Aquaculture Reports 41 (2025) 102652

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

First insight into temporal variation of digestive enzyme activities in flathead grey mullet (*Mugil cephalus)* during the ongrowing phase

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ARTICLE INFO

Keywords:
Grey mullet
Mugil cephalus
Digestive enzyme activity
Digestive physiology
Amylase/protease ratio
Pept1 expression

ABSTRACT

Digestive enzymes break down the complex nutrients of food into easily absorbable molecules. Understanding their temporal variation during food transit through the gastrointestinal tract is crucial for developing effective nutritional strategies. This study provides the first insight on the digestive capacity of captive flathead grey mullet (*Mugil cephalus*), a promising omnivorous/detritivorous species for the sustainable diversification of aquaculture in EU. Fish (initial weight: 67.90 ± 15.46 g) were reared in captivity and fed a low fish meal-based diet for 113 days. At the end of the trial, the activity of pancreatic and intestinal enzymes were analysed at three different times: 0, 6 and 12 hours post-prandial. Additionally, the gene expression of *pept1* was determined in the intestine at all considered times to provide a reference to peptide absorption. Alkaline proteases, trypsin and chymotrypsin showed a significant increase at 6 hours post-prandial, with values markedly lower than those of α-amylase. Bile salt-activated lipase and leucine-alanine aminopeptidase increased at 12 hours post-prandial. In the anterior intestine, aminopeptidase presented higher activity at 6 hours post-prandial while alkaline phosphatase showed no significant differences in both anterior and posterior tracts. *Pept1* expression was significantly higher at 12 hours post-prandial in the posterior intestine. This study provides essential insights into the digestive physiology of captive-reared, ongrowing flathead grey mullet (*Mugil cephalus*), emphasizing the importance of aligning feeding practices with the species' unique digestive adaptations. These findings establish a foundation for optimizing feeding schedules and diet composition to significantly enhance nutrient absorption and growth efficiency.

1. Introduction

Flathead grey mullet (*Mugil cephalus*) is a euryhaline finfish with a circumglobal distribution (Loi et al., 2020; Vallainc et al., 2021). The species is harvested and consumed in various European countries with a good marketability of its flesh and a very high price of its salted and dried roe, also called "bottarga". Being consumers of the lower trophic layers, such as algae, plants and zooplankton (Tacon et al., 2011), flathead grey mullet is considered a high-quality source of protein with little or no dietary fishmeal requirements (Bertini et al., 2023). Moreover, it has been demonstrated that this species copes well with affordable aquafeed formulations, showing dietary protein and fat requirements as low as 30 % and 8 %, respectively (Bertini et al., 2023; Rosas et al., 2019; Talukdar et al., 2020b). It is generally accepted that reducing fishmeal dependency and optimizing nutrients are essential for the environmental and economic sustainability of the aquaculture industry (Glencross et al., 2016; Marchi et al., 2023; Perez-Velazquez et al., 2019). As a result, the research line into flathead grey mullet is an increasingly attractive area in which to expand basic knowledge of its digestive physiology and nutrient requirements.

Digestive enzymes play a key role in the transformation of complex nutrients into assimilable forms, directly affecting the growth, health, and feed efficiency of fish. A detailed analysis of digestive enzyme activity in fish after feeding is becoming essential in aquaculture. Such knowledge is key to refining feeding practices, improving sustainability by lowering feed conversion (Tian et al., 2015), and supporting fish

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https://doi.org/10.1016/j.aqrep.2025.102652
Received 15 November 2024; Received in revised form 9 January 2025; Accepted 22 January 2025
Available online 1 February 2025

welfare (Pedrosa et al., 2019).

Flathead grey mullet presents peculiar anatomical, physiological and feeding behavioural features that only recently have been highlighted. For example, it has been found that in captivity, this species displays a particular feeding behaviour with pelleted diets when compared to other farmed fish species, involving more steps for capturing and spitting out from the oral cavity before finally consuming food particles (Ramos-Júdez and Duncan, 2022).

