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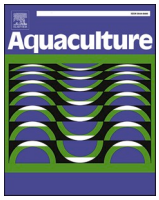

Nutritional innovations in superior gilthead seabream (*Sparus aurata*) genotypes: Implications in the utilization of emerging new ingredients through the study of the patterns of secretion of digestive enzymes

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ABSTRACT

To promote sustainability and efficiency for aquaculture production, there are two key actions: i) The implementation of novel nutritional strategies with more sustainable raw materials, that aim to reduce the dependence on fishmeal (FM) and fish oil (FO) in the feeds, and ii) the implementation of a successful breeding program addressed to improve fish growth, feed utilization, and health. Different studies performed on salmonids and European sea bass (*Dicentrarchus labrax*) support the existence of genetic variability for the utilization of plant or non-marine-based diets. Nevertheless, those studies do not consider that the better zootechnical performance obtained in selected fish can be associated with differences in digestive biochemistry. Therefore, the present study aimed to evaluate the changes in the levels of the activities of different digestive enzymes present in two populations of gilthead seabream (*Sparus aurata*): reference or selected for high growth. Furthermore, the effect of different diets including a variety of protein ingredients partially substituting FM (poultry meal, insect meal, and bacterial single-cell protein) was assessed on the digestive enzyme profile of both fish genotypes, after 90 days of feeding. Higher levels of certain protein-related enzymes (pepsin and chymotrypsin) were found in selected sea bream fish compared with reference fish. Selected fish also revealed higher ADCs for dietary amino acids, irrespective of the diet fed, compared with the reference group. These results, added to the better growth and feed utilization of selected sea bream compared with reference fish, suggest that selected fish are more able to utilize the combination of emergent ingredients for aquafeeds, showing changes in the pattern of digestive enzymes to face the different ingredients in diets, which indicates a higher plasticity of the digestive enzymes to face changes in dietary ingredients. Those changes could be reflecting a compensatory mechanism to improve the digestibility of the ingredient.

1. Introduction

Reducing the ecological impact and improving net proficiency of aquaculture has been in the spotlight in the last years. In this sense, the implementation of novel nutritional strategies with more sustainable raw materials, that aim to reduce the dependence on fishmeal (FM) and fish oil (FO) in the feeds, added to the implementation of a successful breeding program addressed to improve fish growth, feed utilization, and health, are two key actions for promoting sustainability and efficiency of aquaculture production. In the last years, several studies have evaluated the potential of different fish species to grow and utilize diets that partially or totally replaced fish meal, mainly with plant ingredients, to induce selection in fish populations and evaluate the heritability of selected traits related to their zootechnical performance (Pierce et al., 2008; Dupont-Nivet et al., 2009; Le Boucher et al., 2011; Kause et al., 2016). However, the results obtained when evaluating genotype x diet (g x d) interactions have been quite variable depending on the species and the general conditions of the study. While some

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studies reported that fish that are selected for fast growth on FM/FO diets display also higher growth on plant-based diets, i.e., no significant g x d interactions, some others, reported a significant g x d interaction, which means that fish that are selected for their fast growth on FM diets may not be the ones that grow faster when facing a challenging diet. For instance, in rainbow trout (*Oncorhynchus mykiss*), contradictory results have been reported when using populations of fish belonging to different strains fed on plant-based and traditional FM-based diets. The different studies reported an absence of g x d interactions (Palti et al., 2006), clear interactions (Pierce et al., 2008), or reduced but significant g x d interactions for several key productive indicators, including body weight or carcass yield (Le Boucher et al., 2011). In European sea bass (*Dicentrarchus labrax*), it was also evidenced low g x d interactions when using very extreme diets (Le Boucher et al., 2012), although the results suggested that selecting fish for growth on a marine diet should be the most efficient way to also increase growth on plant-based diets, meaning that indirect selection should be more efficient than direct selection. More recently, in two genotypes of gilthead sea bream (*Sparus aurata*), one of them selected for faster growth during winter and fed on either a FM or a plant meal-based diet, no main dietary effects on growth rates or condition factor were observed, but an effect was found on intestinal morphology, that increased the digestive/absorptive capacity of the selected group when fed on the plant-based diet (Perera et al., 2019). Thus, genetic selection in carnivorous farmed fish has been proposed to improve intestinal plasticity and diet flexibility by promoting genetic adaptation to feeds including high levels of non-marine ingredients. Recently, Naya-Català et al. (2022) found that genetically improved gilthead seabream showed some metabolic and functional advantages to better adapt to environmental changes, including enhanced intestinal phospholipid metabolism, epithelial turnover, intestinal motility, and improved microbiota plasticity.

Independently of the existence or not of such interactions between genotype and diet, all those studies agree that genetic improvement can be impacted by extreme changes in diet and that it is possible to identify fish with a global ability to grow better on feeds low in FM/FO and including alternative ingredients. However, most of those studies have been conducted with plant sources as dietary alternatives to FM or FO, and other raw materials like poultry by-products, single-cell proteins from bacterial or microalgal sources as well as insect meals have been emerging more recently for being more sustainable and apparently more interesting from the nutritional point of view compared with some plant feedstuffs and that promise to replace high dietary percentages of FM/FO in aquaculture feeds (Sprague et al., 2017; Galkanda-Arachchige et al., 2020; Glencross, 2020; Tran et al., 2022). Therefore, it is necessary to conduct studies to fill the gap on the potential impact of the genetic selection of farmed fish on their adaptability to novel diets with emergent ingredients that will be the future of aquafeeds.

Furthermore, in the currently available studies, mostly with plant alternative diets, the biological responses used to evaluate the potential different aptitude of genotypes for better use of such alternative ingredients were mainly finalistic, assessing total growth, survival, body composition, and morphometric parameters. Nevertheless, differential adaption of fish genotypes for more efficient use of nutrients can be also evaluated by other physiological and biochemical traits, i.e. those related to the efficiency in the digestive and/or metabolic processing of such nutrients. In this sense, the evaluation of total activities and secretion patterns of digestive enzymes may offer highly valuable information due to their key role within the entire process of feed transformation. The evaluation of the activity of the main digestive enzymes has been extensively used in fish species for different purposes, i.e. to assess changes in their digestive capabilities with development, to support interspecific differences in feeding habits, or to evaluate adaptations of cultured species to the variations in nutrient composition and type of ingredients used in artificial diets (Infante and Cahu, 2007). Concerning the latter, two key points still have not been extensively addressed and that may have a significant impact on the biological efficiency of cultured species: 1) can selective breeding of fish species influence the total amount and relative composition of digestive enzymes? and 2) to what extent cultured fish may adapt their production of digestive enzymes to the nutritional composition of manufactured feeds, so they can maximize the efficiency of the digestion process?. To our knowledge, only one study from Yamamoto et al. (2023), recently reported that rainbow trout juveniles selectively bred for 3 generations on plant-based diets showed increased secretion of some digestive enzymes (lipase and chymotrypsin). Therefore, the two main aims of the present study were to evaluate: a) the changes in the levels of the activities of different digestive enzymes present in two populations (reference or, selected for high growth) gilthead seabream with different growth trajectories over the production cycle, and b) the effect of different diets including a variety of protein ingredients partially substituting FM, on the digestive enzyme profile of both populations of fish. Additionally, the different responses of genetically-selected and reference sea bream to the different dietary formulations, which included poultry meal, single-cell protein meal, insect meal, and microalgal oil, were also assessed on a few growth parameters and the apparent digestibility coefficients of the dietary nutrients.

