehydrogenase*; fatty acid transporters: *cd36*, *fatty acid translocase/cluster of differentiation 36*; and *fatp1*, *fatty acid transport protein 1*; appetite regulation-related gene: *lepr*, *leptin receptor*.

to conclude that preadipocytes have a higher capacity for FA *β*-oxidation, while mature cells are more specialized in lipid storage. However, in the present study the gene expression of *hoad*, another enzyme involved in mitochondrial *β*-oxidation, increased during adipocyte culture development. In agreement with these observations, Hoad presence in adipose tissue was also reported for a few fish species, including gilthead seabream (Polakof et al., 2011; Bou et al., 2017; Sánchez-Moya et al., 2020), suggesting that it may have an important role both in adipogenesis and fully mature adipocytes.

The gene expression during adipocyte development of two membrane-associated FA transporters: *fatp1* and *cd36,* was also analyzed. According to Sánchez-Gurmaches et al. (2012), in rainbow trout Fatp1 is mainly produced in the adipose tissue, while *cd36* is expressed at a higher level in the liver although it is also expressed in the adipose tissue. Both, in Atlantic salmon and rainbow trout adipocytes, *fatp1* transcript levels were induced during adipogenesis, in parallel to lipid accumulation (Todorčević et al., 2008; Sánchez-Gurmaches et al., 2012), whereas *cd36* expression was not affected along the process (Sánchez-Gurmaches et al., 2012). Similarly, in the present study, *cd36* gene expression remained unaltered during adipocyte differentiation; however, differently to what was observed in previous studies, *fatp1* gene expression decreased during adipogenesis. This seems to indicate that differences may exist between species in the regulation of FA transporters expression throughout cell differentiation, which is in agreement with the complex regulation of these transporters in fish (Sánchez-Gurmaches et al., 2011, 2012).

Although Vegusdal et al. (2003) and Salmerón et al. (2015) described an increase of *leptin* expression during adipocyte cell differentiation in Atlantic salmon and rainbow trout, respectively, in the present study, undetectable expression levels were observed for *leptin* during *in vitro* development of gilthead seabream adipocytes. Similar results were also found *in vivo* in the same species (Basto-Silva unpublished observations), where *leptin* expression in the adipose tissue was not detected, suggesting that leptin may be none or poorly produced by gilthead seabream adipocytes, although in the same study, *leptin* mRNA was detected in brain and liver. Indeed, while in mammals the adipose tissue is the major producer of leptin (Harris, 2014), in fish, leptin is mainly expressed and produced in the liver (Zhang et al., 2013; Volkoff et al., 2017).

Nonetheless, the presence of a lepr in the adipose tissue was already reported for a few fish species, such as Atlantic salmon (Rønnestad et al., 2010), rainbow trout (Gong et al., 2013), orange-spotted grouper (Zhang et al., 2013), and Nile tilapia (*Oreochromis niloticus*) (Shpilman et al., 2014). The present study confirmed, for the first time in gilthead seabream adipocyte cells, the expression of a *lepr*. Although, Chisada et al. (2014) suggested that this hormone modulates lipogenesis in adult medaka (*Oryzias latipes*), the lepr relevance during adipogenesis is not completely understood for gilthead seabream. In the present study, *lepr* expression was unaltered during adipocyte differentiation and, mRNA levels of *leptin* were undetectable, raising doubts about the regulation of seabream adipose tissue growth and metabolism by leptin. Nevertheless, as previously mentioned, in another *in vivo* trial from our group also in gilthead seabream (Basto-Silva et al., unpublished observations), although *leptin* was neither detected in the adipose tissue, maybe due to very low levels of expression, it was found in brain and liver, supporting a role for leptin in adipocytes regulation.

Concerning the endocrine regulation of the adipogenic process, in the present study leptin treatment significantly reduced *pparγ* and *cd36* gene expression, both in early differentiating and mature adipocytes, suggesting an anti-adipogenic role of this hormone. These data are also supported by the lower accumulation of lipids in the leptin-treated gilthead seabream cells. Similarly, leptin treatment reduced intracellular TG content and *pparγ* gene expression in yellow catfish hepatocytes (Song et al., 2015) and decreased *lpl* and *fatp1* gene expression during rainbow trout adipocytes differentiation (Salmerón et al., 2015). Although in the present study a trend was also noticed for a decrease in *lpl* and *fatp1* gene expression, due to the high variability between samples this decrease was not statistically significant. These

Table 4
Normalized gene expression in gilthead seabream adipocyte cells at day 12 after 6 h of lipid mixture (5 μL mL^{-1}), leptin (100 nM), ghrelin (10 nM) and insulin (1000 nM) treatments.