The bicameral stomach is divided into a cardiac stomach, which allows food to be stored and prepared before the passage into the pyloric stomach, called the "gizzard", where the food particles are mechanically broken down (Hickling, 1970; Payne, 1978; Lobel, 1981). The intestine of grey mullet is very long and is used to digest plant materials (Crosetti and Blaber, 2016). Specifically, the length of the flathead grey mullet intestine is 3.2–4.6 the total body length (Crosetti and Blaber, 2016; Dankwa et al., 2009). This particular anatomy of the flathead grey mullet influences enzyme activity. The species is also devoid of acidic digestion in the stomach (Solovyev and Gisbert, 2022). As a result, digestion and absorption of nutrients occurs mainly in the intestine. In particular, protein absorption occurs throughout the intestinal tract while lipid absorption occurs predominantly in the anterior intestine (Crosetti and Blaber, 2016). Several studies on the enzymatic activity conducted on other herbivorous, omnivorous, and detritivorous fish have shown that these species can cope well with higher dietary carbohydrate/protein ratio compared to carnivorous fish (Falcón-Hidalgo et al., 2011; Fernandez et al., 2001; Gisbert et al., 2018; Hidalgo et al., 1999). Insights into intestinal transporters, such as peptide transporter 1 (PepT-1), can further deepen our understanding of digestive function, as *pept-1* is essential for protein assimilation, contributing not only to the breakdown of proteins but also to efficient nutrient absorption (D'Souza et al., 2003; Li et al., 2021). Pept1 is essential for oligopeptide absorption, acting as a co-transporter (Li et al., 2021). Its role in di- and tri-peptide uptake is directly influenced by enzymatic activities, notably from trypsin, chymotrypsin, and total alkaline proteases (Terova et al., 2013; Solovyev et al., 2023).

The aim of this study was to explore the digestive physiology of the species through the analyses of digestive enzymes and gene expression at three different times: T0 (0 h post-prandial), T1 (6 h post-prandial), T2 (12 h post-prandial).

2. Materials and methods

2.1. Experimental diets

The experimental diet was produced via extrusion technology by SPAROS Lda, Portugal. The diameter of the pellet was 1.5 mm. Diets included a low level of crude protein (31 %) and crude lipids (9 %) in the proximate composition. This amount of crude protein and lipids has been previously validated by several studies (De et al., 2012; Talukdar et al., 2020). Ingredients and proximate composition are presented in Table 1.

Table 1
Ingredients and proximate composition of the experimental diet (dry matter basis) administrated to flathead grey mullet (*Mugil cephalus*) specimens over 113 days.

Ingredients as % of the diet	
Wheat meal[a]	39.90
Soybean meal[b]	30.00
Poultry meal[c]	9.74
Feather meal hydrolysate[d]	5.00
Fish meal[e]	3.00
Fish oil[f]	3.45
Soybean oil[g]	3.45
Dicalcium phosphate[h]	2.99
L-Lysine[i]	0.82
DL-Methionine[j]	0.30
L-Tryptophan[i]	0.10
Vit & Min Premix[k]	1.00
Vitamin E50[l]	0.05
Antioxidant[m]	0.20
Proximate composition of the diet as % on a wet weight basis	
Crude protein	31.87
Crude fat	9.41
Moisture	8.88
Ash	7.16

[a] Wheat meal, 11.7 % CP, 1.6 % CF, Molisur, Spain;
[b] Soybean meal, 44 % CP, 3.5 % CF, Ribeiro & Sousa Lda, Portugal;
[c] Poultry meal, 62.4 % CP, 12.5 CF, SAVINOR UTS, Portugal;
[d] Feather meal hydrolysate, 88.8 % CP, 1.6 % CF, Empro Europe NV, The Netherlands;
[e] Fishmeal, 61.2 % CP, 8.4 % CF, Conserveros Reunidos S.A., Spain;
[f] Fish oil, 98.1 % CF, Sopropêche, France;
[g] Soybean oil, 98.6 % CF, JC Coimbra, Portugal;
[h] Dicalcium phosphate, Premix Lda, Portugal;
[i] L-Lysine and L-Tryptophan, Ajinomoto EUROLYSINE S.A.S, France;
[j] DL-Methionine, RHODIMET NP99, ADISSEO, France;
[k] Vitamin and Mineral Premix, Vitamins (IU or mg kg-1 diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DLcholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg-1 diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; Premix Lda, Portugal;
[l] Vitamin E50, ROVIMIX E50, DSM nutrition products, Switzerland;
[m] Antioxidant, VERDILOX, Kemin Europe NV