Table 1
Ingredients and proximate composition of the experimental diets.

Ingredients (%)	Control (C)	Future (F)	Insect (INS)	Single-cell protein (SCP)
Corn gluten	6	5	6	6
Hi Pro Soy bean meal [1]	–	5.08	–	–
Wheat gluten	20	14.4	20	17.0
Faba bean dehulled [2]	8	8	8	8
Wheat	23.8	19.0	21.9	20.5
Soy protein concentrate[3]	12.6	17	14.7	18
Fish oil [4]	6.9	–	–	–
Fish meal [5]	15.0	7.5	10	5
Rapeseed oil	4.6	6.5	5.8	6.3
Phosphate	1.0	0.4	1.1	1.4
Vitamin & mineral mix [6]	0.3	0.3	0.3	0.3
Poultry meal [7]	–	10	–	–
Poultry oil [8]	–	2.1	2.6	2.7
DHA oil [9]	–	2.5	2.6	2.9
Insect meal[10]	–	–	5	–
Single-cell protein[11]	–	–	–	10
Lecithin	2.0	2.0	2.0	2.0
Yttrium premix	0.1	0.1	0.1	0.1
Proximate composition (% wet weight)				
Moisture	8.51	7.71	8.17	8.25
Crude protein	46.94	49.85	46.79	48.30
Ash	4.65	4.88	4.59	4.60
Crude fat	16.60	17.34	16.94	17.18

[1] Soya bean meal: CJ Selecta S.A (Brasil).
[2] Faba beans: Cefetra BV (The Netherlands).
[3] Soya protein concentrate: CJ Selecta S.A (Brasil).
[4] Fish oil: Copeinca, S. A. (Perú).
[5] Fish meal: Norsildmel AS (Norway).
[6] Mineral and Vitamin premix: Trouw Nutrition (The Netherlands).
[7] Poultry meal: Sonac (Belgium) (Protein: 60%; Lipids: 19%; Ash: 10.5%; Moisture: 4.5%).
[8] Poultry oil: Sonac (Belgium).
[9] DHA: Veramaris (Evonik).
[10] Produced from *Hermetia illucens*. InnovaFeed (France) (Protein: 57–62%; Lipids: 8–11; Ash: 8–10; Moisture: 2.5).
[11] FeedKind© produced from *Methylococcus capsulatus* fermentation. Calysta (USA) (Protein: 70.6%; Lipids: 9.8; Ash: 7.1; Moisture: 6).

2. Materials and methods

2.1. Production of initial fish populations and fish used in the present study

A total of 192 breeders were selected based on their estimated

Table 2
Amino acid composition (g/100 g feed) and main fatty acid composition (g/100 g fatty acid identified) of the experimental diets.

Amino acid	C	F	INS	SCP
Arginine	2.28	2.17	2.27	2.29
Histidine	1.03	1.01	1.06	1.02
Isoleucine	1.66	1.63	1.69	1.69
Leucine	3.26	3.26	3.34	3.37
Lysine	2.06	2.11	2.23	2.13
Methionine	0.74	0.75	0.78	0.79
Cysteine	0.56	0.59	0.60	0.57
Valine	1.82	1.81	1.91	1.91
Phenylalanine	2.02	2.03	2.12	2.13
Threonine	1.45	1.4	1.44	1.48
Tyrosine	1.15	1.14	1.30	1.22
Alanine	1.93	1.89	1.95	2.01
Glutamic acid	9.2	9.83	10.02	9.82
Glycine	1.78	1.77	1.79	1.75
Aspartic acid	3.31	3.04	3.18	3.18
Proline	2.94	3.29	3.45	3.26
Serine	1.99	1.98	2.04	2.00
Main Fatty acids (g/100 g FA)				
20:4n-6 (arachidonic acid)	0.3	0.4	0.5	0.5
20:5n-3 (eicosapenatanoic acid)	3.4	3.3	3.0	2.9
22:6n-3 (docosahexaenoic acid)	5.3	5.2	6.8	7.2
Σ SFA	15.2	17.5	17.2	16.8
ΣMUFA	51.9	44.5	44.8	45.1
Σ n-3	15.3	13.8	15.6	16.0
Σ n-6	17.0	24.0	22.1	21.9
Σ n-3 PUFA	9.5	9.5	10.3	10.6
n-3/n-6	0.9	0.6	0.7	0.7

C: Control diet; F: Future diet; INS: Insect diet; SCP: Single-cell protein diet. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

breeding values (EBV) and relationship coefficient. Two groups of breeders were established: the High Growth-selected group (HG) (2×, with 46 and 48 breeders per broodstock) and the reference population group (REF). These breeders with opposite values in their EBV, which corresponds to a value of +39.68 in the group of HG breeders, and −25.95 in the REF breeders. Between EBV values of HG and REF breeders was contained almost 47% of the evaluated population.

Fertilized eggs from spontaneous spawning from the two different broodstock groups were collected. The populations resulting from either selected fish (HG) or reference fish (REF), were incubated separately until hatching. Hatched larvae were kept in separate tanks, and larvae were reared using a standardized protocol at the ULPGC facilities (Eryalçin et al., 2020). Progeny from both fish groups was kept at similar conditions during the pre-weaning, weaning, and early juvenile growing phases, being fed on an experimental diet based on low FM/FO formulation until reached 240 days after hatching (dah). At that moment, the dietary challenge period with the experimental diets (Table 1) started (nutritional challenge test). At this point, a total of 1080 fish (540 fish from each genotype) were randomly distributed into 24 tanks from 500 L (45 fish per tank, per genotype and diet, triplicates for each treatment), where they were maintained for 3 months. All tanks were provided with filtered seawater in a flow-through system under natural photoperiod (12 h light: 12 h dark). Dissolved oxygen and water temperature ranged between 6 and 8 ppm and 19.2 to 21 °C, respectively. Salinity was 37 g/L. In the entire growth trial, fish were fed the experimental diets until apparent satiation, 4 times a day and 6 days a week. Unfed pellets were collected after meal to evaluate the total ingested food in each tank. After receiving the experimental diets for 2 months, a random selection of 25 fish per treatment (5 fish per tank and 5 tanks per genotype and diet) was separated from the initial groups, placed in additional 500-L tanks, and continued feeding the experimental diets for one month more. Those fish were used for the determination of variations in the pattern of secretion of several digestive enzymes, while the remaining fish were used for evaluation of growth, feed efficiency, and determination of apparent digestibility coefficients.

2.2. Feeds

The initial low FM/FO diet used during early growing was formulated to meet the nutritional requirements of gilthead sea bream. From 240 dah onwards, four diets were used, both in the complete growth experiment and the digestive enzyme experiment: i) a low content of FM/FO control diet (C) based on current commercial formulation, manufactured by Skretting (Skretting ARC, Stavanger, Norway); (ii) a "future" diet (F) including poultry meal as partial replacer of FM (50%); (iii) a diet using insect meal (INS) at 5% dietary inclusion level as partial replacer of FM (33%) and (iv) a diet using single cell protein meal from bacterial origin (SCP) as partial replacer of FM (66%). Diets F, INS, and SCP also totally replaced FO with a combination of poultry oil and DHA-rich microalgal oil.