	Treatments			
	Lipid	Leptin	Ghrelin	Insulin
pparγ	0.0043 ± 0.0006	0.0019 ± 0.0004*	0.0045 ± 0.0011	0.0029 ± 0.0003
fas	0.081 ± 0.014	0.067 ± 0.012	0.087 ± 0.017	0.072 ± 0.011
lpl-lk	0.043 ± 0.024	0.009 ± 0.004	0.017 ± 0.007	0.057 ± 0.034
lipa	0.087 ± 0.010	0.086 ± 0.015	0.077 ± 0.007	0.079 ± 0.019
hoad	0.181 ± 0.038	0.105 ± 0.027	0.173 ± 0.042	0.139 ± 0.018
cd36	0.406 ± 0.099	0.124 ± 0.027*	0.218 ± 0.032	0.216 ± 0.058
fatp1	0.0019 ± 0.0003	0.0012 ± 0.0001	0.0027 ± 0.0006	0.0017 ± 0.0003
lepr	0.00016 ± 0.00004	0.00006 ± 0.00002	0.00016 ± 0.00005	0.00015 ± 0.00002

Values are presented as means (n = 7) ± standard error (SE). Results were analyzed by one-way ANOVA, followed by Dunnett's test. Significant differences between the lipid (= positive control) and each one of the treatments tested are indicated by $^*P \leq 0.05$. Transcription factor: *pparγ*, *peroxisome proliferator-activated receptor-γ*; lipogenesis markers: *fas*, *fatty acid synthase*; and *lpl-lk*, *lipoprotein lipase like*; lipolysis marker: *lipa*, *lysosomal acid lipase*; *β*-oxidation marker: *hoad*, *3-hydroxyacyl-CoA dehydrogenase*; fatty acid transporters: *cd36*, *fatty acid translocase/cluster of differentiation 36*; and *fatp1*, *fatty acid transport protein 1*; appetite regulation-related gene: *lepr*, *leptin receptor*.

results are in agreement with the anti-adipogenic and anti-obesogenic actions of leptin described in mammals (Friedman and Halaas, 1998). Also in fish, intracerebroventricular and intraperitoneal injections of leptin inhibited feed intake (Murashita et al., 2008; Won et al., 2012), suggesting a decrease of energy intake which in turn could be converted into adipose tissue.

In rainbow trout, ghrelin seemed to influence adipogenesis, promoting simultaneously the synthesis of TG and their mobilization into adipocytes, accelerating lipid turnover (Salmerón et al., 2015). Similar results were observed in Mozambique tilapia, where long-term ghrelin treatment with micro-osmotic pumps promoted an increase of liver and muscle lipid content (Riley et al., 2005). However, different results were obtained in previous *in vivo* studies in rainbow and brown trout. In rainbow trout, Jönsson et al. (2010) did not observe significant differences in mesenteric adipose stores and liver or muscle lipid content between the control and the ghrelin-treated fish after a 14-days treatment period. In brown trout, a ghrelin intraperitoneal injection did not affect lipid metabolism or deposition, since the hepatosomatic index, TG content and Lpl activity in liver and muscle were not affected when compared with control fish (Tinoco et al., 2014). In the present study, although ghrelin treatment significantly decreased the gene expression of the key adipogenic transcription factor *pparγ* in gilthead seabream preadipocytes, significant effects on lipid accumulation during the differentiation phase, compared to the control condition were not observed. Moreover, the lack of significant effects on the expression of any of the genes analyzed in mature cultured adipocytes, suggested that ghrelin does not affect adipogenesis progression in this species. Notwithstanding, further studies would be required to confirm this hypothesis as ghrelin effects on adipogenesis remain controversial, both in fish and in mammals, since its effect appears to be influenced not only by the life cycle phase of the adipocytes, but also by the ghrelin concentration applied. For instance, in a mouse 3T3-L1 preadipocyte line, a 10^{-6} M ghrelin treatment inhibited differentiation but promoted the proliferation step (Zhang et al., 2004), while a 10^{-7}–10^{-15} M ghrelin treatment induced both proliferation and differentiation (Liu et al., 2009).

In fish, as in mammals (Géloën et al., 1989; Zhou et al., 2009), insulin promotes lipid accumulation and adipogenesis-related genes expression during differentiation in several species, such as red sea bream, Atlantic salmon, or large yellow croaker (Oku et al., 2006; Sánchez-Gurmaches et al., 2011; Wang et al., 2012). However, in the present study, lipid accumulation and the differentiation step were not affected when additional 1000 nM insulin was added to the cells, which were already exposed to 1700 nM insulin present in the DM hormonal cocktail. Similar results were also reported in this species by Salmerón et al. (2013), which concluded that the differentiation could be triggered by insulin, but once switched on by a DM containing hormones and a lipid mixture, insulin did not further induce lipid synthesis and accumulation. Accordingly, Bouraoui et al. (2012) also reported in rainbow trout adipocytes that the extra addition of a 1 μM insulin did not affect *lpl* gene expression nor lipid content levels. Despite this, in the same study, a combination of 1 μM insulin plus 1 μM troglitazone, an anti-diabetic agent that enhances insulin sensitivity, increased the lipid content in the cells, leading the authors to conclude that the combination of various adipogenic factors can lead to an optimal medium to induce adipocyte differentiation in rainbow trout. Thus, a better understanding of the influence of insulin in the adipogenic process, as well as its interactions with other factors, may help to understand the mechanisms of fish adipose tissue growth.