2.2. Fish rearing and feeding trial

Flathead grey mullet born in captivity at International Marine Centre (Oristano, Italy) were delivered to the Laboratory of Aquaculture at the University of Bologna (Cesenatico, Italy). At the beginning of the trial, fish (average body weight: 67.90 ± 15.46 g) were randomly distributed into 3 × 800 L fiberglass square tanks (45 fish/tank), connected to a closed recirculation aquaculture system. This system consisted of a mechanical sand filter (PTK 1200; Astralpool, Barcelona, Spain), ultraviolet lights (UV PE 45; Sita Srl, Barcelona, Spain) and a biofilter (PTK 1200; Astralpool, Barcelona, Spain). Water consisted of a mixture of marine and freshwater (salinity 7 ± 2 ‰) (Cardona, 2006), the water exchange rate was kept at 100 % / h in each tank and the overall water renewal in the system at 5 % daily according to standard rearing management procedures for Mediterranean fish species during feeding trial (Busti et al., 2020; Bertini et al., 2023). Temperature was kept at 26.8 ± 2.5 °C, photoperiod was maintained at 12 h light / 12 h dark through artificial light and oxygen saturation at 100 % by an integrated control system. Ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}) and nitrite (≤ 0.2 mg L^{-1}) were spectrophotometrically monitored once a day (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Fish were fed to apparent satiation by oversupplying the feed (10 % of the daily ration) by automatic feeders, once a day for six days a week (Bertini et al., 2023). Each meal lasted 6 hours to provide fish a continuous feed supply over daily hours. The uneaten pellets of each tank were collected three times during each meal (2, 4 and 6 hours after the beginning of each meal), dried overnight at 105 °C, and deducted from overall calculation (Bertini et al., 2023)

2.3. Samplings

Before each sampling, fish were either anaesthetised (100 mg L^{-1}) or euthanised (300 mg L^{-1}) by tricaine methanesulfonate MS-222 (Sigma Aldrich, Germany). At the end of the experiment (day 113) all fish were anesthetised and individually weighed (final average body weight: 182.13 ± 8.27 g). In order to assess the activity of digestive enzymes and

the expression of *pept1*, fish were sampled every 6 hours after the meal (T0, 0 h before feeding; T1, 6 h post-prandial; T2, 12 h after feeding). Digestive enzymes activity (total alkaline protease, trypsin, chymotrypsin, α-amylase and bile salt-activated lipase) was assessed on the whole gastro-intestinal tract from 2 fish per tank (n = 6) at each sampling point (T0, T1, T2). Tissue was gently dissected from each fish and immediately snap-frozen at −80 °C inside resealable bags. The enzyme activity of the intestinal mucosa (alkaline phosphatase, leucine-alanine peptidase and aminopeptidase) was assessed on a 2 cm portion of both anterior and posterior intestine from an additional 3 fish per tank (n = 9) at each sampling point (T0, T1, T2) for alkaline phosphatase and aminopeptidase and was assessed on the intestinal tract from 2 fish per tank (n = 6) at each sampling point (T0, T1, T2) for leucine-alanine aminopeptidase. Samples of intestine were gently dissected as shown in Fig. 1 and immediately snap-frozen at −80 °C into separate Eppendorf tubes. From the same 3 fish from which samples were taken for alkaline phosphatase and aminopeptidase, a 100 mg sample of both anterior and posterior intestine was dissected and placed into Eppendorf tubes containing 10 volumes of RNAlater (Invitrogen™, ref AM7021) for the gene expression analysis of the *pept1* transporter. In total 9 samples at each sampling point (T0, T1, T2). All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.4. Digestive enzyme activity

Samples were shipped and processed at IRTA (Institute of Agrifood Research and Technology) according to the standard procedures given below. For quantifying the activity of pancreatic (total alkaline proteases, trypsin, chymotrypsin, α-amylase and bile salt-activated lipase), and intestinal (leucine-alanine peptidase, aminopeptidase and alkaline phosphatase) digestive enzymes samples were frozen at 80 °C until their analysis, and the protocols were performed as recommended in Cahu and Infante (1994) and in Crane et al. (1979). In order to prevent sample deterioration as described in Solovyev and Gisbert (2016), samples were analysed 210 days after sampling. Gut samples (*ca.* 0.5 g) were homogenized (Ultra-Turrax T25 basic, IKA©-Werke, Germany) in 30 volumes (*v/w*) of ice-cold mannitol buffer (50 mM mannitol, 2 mM Tris-HCl buffer; pH 7.0), followed by addition of 0.1 M of $CaCl_2$. After that, samples were re-homogenised for 30 seconds, after which they were sonicated for 90 s. The homogenised samples were centrifuged at 9000 × *g* (10 min at 4 °C), then 1 mL of supernatant was removed for quantification of pancreatic and cytosolic intestinal enzymes and stored in a new Eppendorf tube (Koven et al., 2019). Samples were stored at −80 °C until further enzyme determination. Then, the crude homogenate was centrifuged at 34,000 × *g* (20 min at 4 °C). After that, the supernatant was discarded and the pellet (containing brush border enzymes) was recovered and dissolved in 1 mL of Tris-Mannitol buffer (50 mM mannitol, 2 mM Tris-HCl buffer; pH 7.0). Samples were stored at −80 °C (Gisbert et al., 2018).