Proximate composition analyses of feeds were carried out accordingly with the standardized procedures described by AOAC (1975). Crude protein content (Nx6.25) was analyzed following the Kjeldahl method. The amino acid composition of feeds was determined according to the principles and methods provided in Commission Regulation (EC) No 152/2009, 2009. Ash content was determined by incineration at 600 °C for 12 h in a muffle furnace, whereas moisture content was determined after drying samples in an oven at 110 °C until constant weight. The total lipid content of the samples was extracted with chloroform/methanol (2:1 *v*/v) (Folch et al., 1957). Fatty acid methyl esters were obtained by transmethylation of total lipids (Christie, 1989) and separated by gas chromatography. The formulation, proximate composition, and amino acid of the diets are shown in Tables 1 and 2, respectively.

2.3. Evaluation of zootechnical performance

Fish used in the growth experiment were used to calculate different indicators related to growth performance, feed utilization, and survival, which were calculated using the following equations: Specific Growth Rate (SGR) = (Ln (final weight)-Ln(initial weight))*100/feeding period (days); Feed Conversion Ratio (FCR) = (total feed fed/total weight gained).

2.4. Sampling of fish used for evaluation of digestive biochemistry

After separation from the initial groups detailed in subsection 2.2., the fish were fed to satiation 3 times a day, 6 days a week for another 30 days with the different experimental diets. After this one month-period, a sequential sampling of fish in each dietary group was organized after receiving the first daily meal. The selected moments were 0, 1, 3, 6 and 9 h after receiving the meal (5 fish/sampling point; 45 fish/diet). Fish were sacrificed by immersion in ice-cold water containing clove oil and immediately dissected to obtain the digestive tract, which was separated into the stomach and the rest of the intestine plus the pyloric ceca. Crude extracts required for the enzyme assays were prepared by mechanical homogenization of the tissues and luminal contents in distilled water (1:10 *w/v*) followed by centrifugation (15.000 X g, 20 min, 4 °C). The obtained supernatants were used for the determination of the different enzyme activities that were evaluated in triplicates from each sample.

2.5. Analysis of digestive enzymes

Acid protease (pepsin activity) was measured following the technique of Anson (1938) using hemoglobin (0.5%) as substrate in Glycine–HCl 50 mmol l^{-1}, pH 3.5. After incubation, the reaction was stopped by adding 20% TCA, and the absorbance of the reaction products was measured at 280 nm. Trypsin activity was measured following Erlanger et al. (1961), using BAPNA (N-a-benzoyl-DL-arginine p-nitroanilide) as substrate in Tris–HCl 50 mmol l^{-1}, pH 8.2 and $CaCl_2$ 10 mmol l^{-1}. Chymotrypsin activity was measured according to Del Mar et al. (1979) using SAPNA (N-succinyl-ala-ala-pro-phe p-nitroanilide) as substrate in

Table 3
Growth and feed utilization of gilthead sea bream from the two different genotypes fed the experimental diets.

	Initial weight (g)	Final weight (g)	SGR (%/day)	FCR
HG				
C	50.03 ± 1.78	133.32 ± 2.91[a]	1.09 ± 0.08	1.45 ± 0.15 [a]
F	50.32 ± 1.16	127.21 ± 1.17[a]	1.03 ± 0.02	1.45 ± 0.06 [a]
INS	49.77 ± 1.08	116.46 ± 2.18[b]	0.94 ± 0.02	1.55 ± 0.05 [ab]
SCP	50.13 ± 0.84	118.48 ± 1.37[b]	0.95 ± 0.04	1.62 ± 0.15 [ab]
REF				
C	50.13 ± 1.31	120.41 ± 1.52[b]	0.97 ± 0.02	1.65 ± 0.04 [ab]
F	50.31 ± 1.45	115.61 ± 1.84[b]	0.92 ± 0.02	1.71 ± 0.09 [ab]
INS	50.70 ± 1.83	114.61 ± 1.93[bc]	0.92 ± 0.01	1.73 ± 0.06 [ab]
SCP	50.20 ± 1.80	112.00 ± 0.60[c]	0.89 ± 0.01	1.76 ± 0.05 [b]
Two-way ANOVA (*p*-value)				
Genotype	n.s.	<0.001	<0.001	<0.001
Diet	n.s.	<0.001	<0.01	n.s.
g x d	n.s.	<0.001	n.s.	n.s.

HG: High growth genotype; REF: Reference genotype; C: Control diet; F: Future diet; INS: Insect diet; SCP: Single-cell protein diet. Two-way ANOVA, $p < 0.05$, Genotype and Diet as fixed factors. Different letters denote significant differences analyzed with one-way ANOVA, $p < 0.05$ for significant g x d interactions. n.s = not significant.

DMSO 10 mmol l-^{1} and Tris–HCl 50 mmol l^{-1}, pH 7.8 and $CaCl_2$ 10 mmol l^{-1}. The α-amylase activity was measured using starch (2%) as substrate in phosphate-citrate 100 mmol l^{-1}, NaCl 50 mmol l^{-1}, pH 7.5, at 600 nm as described by Robyt and Whelan (1968). Lipase activity was quantified according to Versaw et al. (1989) using β-naphthyl caprylate (200 mmol l^{-1}) as substrate in Tris–HCl 50 mmol l^{-1}, pH 7.2 and sodium taurocholate (100 mmol l^{-1}). Incubation lasted 30 min after which the reaction was stopped with TCA (0.72 N); fast blue (100 mmol l^{-1}) was added and ethanol/ethyl acetate (1:1 *v*/v) was added to clarify. The alkaline phosphatase was estimated using 4-nitrophenyl phosphate as substrate in NaOH-glycine 100 mmol l^{-1} buffer pH 10.1, according to Bergmeyer (1974). Leucine aminopeptidase activity was measured with Leucine p-nitroanilide in DMSO 0.1 M and 50 mmol l^{-1} sodium phosphate, pH 7.2 (Maraux et al., 1973).

2.6. Apparent digestibility coefficients (ADC)

The remaining fish that were not used for enzyme determinations (75 fish per genotype and diet) were kept in the tanks to obtain feces required for the determination of ADC of protein and amino acids. Fish feces were obtained by dissection of fish guts and remotion of the digested material, which were frozen at −80 °C until analysis. These were obtained by dissection of fish guts and remotion of the digested material. The determination was based on the following formula as described by Cho et al. (1982):

$$ADC_{test\ diet}(\%) = 100\text{-}100\times(\%Y_{feed}/\%Y_{faeces})\ x\ (\%Nutrient_{faeces}/\%Nutrient_{feed})$$

where Y_{feed} and Y_{faeces} are the dietary and fecal yttrium oxide content, and $Nutrient_{faeces}$ and $Nutrient_{feed}$ are the fecal and dietary nutrient content (% on dry matter basis).

2.7. Data analysis

Data are presented as mean ± SD. Growth and feed utilization parameters as well as ADCs were also analyzed with a two-way ANOVA, with genotype and diet as fixed factors, using a 95%-confidence level (p<0.05), and followed by Tukey *posthoc* test when significant differences were detected. The effect of genotype and diet on total enzyme production, as well as the effect of sampling point and diet on the time pattern of enzyme production within each genotype, were also evaluated by a two-way ANOVA followed by Bonferroni *posthoc* test when significant differences were found at p<0.05. One-way ANOVA was also applied when significant genotype x diet interactions were detected (p<0.05). The differences in values of enzyme activity obtained at different sampling moments for a given diet were evaluated by one-way ANOVA also followed by Bonferroni *posthoc* test. All those analyses were carried out using the software Statgraphics Centurion (Statgraphics Technologies, The Plains, VI.EE. UU.) or SPSS Statistical Software

Table 4
Effect of genotype and diet on the values of the different enzyme activities (units/100 g fish) irrespective of the sampling moment.