5. Conclusions

In vitro cultured preadipocytes and mature adipocytes of gilthead seabream exhibited a normal morphological evolution. The gene expression of *pparγ*, *fas*, *hoad*, and *fatp1* was affected during culture development, while *lpl-lk*, *lipa*, *cd36*, and *lepr* remained unaltered. Leptin appeared to have an anti-adipogenic function in gilthead seabream differentiating preadipocytes while ghrelin had a minor effect only downregulating *pparγ*. In mature adipocytes, leptin seemed to continue exerting its anti-adipogenic role, while ghrelin and insulin did not further affect adipogenesis progression. Notwithstanding, a better understanding of leptin, ghrelin, and insulin influences in the adipogenic process, either in this as in other species, could help the prevention of fat accumulation, improving aquaculture fish production and quality.

Declaration of Competing Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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CHAPTER 8| GENERAL DISCUSSION

Feed and feeding practices influence fish growth and feed utilization, having economic, environmental, and social implications, which may compromise aquaculture profitability and sustainability (Kaushik 2013). Thus, an integrated view of the dietary composition and FF effects on gilthead seabream (one of the most important marine species produced in Europe) is of utmost importance to ensure and enhance the future of this industry. In general, in the present thesis, no major interactions were found between the use of PF-based diets and dietary P/CH ratios (P50/CH10, P40/CH20; Chapters 2 and 5), neither between P/CH ratios and FF protocols (1, 2, or 3 meals per day; Chapters 3, 4, and 6) on gilthead seabream appetite regulation, metabolism, and intestine functionality and health, but effects related to the dietary protein source, P/CH ratios, and FF protocol were observed, as discussed below.

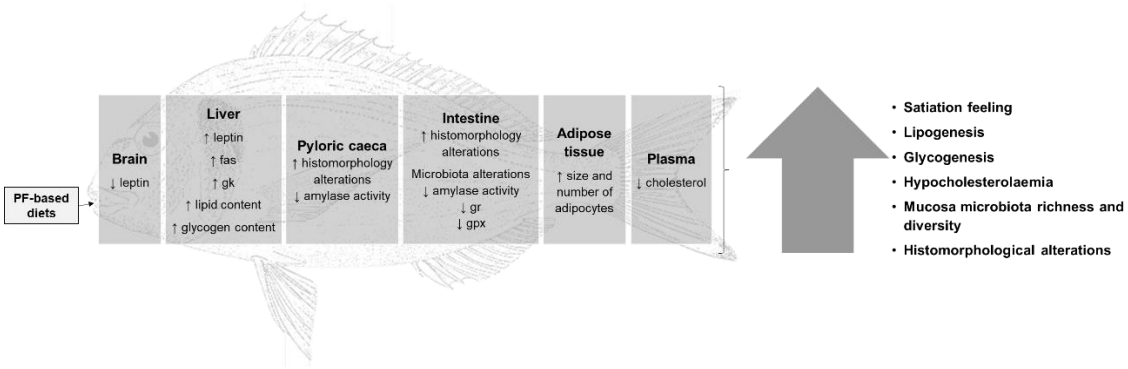

Figure 1. Schematic representation of the effects of PF-based diets on gilthead seabream appetite regulation, metabolism, and intestine functionality and health. fas: fatty acid synthase; gk: glucokinase; gpx: glutathione peroxidase; gr: glutathione reductase; PF: plant-feedstuffs.

In general, PF-based diets promoted a longer satiation feeling, an enhancement of lipogenesis and glycogenesis, and hypocholesterolemia in gilthead seabream (**Figure 4**, Chapter 2). Although FI was not affected by the dietary protein source, the longer satiation feeling in fish fed with the PF-based diets was supported by the reduction of brain *leptin* expression, and increased hepatic *leptin* expression, which seemed to have an orexigenic and anorexigenic behavior, respectively. Indeed, as reported previously, leptin seems to have a tissue and species-specific behavior. For instance, in goldfish, brain *leptin* expression was not affected by a short-term fasting period of up to 1 day, but hepatic *leptin* expression increased 12 h after fasting, suggesting an orexigenic function (Tinoco et al. 2014b). In the present thesis, different results were reported for gilthead seabream, since brain *leptin* appeared to have an orexigenic function, presenting a higher expression at 24 h than at 5 h AF, while hepatic *leptin* seemed to have an

anorexigenic role, with higher expression at 5 h than at 24 h AF. As our results are the first to report the effects of short-term fasting on gilthead seabream *leptin* expression, further studies are needed to support present findings.