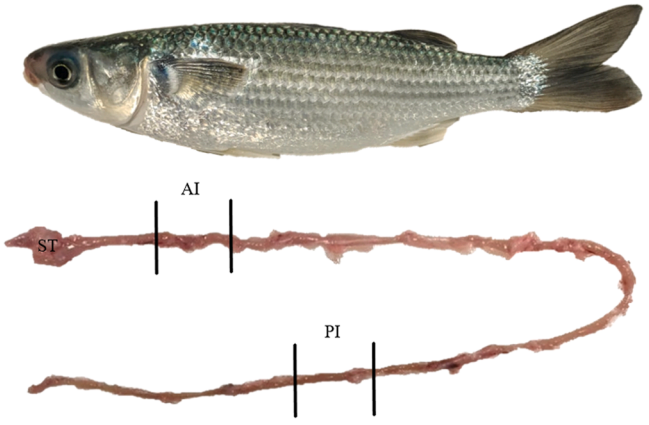

Fig. 1. Photograph of the gastrointestinal tract of grey mullet (*Mugil cephalus*) (weight 102.30 g and length 23.4 cm): stomach (ST), anterior intestine (AI) and posterior intestine (PI) (total length of intestine: 50.8 cm). Sample sites of AI and PI for enzyme activity and gene expression shown between bars.

Total alkaline proteases were determined after 1 h of incubation at 25 °C using 0.5 % (*w/v*) azocasein as substrate and Tris-HCl 50 mM (pH 8.0) as a buffer. The reaction was stopped with trichloroacetic acid (TCA) (20 % w/v). When the reaction was stopped, samples were centrifuged at 10,000 × *g* (5 min at 4 °C). The absorbance of the supernatant was read at λ = 366 nm. One unit of total alkaline proteases per mL (U) was defined as 1 μM azocasein hydrolysed per min^{-1} mL^{-1} of homogenate (García-Carreño and Haard, 1993). The quantification of trypsin was carried out using BAPNA (Benzoyl-DL-arginine 4-nitroanilide hydrochloride) as a substrate. One unit of trypsin per mL (U) was defined as 1 μmol BAPNA hydrolysed min^{-1} mL^{-1} of enzyme extract at λ = 405 nm for 5 min (Holm et al., 1988). The quantification of chymotrypsin was assayed using Suc-AAPF-pNA (N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide) as a substrate and the absorbance read at λ = 405 nm for 5 min (Decker, 1977). α-Amylase was quantified using soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate. A mixture of 50 μl of homogenised sample with reagents and substrate were incubated for 35 min at 25 °C and absorbance read at λ = 580 nm (Métais and Bieth, 1968). Bile salt-activated lipase was analysed using pNPM (*p*-nitrophenyl myristate), a C14 long chain substrate, and read at λ = 405 nm. Lipase activity was defined as the amount (nmol) of substrate hydrolysed min^{-1} mL^{-1} of enzyme extract (Iijima et al., 1998). Leucine-alanine peptidase (LAP) is measured by its absorbance at λ = 530 nm. One unit of enzyme activity (U) was defined as 1 nmol of the hydrolysed substrate min^{-1} mL^{-1} of tissue homogenate (Nicholson and Kim, 1975). Leucine-alanine (Leu-Ala) has been used as substrate and Tris-HCl (100 mM, pH 8.0) as buffer and with an incubation time of 20 min at 25°C (Maroux et al., 1973). The reaction was stopped with H_2SO_4 50 %. Aminopeptidase-N was quantified using a L-leucine p-nitroanilide as a substrate and a phosphate buffer solution 1 M ($NaH_2PO_4 + H_2O$). One unit of enzyme activity (U) was defined as 1 μg nitroanilide released min^{-1} mL^{-1} of brush border homogenate at λ = 410 nm (Maroux et al., 1973). Alkaline phosphatase was determined using P-nitrophenyl phosphate (PNPP) as a substrate and Tris-HCl 100 mM (pH 8,0) as a buffer and read at λ = 405 nm, 5 min (Bessey et al., 1946). Determination of soluble proteins were based on methods previously described by Bradford (1976). Soluble protein quantification was performed by spectrophotometry (λ = 595) using bovine serum albumin (BSA) as a standard. Soluble protein extraction is needed for the quantification of enzymes. Tissue proteins are obtained after their union with the pigment (Coomassie Brilliant Blue G250) forming a blue complex that remains stable in solution for 1 hour. Enzymes activities were measured in a spectrophotometer using a microplate or a quartz microplate (Infinite® 200 PRO, TECAN, Switzerland) and expressed as specific activity units (U mg $protein^{-1}$ or mU mg $protein^{-1}$). All analyses were performed in triplicate (methodological replicates).