	PEPSIN		TRYPSIN		CHYMOTRYPSIN		AMYLASE	
	mean ± SD	*p value*	*mean ± SD*	*p value*	*mean ± SD*	*p value*	*mean ± SD*	*p value*
Genotype								
HG	12.64 ± 3.73	0.011	0.44 ± 0.37	n.s	4.16 ± 2.44	0.018	4.10 ± 2.48	0.074
REF	6.91 ± 1.72		0.40 ± 0.17		2.87 ± 1.62		2.85 ± 1.53	
Diet								
C	9.24 ± 3.25	0.422	0.37 ± 0.16	0.089	3.45 ± 1.88	0.052	3.27 ± 1.79	0.061
F	8.63 ± 2.70		0.32 ± 0.19		2.57 ± 1.72		1.95 ± 1.39	
INS	9.94 ± 4.09		0.45 ± 0.23		3.48 ± 1.73		3.51 ± 1.67	
SCP	11.28 ± 5.77		0.54 ± 0.42		4.58 ± 2.86		5.16 ± 2.45	

	LIPASE		AMINOPEPTIDASE		ALKALINE PHOSPHATASE	
	mean ± SD	*p value*	*mean ± SD*	*p value*	*mean ± SD*	*p value*
Genotype						
HG	0.29 ± 0.10	0.096	0.61 ± 0.28	0.586	10.98 ± 5.16	0.122
REF	0.34 ± 0.15		0.68 ± 0.36		13.40 ± 7.07	
Diet						
C	0.32 ± 0.12	0.220	0.59[a] ± 0.26	0.043	11.08 ± 5.27	0.153
F	0.35 ± 0.16		0.63[b] ± 0.29		11.89 ± 5.66	
INS	0.30 ± 0.10		0.51[ab] ± 0.21		10.45 ± 4.32	
SCP	0.29 ± 0.16		0.83[c] ± 0.43		15.36 ± 8.57	

HG: High growth genotype; REF: Reference genotype; C: Control diet; F: Future diet; INS: Insect diet; SCP: Single-cell protein diet. Two-way ANOVA, $p < 0.05$, Genotype and Diet as fixed factors. Different letters denote significant differences analyzed with one-way ANOVA, $p < 0.05$ for significant g x d interactions. n.s = not significant.

Table 5
P-values of the statistical comparisons evaluating the effect of time and diet on the values of the different enzyme activities measured in the two genotypes of fish.

	HG	REF
Pepsin		
Time	0.001	n.s
Diet	0.012	n.s
Trypsin		
Time	0.004	0.029
Diet	n.s	n.s
Chymotrypsin		
Time	0.005	0.008
Diet	n.s	n.s
Amylase		
Time	0.026	0.021
Diet	0.011	0.024
Lipase		
Time	n.s	0.000
Diet	n.s	n.s
Aminopeptidase		
Time	0.001	0.000
Diet	0.024	n.s
Alk. phosphatase		
Time	n.s	0.002
Diet	n.s	n.s

HG: High growth genotype; REF: Reference genotype. n.s = not significant.

System v24.0 (SPSS, Chicago, IL, USA).

Additionally, principal components (PCA) were carried out to integrate the information obtained from the different enzyme activities and to potentially discriminate between genotypes. This analysis was conducted using the R Project for Statistical Computing software, using the statistical packages "FactoMiner 2.4" for data analysis and "Factoextra 1.0.7" for graphical representation. As a prerequisite for the analysis, data normality was verified through multivariate skewness and Kurtosis analysis (Wang and Du, 2000).

3. Results

3.1. Fish performance

A significant effect of genotype on growth (final weight and SGR) and feed conversion ratio was evidenced since higher and lower values, respectively, of both indicators were obtained for all diets in the HG group (p<0.05; Table 3). Diet showed a significant effect on SGR and a significant g x d interaction was observed in fish final weight, with INS and SCP diets leading to lower growth compared with C or F diets (p<0.05; Table 3).

3.2. Digestive biochemistry

Enzyme production data were analyzed in two forms: a) Comparing the average values of enzyme production measured during the whole sampling period considering the two factors involved (genotype and