It is important to mention that, in agreement with the present results, Pulido-Rodriguez et al. (2021) also reported that brain *npy* and intestine *ghrelin* gene expression were not affected by PF-based diets on gilthead seabream, and *cart* expression was not affected by PF-based diets in the majority of fish species evaluated, such as Atlantic cod and pacu (Tuziak et al. 2014; Volkoff et al. 2017).

Results of the present thesis indicate that PF-based diets did not affect FE nor PER, which is in agreement with what was previously reported for this species (Gómez-Requeni et al. 2003; Bonaldo et al. 2008). Concerning fish intermediary metabolism, the lipogenesis increase in fish fed with PF-based diets was also supported by the increase of adipocytes size and number, and by the increase of hepatic lipid content and *fas* gene expression. Similar evidence was already reported for gilthead seabream (Sitjà-Bobadilla et al. 2005; De Francesco et al. 2007), since in both studies the authors observed an increase of hepatic lipid content with the use of PF-based diets in comparison with fish fed FM-based diets. PF-based diets also seem to have promoted glycogenesis and hypocholesterolemia, as suggested by the increase of hepatic *gk* gene expression and glycogen content, and the decrease of plasmatic cholesterol levels, respectively. Hypocholesterolemia was previously reported in gilthead seabream fed PF-based diets (Gómez-Requeni et al. 2004), and it might be related to precipitation by plant sterols of the marginally soluble cholesterol into a non-absorbable state or by the displacement of cholesterol from micelles, which assist its absorption into the enterocytes (Hicks and Moreau 2001). A glycogenesis increase was, however, not expectable since neither in our study nor in others, plasma glucose levels were affected by FP-based diets (Gómez-Requeni et al. 2003; Gómez-Requeni et al. 2004; Sitjà-Bobadilla et al. 2005; Benedito-Palos et al. 2016).

Concerning intestine functionality and health, some authors reported a synchronism between the immune and oxidative stress responses and histomorphological alterations of the intestine of gilthead seabream fed PF-based diets (Sitjà-Bobadilla et al. 2005; Kokou et al. 2015; Kokou et al. 2017). A similar relationship between intestine histomorphological alterations and oxidative stress response was observed in the present thesis (Chapter 5), although no effects were observed in the immune-related genes evaluated. The downregulation of oxidative-stress gene expression (namely of *gr* and *gpx*) and histomorphological alterations can be related to the presence of ANF in

soybean meal, such as soy saponins and phytosterols (Sitjà-Bobadilla et al. 2005; Bonaldo et al. 2008; Kokou et al. 2015; Monge-Ortiz et al. 2016; Kokou et al. 2017).

Regarding intestine microbiota, the use of PF-based diets did not affect the digesta but influenced mucosa composition, namely, it led to an increase in the number of OTUs, richness, and diversity indices. The absence of effects on digesta microbiota in fish fed different dietary compositions was previously observed in gilthead seabream (Guerreiro et al. 2016; Castro et al. 2019), and could be expected since digesta microbiota comprises transient microorganisms, which are often surrounded by the resident microbiota in the intestine wall and thus do not last a long time (Yukgehnaish et al. 2020). The observed effects in mucosa microbiota of gilthead seabream fed PF-based diets agree with what was previously reported in the same species in fish fed soybean meal-based diets compared with those fed FM-based diets (Dimitroglou et al. 2010) and can be explained by the presence of non-digestible CH on PF, which provides the required substrate for intestine bacteria proliferation (Scott et al. 2013; Villasante et al. 2019).

Some bacteria can produce amylase into the fish intestine lumen (Ray et al. 2012), which may explain the significant differences observed in amylase activity in fish fed the different protein sources. However, as only a DGGE microbial analysis was used in the present thesis, no link between the presence of amylase-producing bacteria and amylase activity can be made. Thus, to deeper knowledge, in future studies, a higher-resolution method, such as next-generation sequencing or FISH, should be used to provide not only the full identification of the species and/or subspecies of bacteria present in the intestine, but also to allow their quantification. It can not be discarded that the decrease of amylase activity in the PC and intestine of fish fed with PF-based diets may be related to the ingredients used in those diets, namely wheat gluten, which is a source of amylase inhibitors (Storebakken et al. 2000; Bakke-McKellep and Refstie 2008).