2.5. Gene expression

Total RNA was extracted from 0.030 g of tissue (anterior and posterior intestines) using the RNeasy Kit (Qiagen), according to the manufacturer's instructions. Samples were diluted and evaluated for concentration and purity by spectrophotometry (NanoDrop2000, Termo Fisher Scientific, Madrid, Spain) measuring the absorbances at λ = 260 and 280 nm (Castro-Ruiz et al., 2021; Masroor et al., 2019). An aliquot of 1 μg of each RNA sample was used to verify RNA integrity by agarose gel electrophoresis. For the purposes of confirming the integrity of the RNA these aliquots were prepared in an Eppendorf tube containing formamide (ratio v/v 1:1.6), and incubated for 5 minutes at 60°C prior

to loading on a 1.5 % agarose gel for denaturing electrophoresis according to (Masek et al., 2005) for visual confirmation of the presence and uniformity among samples of the ribosomal RNA bands. Afterwards the RNA was reverse transcribed to cDNA using the Quantitect cDNA synthesis kit (Qiagen). All cDNA was then diluted 1:20 for qPCR analysis (Bustin et al., 2009). Real time PCR were conducted with c10000 touch Thermal Cycler instrument (BIO-RAD) for the amplification of the housekeeping gene Elongations Factor 1a (*ef1a*) and the target gene Peptide Transporter 1 (Skalli et al., 2014).

Primary sequence of each gene was obtained using primers identified as being conserved by inspection of alignments of each gene previously obtained from extant teleost species and recovered from Genbank [*Gadus morhua*: AY921634.1; *Dicentrarchus labrax*, FJ237043.2; *Sparus aurata*, GU733710.1; *Salmo salar*, AB455540.1]. These primers amplified large fragments (*ef-1* = ~470 bp; *pept1*= ~910 bp). These sequences, after alignment with the above mentioned extant species sequences enabled identification of sequences more unique to *M. cephalus* for design of the gene-specific primers. The forward and reverse primers for *ef-1* and *pept1* were respectively,

ef-1 F: 5´CACTGAGGTCAAGTCCGTTGAG 3´;
ef-1 R: 5´CGTTCTTGACGTTGAAGCCGAC 3´, and
pept1 F: 5´TTGGCTTCGGGAGTTCGTACAC 3´;
pept1 R: 5´GTGCCTGTGAGTAAGAGAACTC 3´.

The specific amplicons obtained using these assays were both ~200 bp for *pept1* and *ef-1*. Each qPCR assay was evaluated individually for efficiency using the slope obtained from a linear regression of amplifications resulting from a serial dilution of cDNA, initially diluted 1:20, then further diluted 1:5 over a series of six dilutions. The E% values obtained were 109.18 % and 102.65 % for *ef-1* and *pept1*, respectively. Melt curves obtained from the SYBER green amplification indicated each assay produced single amplicons.

All samples were tested in duplicate. For each sample, the gene expression was normalised to the *ef1a* and expressed as 2-ΔΔCt. ΔCt was obtained by subtracting the cycle threshold (Ct) of the *ef1a* gene from the Ct value of the target gene (*pept1*) (Livak and Schmittgen, 2001). Gene expression in samples collected at times T1 and T2 was expressed relating to the samples collected at time zero (T0).