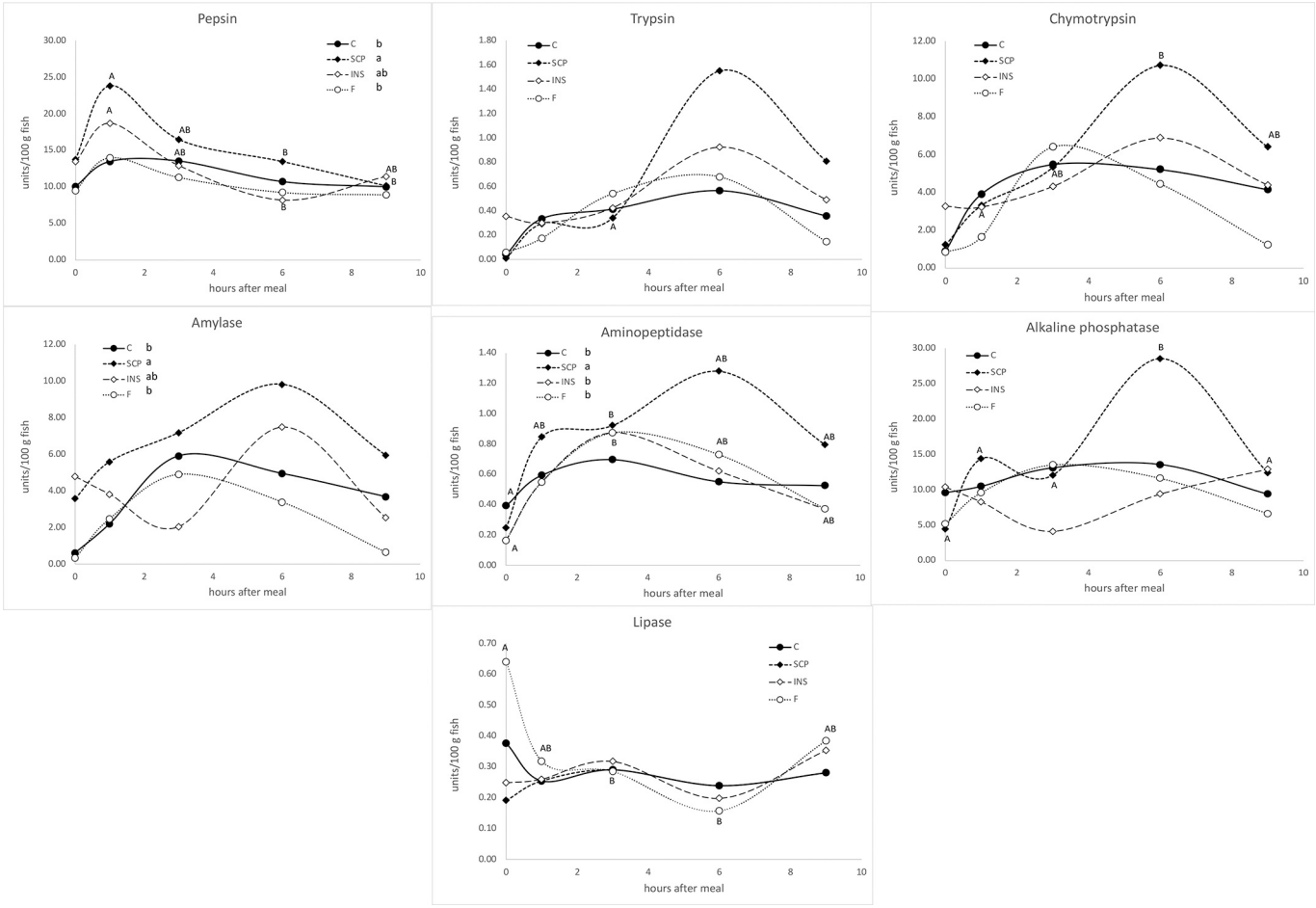

Fig. 1. Time patterns of enzyme secretion in fish of the HG genotype fed on the different diets. When present, significant differences ($p < 0.05$) between diets are indicated with low case letters in the legends corresponding to each enzyme. Significant differences in values of activity measured at different sampling moments for a given diet are indicated with capital letters.

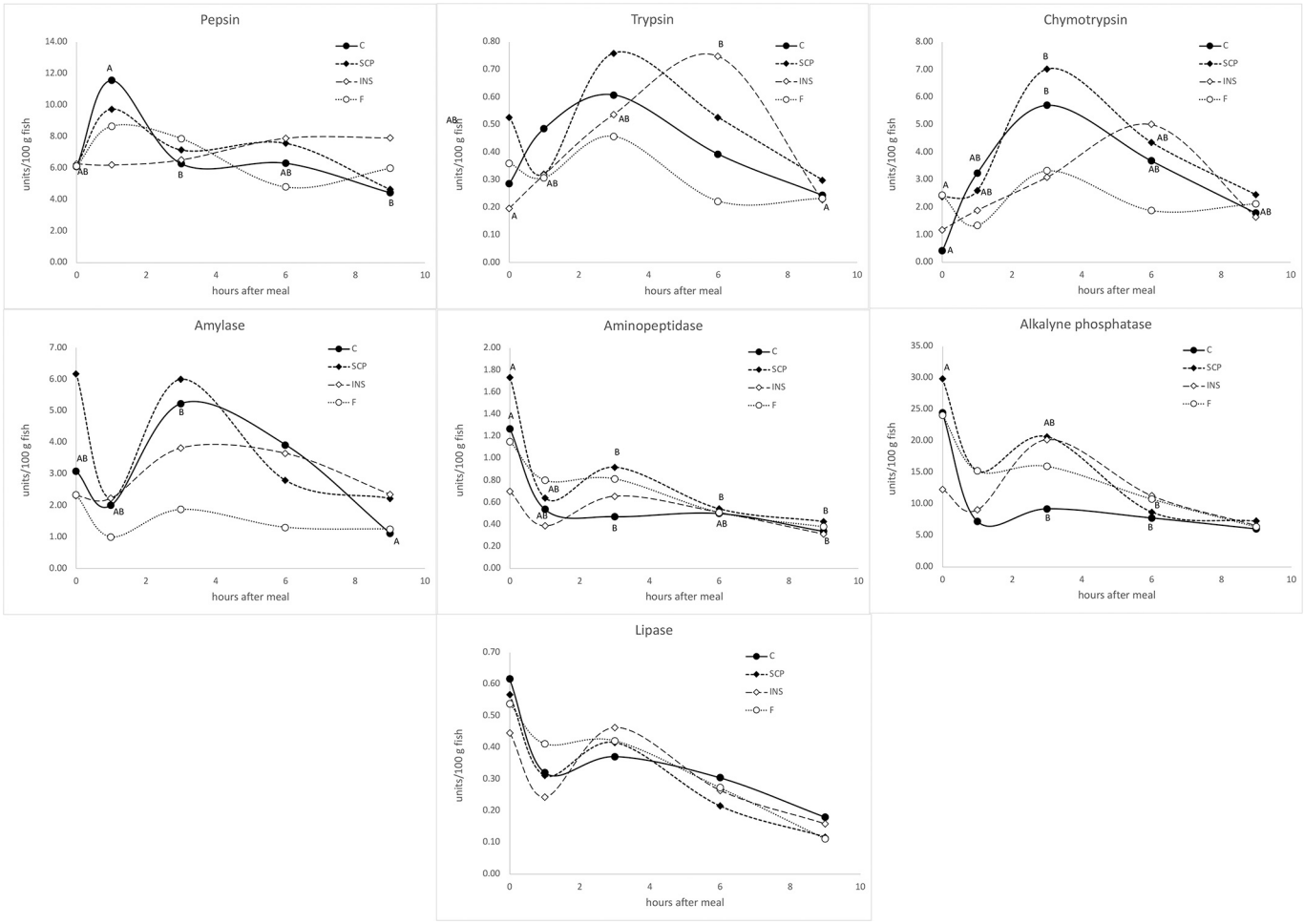

Fig. 2. Time patterns of enzyme secretion in fish of the REF genotype fed on the different diets. When present, significant differences ($p < 0.05$) between diets are indicated with low case letters in the legends corresponding to each enzyme. Significant differences in values of activity measured at different sampling moments for a given diet are indicated with capital letters

diet) (Table 4), and b) Evaluating the effect of time on the secretion patterns of the different enzymes within each genotype (Table 5).

Significantly higher values of pepsin and chymotrypsin ($p < 0.05$) were obtained in HG fish compared to REF. The effect of the diet was only significant ($p < 0.05$) in the case of aminopeptidase, with higher values measured in fish fed the SCP when compared to those in fish fed the rest of the dietary treatments, irrespectively of the genotype. However, although not statistically significant, a tendency ($p < 0.1$) in SCP to increase other enzyme activities like trypsin, chymotrypsin, and amylase was also noted. Results of the second analysis are presented in Figs. 1 and 2 and Table 4. Roughly, three different patterns of enzyme secretion could be observed as a response to one meal intake in either HG or REF fish fed on the different diets:

Quick response- This was characterized by a sharp increase of activities measured 1 h after meal supply, followed by a steady decrease and maintenance of values for the rest of the sampling time. In the case of the HG group, this pattern was observed for pepsin in fish receiving diets SCP, F and INS, but not in those fed on C diet. In the case of the REF group, this profile was observed also for pepsin in fish fed on all diets except in those fed on INS.

Delayed response- This was characterized by great variations and peaks of secretion observed several hours after feed intake. In the case of the HG group, a peak was identified 6 h after feed intake for trypsin, chymotrypsin, alkaline phosphatase and to a lesser extent for amylase and aminopeptidase in fish fed on the SCP and INS diets, being not so evident in fish fed on diets C and F. In contrast, variations and peaks were generally measured much earlier in the REF group, around 3 h after food intake. This was the case for trypsin, chymotrypsin and amylase, although the response was not equivalent for all the diets, since fish fed on INS diet presented a delayed pattern resembling that observed in the HG group.

No variation- Characterized by the absence of significant time variations in enzyme activity measured as a response to meal supply. In the HG group, this was the case for lipase, irrespective of the diet (except for fish fed on the F diet, which presented very high values of activity before meal intake). In the REF group, this profile was also observed for lipase, as well as for aminopeptidase and alkaline phosphatase. In these latter enzymes, the pattern was characterized by a decrease in the values of activity during digestion time from those measured before meal supply.