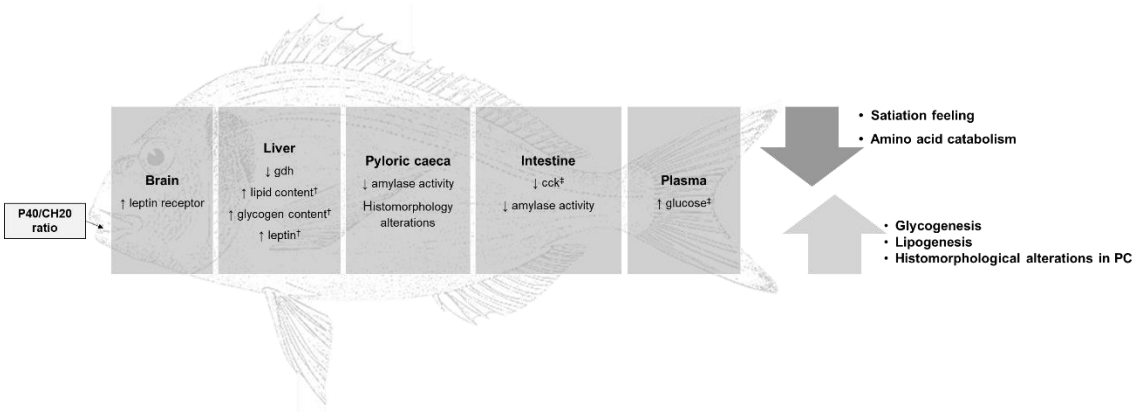

Figure 2. Schematic representation of the effects of P40/CH20 diets on gilthead seabream appetite regulation, metabolism, and intestine functionality and health. cck: cholecystokinin; gdh: glutamate dehydrogenase; P/CH: protein/carbohydrate. †, data reported only for gilthead seabream fed the diets on Chapter 3; ‡, data reported only for gilthead seabream fed the diets on Chapter 2.

Overall, compared to a P50/CH10 diet, a P40/CH20 diet seemed to promote a shorter satiety feeling (**Figure 5**, Chapter 2 and 3), a decrease in AA catabolism, an enhancement of glycogenesis and lipogenesis (Chapters 2 and 4), and some histomorphological changes in the PC (Chapter 5 and 6).

A shorter satiation feeling in gilthead seabream juveniles fed diets with lower dietary P/CH ratio is supported by the increase of FI (Couto et al. 2008) and by the expression of some appetite regulation-related genes, such as the decrease of intestine *cck*, and *ghrelin* expression in the GI (but an increase of brain *ghrelin* expression) (Babaei et al. 2017). However, in on-growing gilthead seabream, no effect of dietary P/CH ratio on FI was observed (Bou et al. 2014). This supports the fact that gilthead seabream juveniles require a higher amount of protein for growth than on-growing fish, leading to an increased FI in fish fed diets with lower P/CH ratios to suppress juveniles' nutritional needs.

In this thesis, the unaffected growth, together with the decrease of FE and PER increase, in fish fed P40/CH20 diets indicate that the inclusion of CH as an energy source spare the use of dietary protein for growth. This is in agreement with what was previously suggested for the species (Fernández et al. 2007; Enes et al. 2011; Castro et al. 2016a; Magalhães et al. 2021). The protein-sparing effect was also confirmed by the reduction of the *gdh* expression in gilthead seabream fed the P40/CH20 diets, which further suggests the reduction of AA catabolism, as previously described for this species (Couto et al. 2008).

The P40/CH20 diets also seemed to promote glycogenesis and lipogenesis pathways. In Chapter 4, gilthead seabream fed P40/CH20 diet presented higher glycogen and lipid

content in the liver as well as a higher area covered by lipid vacuoles than those fed P50/CH10 diet. However, no changes in *fas* gene expression were observed. This suggests that glucose used for energy purposes also spared dietary lipids, which might have been directly deposited in the liver. Similar evidence was recently reported for Nile tilapia (Chen et al. 2020). The lack of *fas* gene expression induction was also observed in Chapter 2, but in this section, fish fed P40/CH20 diets presented higher HSI and VSI, and a tendency for higher glycogen content in the liver, which suggests an increase of lipogenesis and glycogenesis, respectively. Indeed, it cannot be disregarded that fas enzyme activity could have been increased if measured, as previously observed by Castro et al. (2016a). These authors also did not observe changes in *fas* gene expression, but hepatic fas enzymatic activity was higher in fish fed P50/CH20 than in those fed a diet with 66% of protein and no CH content. The increase of glycogenesis with the decrease of dietary P/CH ratio was already reported for this species by other authors (Enes et al. 2008; Castro et al. 2016a; Magalhães et al. 2021).

Present results confirmed the absence of effects on *g6pase* expression, as already reported by Enes et al. (2008) for the same fish species fed with different levels and sources of starch. This suggests that endogenous glucose synthesis was not particularly depressed by increasing the dietary starch content.

The dietary P/CH ratios evaluated did not significantly affect intestine histomorphology, digestive enzymes activity, immunological and oxidative stress-related markers, except for some minor data. For instance, amylase activity in the intestine and PC was lower in fish fed P40/CH20 diets than P50/CH10 diets. This agrees with what was already reported for gilthead seabream (García-Meilán et al. 2020) and can be related to the adsorption of amylase molecules to the dietary crude starch (Spannhof and Plantikow 1983). PC histomorphology and *cox2* and *sod* expressions were also slightly affected by the dietary P/CH ratios tested. However, as no further changes were observed in the immunological and oxidative stress-related parameters measured, it might be that these effects had no biological meaning.