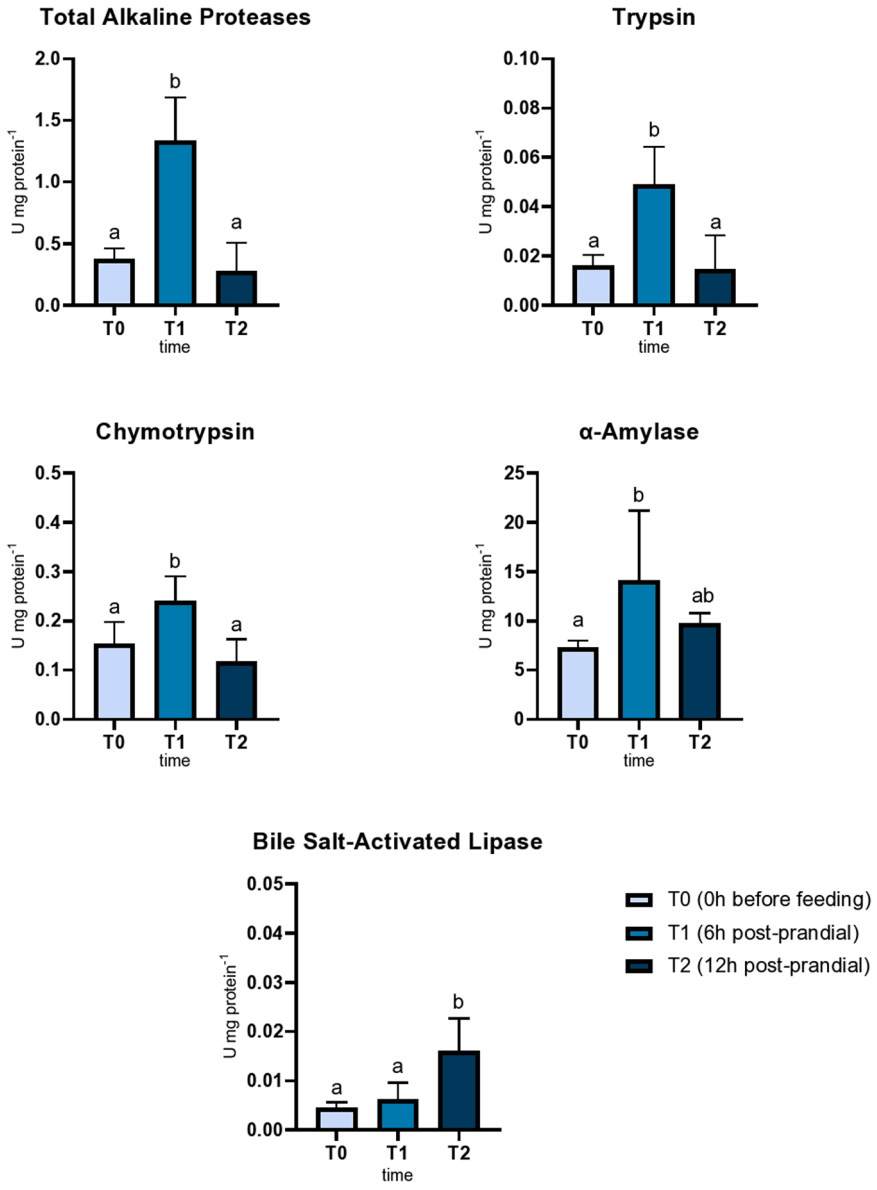

Fig. 2. Specific (U mg protein^{-1}) digestive enzyme activities of pancreatic of *Mugil cephalus* at T0 (0 h before feeding), T1 (6 h post-prandial) and T2 (12 h post-prandial) activity. Different letters indicate significant difference ($P \leq 0.05$) between times.

2.6. Statistical analysis

All data are presented as mean ± standard deviation (SD). Data of enzyme activity and gene expression were analysed by a one-way analysis of variance (ANOVA) and in case of significance ($p \leq 0.05$) the Tukey's post hoc test was performed. The normality and/or homogeneity of variance assumptions were validated for all data preceding ANOVA. If these tests were not passed, it is continued with a non-parametric test with a Kruskal-Wallis test and Dunn's multiple comparison test. Outliers Peirce Chauvenet and Graph Pad 8.0 were used to perform all statistical analysis.