The statistical significance of the effects of sampling moment and diet composition on the values of activity for each enzyme and genotype are resumed in Table 5. A great diversity of responses could be observed, some of the more remarkable:

- Secretion of pepsin showed significant variations due to diet type and sampling moment in fish in the HG group, while none of the two factors influenced its secretion in the REF group. In contrast, values of lipase or alkaline phosphatase activities were not affected by such factors in the HG group but evidenced a significant effect of time in the REF group.
- Values of trypsin and chymotrypsin significantly varied with time in both genotypes, but not in relation to diet. In contrast, values of

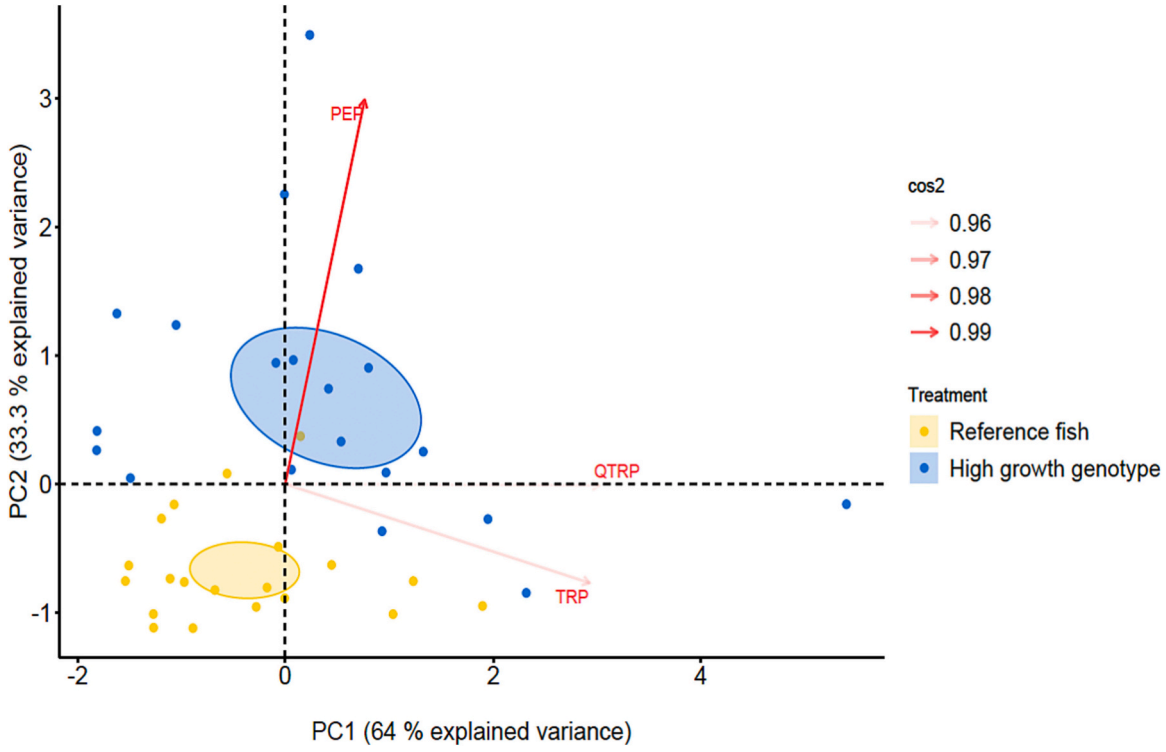

Fig. 3. Principal component analysis (PCA) correlation biplot based on the first two principal components (PC1 and PC2) generated from average values of activity of the different digestive enzymes in the two fish genotypes (high growth and reference).

amylase activity were significantly influenced by the two factors, irrespective of the genotype.

In addition, the results of the PCA carried out using average values of the different enzyme activities measured along the whole sampling period are summarized in Fig. 3. The combination of several variables into two principal components explained 97.3% of total variability observed among data. There was no overlap between the different genotypes. Interestingly, protein-related digestive enzymes, and specially pepsin, were highly correlated with PC1 and PC2 and thus characterizing the digestive enzymes of the HG fish, which formed a well-defined group towards the upper-right of the plot (Fig. 3). In contrast, the PCA analysis performed within each genotype showed no clear differences among diets since values were overlapped. In the case of HG fish, great variability was obtained in those fish fed SCP diet (Fig. 4B).

3.3. Apparent digestibility coefficients (ADC)

The ADC of protein was not affected by genotype, but ADCs of all amino acids were significantly higher in fish from HG genotype compared with REF fish ($p < 0.05$; Table 6). In contrast, diet did not significantly affect ADCs of protein or amino acids, and neither an interaction g x d was noted in ADCs (Table 6).

4. Discussion

Results obtained in the present study pointed out the existence of significant differences between the two fish genotypes in terms of total production of several digestive enzymes, as well as in the patterns of their secretion after feeding. It was clear that genetically selected for fast growth sea bream (HG genotype) showed an enhanced ability to digest protein, indicated by the significantly higher average values of two key digestive proteases: stomach pepsin and intestinal chymotrypsin. Accordingly, the ADC of protein was not significantly affected by genotype, although a trend to present higher ADC values for protein, as well as significantly higher ADC values for all amino acids, were observed in HG genotype fish. Furthermore, these results are also correlated with the better growth performance and feed conversion ratio obtained in selected fish. The key role of acid digestion in protein bioavailability for most fish species is widely recognized since the development of a functional stomach is a milestone that determines the onset of adult digestion mode and also determines the efficiency of the whole digestion process for protein (Yúfera et al., 2004; Márquez et al., 2012). In addition, the potential enhancement of protein digestion in fish belonging to the HG group involved chymotrypsin, an endoprotease that is secreted in the anterior portion of the intestine and plays a major role in protein digestion. These differences in the activity of proteases clearly separated the two genotypes when combined and plotted in the PCA (Fig. 3), with the main vector discriminating both populations being the average activity of stomach pepsin. All these results indicate that genetic selection for high growth in gilthead sea bream had a positive effect on their digestive biochemistry. The impact of genetic selection on digestive enzymes has been assessed in several domestic animals, which showed that dietary changes associated with the use of artificial diets seem to actively promote adaptations to increase the use of specific nutrients. For instance, copy numbers of alpha-amylase genes (*AMY*), which encode starch-digesting enzymes, are markedly increased in modern domesticated dogs as an adaptive evolutionary mechanism, in response to increased consumption of starch-rich foods (Axelsson et al., 2013). Cats developed a hypercarnivorous diet due to the positively selected genes enriched in lipid metabolism through the domestication process (Montague et al., 2014). Domesticated ducks present elevated digestive enzyme activity and a greater intestinal absorptive surface area than mallards to compensate for the increased body mass and growth rate maintaining the same mass of digestive organs (Watkins et al., 2004).