It is also important to mention that the autochthonous microbiota composition was affected by diets used in Chapter 6 but not by those diets used in Chapter 5. These results can be due to the dietary CH source used. In fact, in Chapter 5, the CH source used was pregelatinized maize starch, while in Chapter 6 it was used wheat meal, which has a higher non-digestible CH content that would be available as a substrate for bacteria proliferation (NRC 2011).

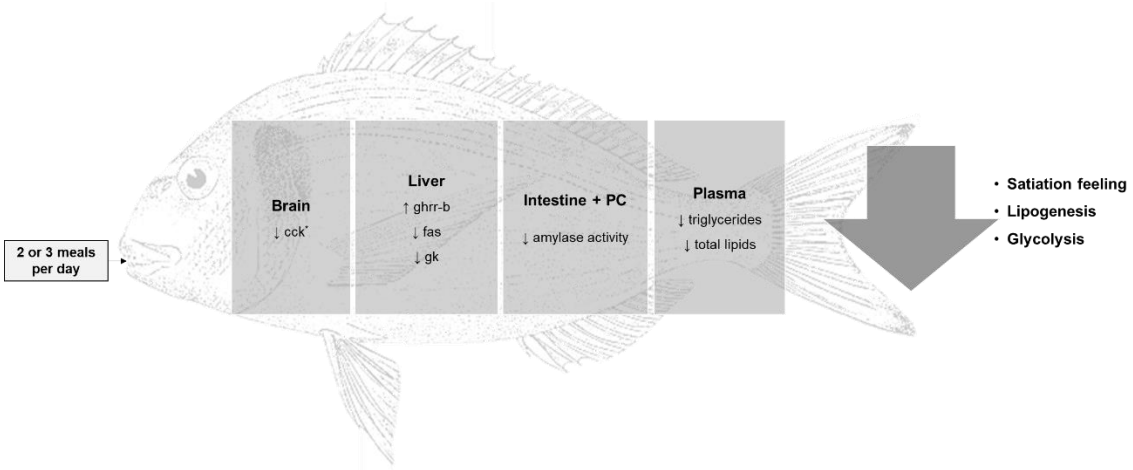

Figure 3. Schematic representation of the effects of higher FF (2 or 3 meals per day) on gilthead seabream appetite regulation, metabolism, and intestine functionality and health. cck: cholecystokinin; fas: fatty acid synthase; ghrr-b: ghrelin receptor-b; gk: glucokinase; PC: pyloric caeca. * the cck expression was lower in fish fed 3 meals per day, when compared with those fed 2 meals per day.

Concerning FF, feeding more meals per day seemed to promote a lesser satiation feeling (Chapter 3), inhibited lipogenesis and glycolysis, and enhanced FI and growth (Chapter 4), as described by **Figure 6**.

The increased FI in fish fed 2 or 3 meals per day can explain the higher FBW observed on those groups and suggests that feeding only 1 meal per day was not enough to fulfill gilthead seabream nutritional requirements. This is possible due to stomach size limitations, indicating that fish fed only 1 meal per day were not able to consume the amount of feed needed to satisfy their nutritional requirements (Ruohonen and Grove 1996; Peterson and Small 2006). However, despite the increase in growth and FI in fish fed 2 and 3 meals per day, FE and PER were lower than in fish fed 1 meal per day. This worse feed utilization in fish fed more than 1 meal per day might be associated with a faster transit time and thus less effective digestion, as also suggested for other species, such as Asian seabass (*Lates calcarifer*), dark-banded rockfish, flounder fish (*Platichthys flesus luscus*), and Korean rockfish (Biswas et al. 2010; Küçük et al. 2014; Md Mizanur and Bai 2014; Oh et al. 2018).

Regarding feed consumption by meal, present results showed that increasing the number of meals from 1 to 2 per day led to a higher FI but increasing to 3 meals per day did not further increase FI or growth. This suggests that 2 meals per day allow meeting the full growth potential, but it can not be disregarded that it can also be due to gut filling

limitations since the amount of feed in the gut limits the FI of the following meal (Peterson and Small 2006; Küçük et al. 2014).

Less satiation feeling in fish fed 3 meals per day than in fish fed 1 meal per day was suggested because fish fed 3 meals per day presented higher FI and hepatic *ghrr-b* expression and lower *cck* expression in the brain. However, this lower satiation feeling can only be considered if ghrr-b and cck have an orexigenic and an anorexigenic role, respectively. The anorexigenic role of cck seems to be well documented 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), but the role of ghrr-b remains controversial. For instance, while in zebrafish, this receptor seems to have an orexigenic function (Eom et al. 2014), in Mozambique tilapia, it had an anorexigenic role (Peddu et al. 2009), and in gilthead seabream, *ghrr-b* function is not conclusive (Perelló-Amorós et al. 2018). Thus, more studies should be done to better understand the role of ghrelin receptors in gilthead seabream.