3. Results

3.1. Pancreatic enzymes activity

The activity of six pancreatic enzymes (trypsin, chymotrypsin, α-amylase, bile salt-activated lipase and total alkaline proteases) are shown in Fig. 2. Trypsin activity was higher in T1 (6 h post-prandial) than T0 (0 h before feeding) and T2 (12 h post-prandial) ($p < 0.05$). Chymotrypsin was statistically different ($p < 0.05$) depending on the post-prandial time; it was higher in T1 than T2 but the results of T1 and T2 were no different from T2. α-Amylase activity at T0 and T1 were different from each other ($p < 0.05$). In particular, T1 was higher than

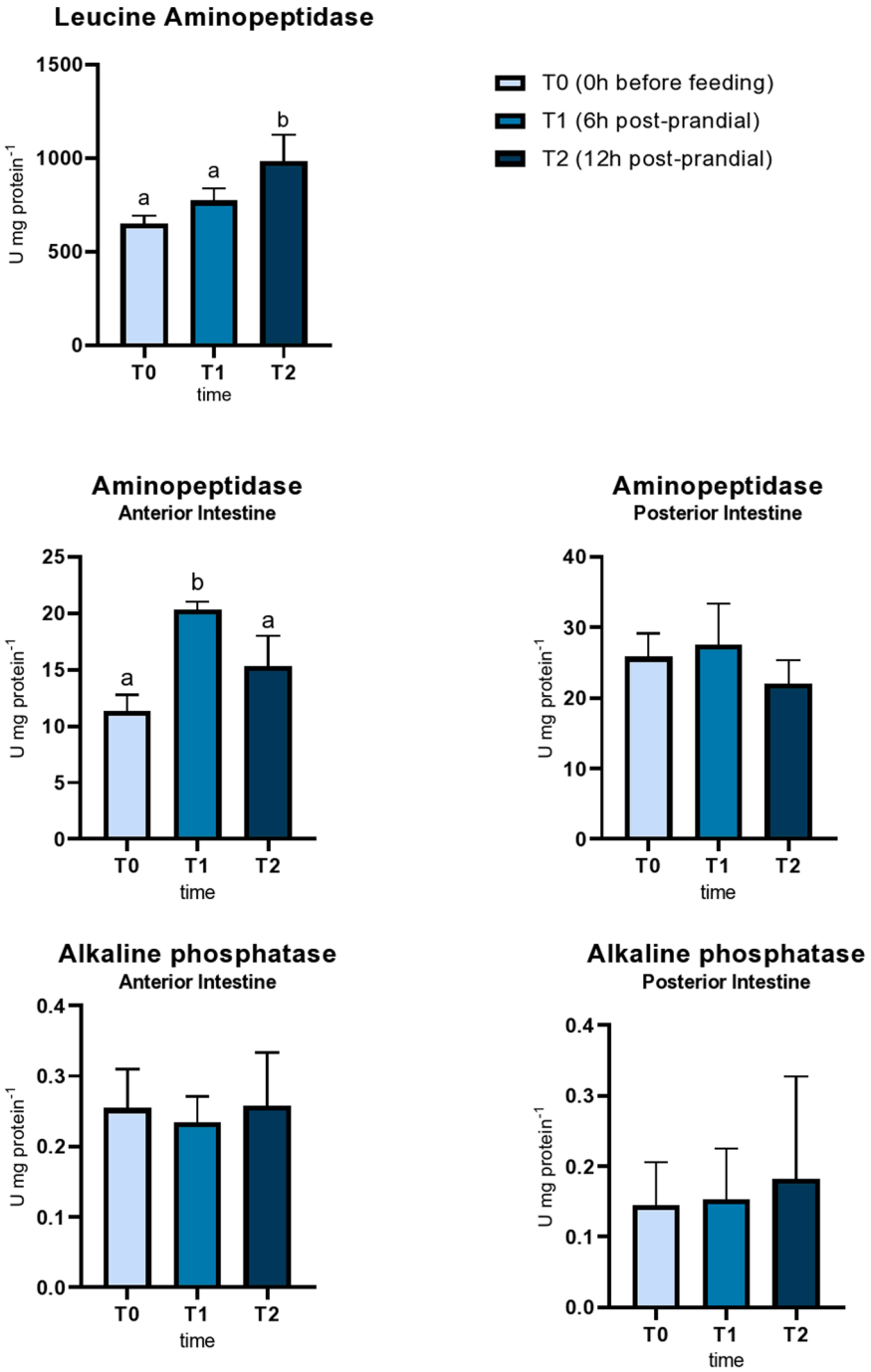

Fig. 3. Specific (U mg protein^{-1}) digestive enzyme activities of cytosolic enzyme within enterocytes (leucine-alanine peptidase) and of intestinal brush border enzymes (anterior intestine (AI) and posterior intestine (PI)) of *Mugil cephalus* at T0 (0 h before feeding), T1 (6 h post-prandial) and T2 (12 h post-prandial) activity. Different letters indicate significant difference ($P \leq 0.05$) between times.