Interestingly, despite its clear influence on growth rate and feed conversion, enhanced production of enzymes has never been considered a major objective of genetic selection in fish, in contrast to other productive traits such as mortality rates, skeletal deformities, disease

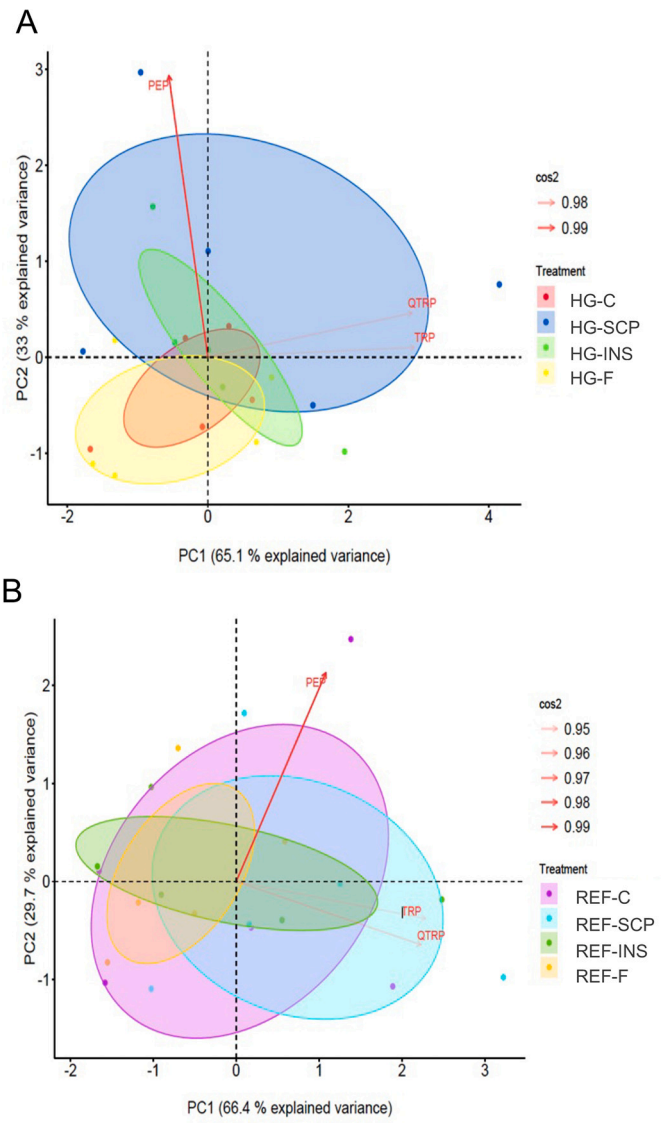

Fig. 4. Principal component analysis (PCA) correlation biplot based on the first two principal components (PC1 and PC2) generated from average values of activity of the different digestive enzymes measured in fish receiving the different diets in A) HG genotype and B) REF genotype.

resistance, fillet yield and flesh and carcass quality (Lee-Montero et al., 2015; García-Celdrán et al., 2015, 2016; Janssen et al., 2017, 2018). For this reason, very few studies have focused on the effect of directed selection on digestive biochemistry associated with domestication in fish, except for some performed in the Eurasian perch (*Perca fluviatilis*), which evidenced that domestication reduced the expression of genes encoding proteolytic enzymes either in larvae (Palińska-Żarska et al., 2020) and juveniles (Chen et al., 2017). The improved protein digestion evidenced in the present study showed an opposite trend that could be explained considering that HG fish were selected based on their improved growth, being this closely related in carnivorous fish to greater efficiency in the use of such nutrient.

In addition to the effect of genotype, the present results also evidenced that the composition of the diets influenced both total production as well as patterns of enzyme release in the digestive tract. Significantly higher activity of aminopeptidase was measured in fish fed on diet SCP, irrespective of the genotype of the fish. Dietary adaptations of the digestive enzyme profile have been extensively studied in wild animals, being supported by two main hypotheses: the Adaptive Modulation Hypothesis (Karasov, 1992) and the Nutrient Balancing Hypothesis (Clissold et al., 2010). The first one suggests a positive correlation between substrate concentrations and enzyme activities, thus an abundant substrate should promote more enzyme activity to ensure its efficient digestion. This has been demonstrated repeatedly in birds (Rott et al., 2017) and also in fish (German et al., 2016). The second suggests that an animal should invest in elevated enzyme activities against limiting nutrients that are low in concentration to ensure their acquisition, and it has been demonstrated in herbivorous fish species for lipases (German et al., 2004). In the case of farmed fish, changes in the digestive enzyme profile have been reported as a result of variations in the total amount of protein (Santos et al., 2020), or in the type of protein (Santigosa et al., 2008; Pérez-Jiménez et al., 2009). Changes in the profile of digestive enzymes associated with modifications in feed ingredients have been also reported in some species, like *Pagrus major* (Murashita et al., 2015), that showed lower activities of trypsin, chymotrypsin, lipase, and amylase when fed on a soybean meal-based diet (SBM) than when fed a FM-based diet. This study also reported lower gene expression levels of the digestive enzymes in the hepatopancreas in the SBM-fed fish compared with the FM-fed fish and suggested that some compounds present in FM diet stimulated the secretion/synthesis of pancreatic digestive enzymes to a greater degree than the SBM diet. Also, in a recent study important changes in the expression of some digestive enzymes were measured in mandarin fish (*Siniperca chuatsi*) as a result of adaptation to feeding on artificial diets (Shen et al., 2021). Results obtained in the present study show a not significant, but clear trend of higher production of almost all enzymes in fish fed on SCP diet, which could be interpreted as compensation to increase the bioavailability of nutrients from a protein source that presents more difficult digestion. Accordingly with the present results, in black sea bream (*Acanthopagrus schlegelii*), the same SCP source (*Methyloccocus capsulatus*) at different dietary inclusion levels also increased the digestive enzymes, including amylase, trypsin, and lipase, in fish gut compared with a C diet with no *M. caspulatus* inclusion (Xu et al., 2021).

It is worthwhile to mention that in most studies oriented to the evaluation of changes in the digestive biochemistry of fish concerning different factors (feeding frequencies, feed composition, etc.), enzyme activities are evaluated using a single sampling point. This usually offers a wrong or very partial picture of the response, taking into account that digestion is a dynamic process that involves a sequential secretion of enzymes and fluids, absorption, and motility that are interdependent and regulated by external factors, such as food composition and availability, light/dark cycle, or temperature, as well as internal factors, including hormones, metabolites, and other sensor molecules, signals (Rønnestad et al., 2013; Isorna et al., 2017). Only in a few fish species, the production of digestive enzymes and/or other factors determining digestive functionality have been examined from the perspective of daily patterns, being these studies mainly focused on larval stages (Mata-Sotres et al., 2016; Gilannejad et al., 2021). In the present study, samplings covered a wide range of time after feeding, thus reinforcing the significance of the observed differences. Both genotypes showed differences in secretion patterns in enzymes like chymotrypsin, amylase, or alkaline phosphatase. These were characterized by a significant delay in the moment of reaching a maximum gut concentration of enzymes in genetically selected sea bream (HG genotype) when compared to non-selected ones (REF genotype), being particularly evident in fish fed on the diet including SCP. In addition, it was observed that patterns of release of some enzymes, like pepsin or amylase, were significantly influenced by diet composition in both genotypes. It must be considered that gut transit rate impacts the hydrolysis of nutrients and hence dictates the exposure of food to digestive enzymes, ultimately influencing the extent of nutrient absorption (Fauconneau et al., 1983; Gilannejad et al., 2019). Notably, delayed production of pepsin in fish fed on SCP could reflect the existence of differences in gut transit rates derived from a higher stomach retention time that could reinforce the previously suggested compensation mechanism to reach more efficient digestion of this protein source.