The less satiation feeling in fish fed 2 or 3 meals per day can be also related to a poorer digestion efficiency, since the shorter interval between meals increases intestine feed transit velocity, and therefore, the digestive process and absorption of nutrients can be compromised (Liu and Liao 1999; Thongprajukaew et al. 2017). This evidence agrees with the decrease of α-amylase activity fish fed 2 or 3 meals per day in comparison with those fed only 1 meal per day (Chapter 6).

The inhibition of lipogenesis in fish fed 2 or 3 meals per day is supported by the decrease of plasmatic TG, total lipids, and liver *fas* expression observed on those fish, and the glycolysis inhibition is supported by the reduction of *gk* expression. This agrees with previous observations in white seabream, where fish fed 2 meals per day presented higher gk activity than those fed 3 or 4 meals per day, which can be explained by the higher glucose load available at each meal in fish fed fewer meals (Enes et al. 2015).

Regarding intestine functionality and health (Chapter 6), only minor histomorphological alterations in the PC of gilthead seabream fed 3 meals per day, and in the intestine glucose-6-phosphate dehydrogenase (G6PD) and CAT activity were observed. However, these small changes may do not have a significant biological value since the histomorphological PC mean score was not affected by the experimental conditions, and no major effects were also found in the activity of the other oxidative stress-related enzymes evaluated.

Hence, overall, based on fish growth, feed utilization, appetite regulation, metabolism, and intestine functionality and health, the results of this thesis suggest that feeding 2 meals per day and using diets with a low P/CH ratio (P40/CH20) can be the best strategy for feeding gilthead seabream juveniles.

Since the role of ghrelin in fish remains controversial and little explored, this thesis also tried to further contribute to the knowledge of this hormone´s role in this species. For the first time, it was detected immunopositive ghrelin cells in the stomach of gilthead seabream through an IHC technique (Chapter 3). As in other fish species (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), the immunopositive ghrelin cells were small and round and were found mainly at the base of the gastric folds in the mucosal layer of the stomach. We also tried to immuno-locate ghrelin cells on the anterior intestine of gilthead seabream but without success, supporting the suggestion that ghrelin is mainly expressed in the stomach of gilthead seabream (Perelló-Amorós et al. 2018).

The present thesis also aimed to further explore the effects of leptin and ghrelin in the adipogenic process using an *in vitro* approach (Chapter 7). Leptin treatment reduced *pparγ* and *cluster of differentiation-36* (*cd36*) expression in both early differentiating and mature adipocytes, and also promoted a lower accumulation of lipids in gilthead seabream adipocytes cells. This suggests an anti-adipogenic role for this hormone. Similar results were observed in yellow catfish hepatocytes and rainbow trout adipocytes, since leptin treatment reduced intracellular TG content and *pparγ* gene expression in catfish hepatocytes, and decreased *lipoprotein lipase* (*lpl*) and *fatty acid transport protein-1* (*fatp1*) gene expression during rainbow trout adipocytes differentiation (Salmerón et al. 2015; Song et al. 2015) These results are also in agreement with *in vivo* studies using icv and ip injections of leptin, which inhibited FI of several fish species (Volkoff et al. 2003; De Pedro et al. 2006; Murashita et al. 2008; Aguilar et al. 2010; Li et al. 2010; Won et al. 2012). This led to a decrease in energy intake by fish, and a concomitant decrease of lipid deposition in the adipose tissue. Regarding ghrelin treatment, no effects were observed on lipid accumulation during the differentiation phase of preadipocytes, neither on the adipogenesis genes evaluated, suggesting that ghrelin does not influence adipogenesis progression. This agrees with what was described for brown trout, where a ghrelin ip injection did not affect lipid metabolism or deposition (Tinoco et al. 2014a). Differently, in Mozambique tilapia, long-term ghrelin treatment with micro-osmotic pumps promoted an increase of liver and muscle lipid content (Riley et al. 2005). Also in rainbow trout ghrelin seemed to influence

adipogenesis, promoting the synthesis of TG and their mobilization into adipocytes, accelerating lipid turnover (Salmerón et al. 2015). These contradictory responses can be due to species-specific differences, but more studies should be done for a better understanding of ghrelin´s effects on lipid adipogenesis progression, as this knowledge can help prevent fat accumulation and consequently improve aquaculture fish production and quality.

CHAPTER 9| GENERAL CONCLUSIONS AND FINAL CONSIDERATIONS

9.1. General conclusions

The results of the present thesis allowed us to formulate the following conclusions:

- No major interactions were observed between the use of PF-based diets or FM-based diets and dietary P/CH ratios or between dietary P/CH ratios and FF protocols on gilthead seabream appetite regulation, metabolism, and intestine functionality and health (Chapters 2, 3, 4, 5, 6).
- Compared to FM-based diets, PF-based diets promoted a longer satiation feeling and led to hypocholesterolemia, and an increase in lipogenesis, and glycogenesis (Chapter 2).
- Compared to FM-based diets, PF-based diets increased the number of OTUs, richness and diversity indices of autochthonous microbiota, but no effect was noticed regarding the allochthonous bacteria (Chapter 5).
- Compared to FM-based diets, PF-based diets did not compromise growth and FI but seemed to influence the intestinal absorptive and digestive metabolism (Chapters 2 and 5).
- Compared to P50/CH10 diets, P40/CH20 diets promoted a shorter satiety sensation (Chapter 2 and 3), reduced the AA catabolism, and enhanced glycogenesis and lipogenesis (Chapters 2 and 4).
- P40/CH20 diets did not compromise fish growth but increased PER confirming that increasing dietary CH level spares the use of dietary protein for growth (Chapters 2 and 4).
- The dietary P/CH ratio did not influence hepatic gluconeogenesis (Chapters 2 and 4).
- P40/CH20 diets led to a reduction of amylase activity in the intestine and PC but did not seem to affect the digestive and absorptive processes (Chapters 2 and 4).
- Different dietary P/CH ratios promoted faster changes in appetite-related genes than the use of different dietary protein sources (Chapter 2).
- Compared to feeding 1 meal per day, 2 or 3 meals per day seemed to promote a lower satiation feeling (Chapter 3), decreased lipogenesis and glycolysis, enhance FI and growth (Chapter 4).
- Amylase activity was lower in fish fed 2 or 3 meals per day than 1 meal per day, and this suggests a decreased of the digestion efficiency in these groups (Chapter 6). This is also supported by the lower FE and PER reported in fish fed 2 or 3 meals per day in comparison with those fed only 1 meal per day (Chapter 4).

- No major effects in intestine histomorphology, microbiota composition, digestive enzymes activity, and oxidative stress-related markers were reported with FF protocols (Chapter 6).
- Present results suggest that 2 meals per day seem to be the best feeding strategy under the experimental conditions tested (Chapters 3, 4, and 6).
- Independently of the dietary composition and FF, the dominant allochthonous and autochthonous bacteria detected were most closely related to the *Firmicutes* and *Proteobacteria* phylum (Chapters 5 and 6).
- Immunopositive ghrelin cells were located for the first time in the stomach of gilthead seabream and appeared as small and round shape cells, located mainly in the gastric folds of the mucosal layer (Chapter 3).
- Leptin has an anti-adipogenic role in early differentiating and mature adipocytes, but ghrelin seems to have only minor effects in gilthead seabream differentiating preadipocytes (Chapter 7).

9.2. Final considerations

The main conclusion of the present thesis is that there were no major interactions between dietary protein source (FM or PF) and dietary P/CH ratios (P50/CH10 and P40/CH20), nor between dietary P/CH ratios (P50/CH10 and P40/CH20) and FF protocols (1, 2 or 3 meals per day) on gilthead seabream appetite regulation, metabolism, and intestine functionality and health.

A better knowledge of the appetite regulation mechanisms can improve aquaculture growth practices, profits, and sustainability. In the present thesis, the appetite control seemed to be influenced by dietary composition and FF protocols, but a deeper knowledge is needed to better characterize the appetite control mechanisms in gilthead seabream, for instance in fish under different life stages or production conditions.

Growth, feed utilization, and intermediary metabolism in gilthead seabream seem to be highly influenced by dietary composition and FF protocols (Guinea and Fernandez 1997; Fernández et al. 2007; Couto et al. 2008; Enes et al. 2011; Gilannejad et al. 2019; Busti et al. 2020; Gilannejad et al. 2021; Magalhães et al. 2021). However, there are still some inconsistencies between gene expression results and the activities of some enzymes related to the intermediary metabolism (Castro et al. 2016a), and then more studies should be performed to take into account these differences.

Some bacteria can produce exoenzymes (Ray et al. 2012), affecting total digestive enzymes activities and fish performance. Furthermore, recently Sherif et al. (2020) also found an association between FF protocols and the abundance and proportions of the microbial community in the Nile tilapia intestine. However, the methods used in the present work to analyze microbiota diversity (DGGE analysis) did not allow to make a similar association. Hence, future studies should include higher-resolution methods, such as next-generation sequencing, proving not only the full identification of bacteria species and /or subspecies, but also allowing their quantification.

The present thesis also aimed to further explore leptin and ghrelin physiological functions. In the in *vitro trial*, the anti-adipogenic role of leptin was confirmed, but ghrelin seemed to have only minor effects in gilthead seabream differentiating preadipocytes, and results were not in agreement with the ones reported in other fish species (Riley et al. 2005; Salmerón et al. 2015). Thus, more studies are needed to elucidate the influence of ghrelin on adipogenesis in gilthead seabream. The *in vitro* studies should be further extended to other cell types, such as hepatocytes, for a better understanding of these appetite hormones' effects on intermediary metabolism.

Finally, as the optimization of the dietary composition and feeding practices can enhance fish performance and feed utilization, reducing aquaculture costs and contributing to a more sustainable industry, long-term effects should be considered and better explored in future works.

CHAPTER 10| REFERENCES

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