Table 6
Apparent digestibility coefficients (%) of gilthead sea bream from the two different genotypes fed the experimental diets.

									Two-way ANOVA (*p*-value)		
	HG-*C*	HG-*F*	HG-*INS*	HG-*SCP*	REF-*C*	REF-*F*	REF-*INS*	REF-*SCP*	genotype	diet	gxd
Protein	88.57 ± 0.07	85.70 ± 3.48	90.34 ± 0.19	89.74 ± 0.14	87.10 ± 1.17	83.45 ± 7.77	87.54 ± 2.43	86.48 ± 1.00	n.s	n.s	n.s
Arginine	90.93 ± 0.17	90.27 ± 0.15	92.77 ± 0.33	91.78 ± 0.27	89.99 ± 1.22	90.50 ± 0.78	89.84 ± 2.33	89.61 ± 0.13	0.019	n.s	n.s
Histidine	88.30 ± 0.03	88.15 ± 0.11	90.78 ± 0.44	89.90 ± 0.27	86.91 ± 2.00	88.93 ± 0.78	87.75 ± 1.93	86.84 ± 0.41	0.013	n.s	n.s
Isoleucine	89.57 ± 0.15	89.29 ± 0.01	91.94 ± 0.44	90.90 ± 0.10	88.45 ± 1.48	89.73 ± 0. 65	88.81 ± 2.18	88.29 ± 0.41	0.011	n.s	n.s
Leucine	91.31 ± 0.11	91.03 ± 0.10	93.08 ± 0.34	92.25 ± 0.18	90.29 ± 1.17	91.21 ± 0.56	90.39 ± 2.01	90.03 ± 0.43	0.011	n.s	n.s
Lysine	88.60 ± 0.45	89.03 ± 0.19	91.60 ± 0.44	91.04 ± 0.35	87.93 ± 1.39	89.87 ± 1.73	88.39 ± 2.26	88.44 ± 0.82	0.045	n.s	n.s
Methionine	88.26 ± 0.35	88.85 ± 0.01	91.20 ± 0.38	90.49 ± 0.01	87.50 ± 1.73	89.20 ± 0.54	88.25 ± 2.07	87.83 ± 0.66	0.018	n.s	n.s
Cysteine	78.93 ± 1.18	80.65 ± 0.32	85.90 ± 0.70	83.63 ± 1.59	75.03 ± 6.18	82.07 ± 1.06	80.35 ± 4.15	76.49 ± 2.34	0.030	n.s	n.s
Valine	87.90 ± 0.12	87.52 ± 0.02	90.14 ± 0.43	89.55 ± 0.00	86.41 ± 0.91	88.04 ± 0.69	86.97 ± 2.08	86.54 ± 0.59	0.008	n.s	n.s
Phenylalanine	91.28 ± 0.09	90.65 ± 0.04	92.57 ± 0.35	91.26 ± 0.74	90.00 ± 2.53	90.32 ± 0.34	90.06 ± 1.67	89.13 ± 1.40	0.008	n.s	n.s
Threonine	85.41 ± 0.02	84.87 ± 0.05	88.29 ± 0.53	87.52 ± 0.14	83.66 ± 6.66	85.49 ± 1.03	84.02 ± 3.14	83.65 ± 0.72	0.015	n.s	n.s
Tyrosine	90.59 ± 0.32	97.17 ± 1.98	98.85 ± 0.45	96.58 ± 2.66	93.80 ± 0.87	93.64 ± 0.51	95.22 ± 3.49	97.09 ± 1.94	n.s	n.s	n.s
Alanine	89.10 ± 0.11	87.59 ± 0.07	90.09 ± 0.31	89.31 ± 0.58	87.30 ± 1.23	87.68 ± 0.33	86.86 ± 2.57	86.40 ± 0.95	0.006	n.s	n.s
Glutamic acid	94.42 ± 0.10	94.85 ± 0.03	96.27 ± 0.13	95.85 ± 0.19	93.66 ± 1.01	95.32 ± 0.75	94.71 ± 1.42	94.51 ± 0.31	n.s	n.s	n.s
Glycine	84.75 ± 0.24	81.68 ± 0.02	86.44 ± 0.34	84.55 ± 0.54	81.08 ± 3.35	82.89 ± 1.65	81.25 ± 4.91	79.71 ± 0.21	0.011	n.s	n.s
Aspartic acid	83.34 ± 0.01	83.01 ± 0.67	88.48 ± 0.34	87.12 ± 0.87	81.61 ± 0.70	85.33 ± 1.32	84.28 ± 3.71	82.65 ± 0.13	n.s	n.s	n.s
Proline	92.89 ± 0.01	92.98 ± 0.01	95.07 ± 0.20	93.91 ± 0.25	91.66 ± 1.95	93.37 ± 0.51	93.03 ± 1.73	92.17 ± 0.03	0.011	0.042	n.s
Serine	88.63 ± 0.03	88.71 ± 0.19	91.60 ± 0.27	90.65 ± 0.27	87.27 ± 1.95	89.68 ± 0.81	88.71 ± 2.41	87.67 ± 0.20	0.026	n.s	n.s
Σamino acids	89.99 ± 0.03	90.12 ± 0.03	92.69 ± 0.31	91.77 ± 0.09	88.82 ± 1.70	90.70 ± 0.90	89.84 ± 2.29	89.26 ± 0.07	0.023	n.s	n.s

HG: High growth genotype; REF: Reference genotype; C: Control diet; F: Future diet; INS: Insect diet; SCP: Single-cell protein diet. Two-way ANOVA, $p < 0.05$, Genotype and Diet as fixed factors. Different letters denote significant differences analyzed with one-way ANOVA, $p < 0.05$ for significant g x d interactions. n.s = not significant.

5. Conclusions

The present study provides partial answers to the two questions initially proposed, demonstrating that:

a) Selected fish grew better and had a better feed utilization, at any of the diets assayed;
b) Higher levels of certain protein-related enzymes found in HG fish could be related to better growth, feed utilization, and protein efficiency;
c) Selected fish are more able to utilize the combination of emergent ingredients for aquafeeds, showing changes in the pattern of digestive enzymes to face the different ingredients in diets. Those changes could be reflecting a compensatory mechanism to improve the digestibility of the ingredient;
d) REF fish did not show changes in digestive enzyme patterns to face the changes in ingredients.

Overall, our data indicate that selection modulates positively fish performance and fish digestive enzymes related to protein, improving sea bream capacity for adaptation to novel or so-called future feed formulations. This should be the first report that points to a direct relation between a modified pattern of digestive enzyme secretion and its potentially beneficial effect on nutrient bioavailability in a fish species.

Ethical statements

The described experiments comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals. The experimental protocol was approved by the Institutional Review Board (or Ethics Committee) of the University of Las Palmas de Gran Canaria (approval no. OEBA_ULPGC_12/2020 and OEBA_ULPGC_26/2019) for studies involving animals.

CRediT authorship contribution statement

D. Montero: Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition. **F.J. Moyano:** Conceptualization, Formal analysis, Investigation, Resources, Writing – original draft. **M. Carvalho:** Formal analysis, Writing – original draft. **S. Sarih:** Methodology. **R. Fontanillas:** Resources, Writing – original draft. **M.J. Zamorano:** Methodology. **S. Torrecillas:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Supervision.

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.

Data availability

Data will be made available on request.

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