T0, and T2 was not statistically different from T0 and T1. Bile salt-activated lipase activity was higher in T2 than T0 and T1 ($p < 0.05$). The activity of total alkaline proteases was higher in T1 ($p < 0.05$) than T0 and T2.

3.2. Intestinal enzymes activity

The activity of cytosolic enzymes within enterocytes (leucine-alanine peptidase) and brush border enzymes (aminopeptidase and alkaline phosphatase) are presented in Fig. 3. Leucine-alanine peptidase activity increased significantly in T2 ($p < 0.05$) as compared to the other group (T0; T1). The aminopeptidase activity of the anterior intestine was statistically different ($p < 0.05$); it was higher in T1 (6 h post-prandial) than T0 and T2. The activity of aminopeptidase in the posterior intestine evinced no significant difference ($p > 0.05$) regardless of the post-prandial time considered. Alkaline phosphatase activity measured in the anterior and posterior intestinal segments did not vary among samples times ($p > 0.05$).

3.3. Gene expression

The expression of *pept1* in the anterior and posterior intestine is presented in Fig. 4. In the anterior intestine, the expression of *pept1* was significantly higher in T0 than in T1 and T2 ($p < 0.05$). Gene expression in the posterior intestine was statistically different, with higher activity observed in T0 and T2 compared to T1.

4. Discussion

The activities of total alkaline proteases, trypsin, chymotrypsin, α-amylase, and bile salt-activated lipase have been previously characterised in other Mugilidae species in response to different dietary ingredients (De et al., 2012; Luzzana et al., 2005; M. Yones et al., 2019; Quirós-Pozo et al., 2021; Talukdar et al., 2020) and dietary additives (El-Sharkawy et al., 2023). Despite this knowledge, limited information has been collected on the temporal variation of enzymatic activity during feeding. Exploring these temporal dynamics could provide a deeper understanding of the species' digestive physiology and nutrient utilisation. In the majority of fish species, trypsin and chymotrypsin stand out as the primary alkaline digestive proteases responsible for initiating the hydrolysis of proteins in the intestine, regardless of whether the fish are gastric or agastric (Day et al., 2011). In this study, protease activity showed a significant increase at 6 hours (T1) post-prandial. In particular, total alkaline proteases and trypsin almost tripled their activity at 6 h (T1) compared to 0 h (T0) and 12 h (T2). These data confirm the role of alkaline proteases as key enzymes in protein digestion for this species (Bezerra et al., 2005; Bkhairia et al., 2015; Chan et al., 2004; Willora et al., 2022). The elevated total alkaline proteases and trypsin activity observed at 6 hours post-prandial likely corresponds to the peak of protein digestion, aligning with the species' rapid gut evacuation rate, which completes around 8 hours post-feeding (unpublished data). Supporting this interpretation, enzyme activity was low at T0 due to the absence of feed in the gut and decreases again at T2 as the feed content in the intestine diminishes, resulting in reduced enzyme secretion. Solovyev and Gisbert (2022) observed that in flathead grey mullet fry fed once or twice daily, peaks in enzyme activity occurred shortly after each meal. However, in fry fed continuously over a 6-hour light period, as in this study, this peak activity trend was not present. It has been suggested that digestive enzyme activities in the gut are regulated by feeding frequency; thus, as meal frequency increases, enzymatic activity decreases. This supports the hypothesis that the digestive system adapts to maximize nutrient absorption while minimizing energy expenditure (Solovyev and Gisbert, 2022). In gilthead sea bream (*Sparus aurata*), different feeding frequencies have been shown to impact enzymatic activity (Busti et al., 2020). Meal timing, feeding frequency, and food characteristics are crucial factors that influence gut evacuation time (Ng and Romano, 2013; López-Olmeda et al., 2012). In natural environments, the continuous feeding behaviour observed in Mugilidae species, as reported by Blaber (1976) and Marais (1980), optimises digestion, especially for species that rely on low-energy food sources and need to feed frequently to meet their nutritional needs. Thus, continuous feeding for 6 hours, as applied in this study, may affect digestive enzyme activity differently than a more typical feeding regime used in other species (e.g., two 1-hour meals) (Marchi et al., 2023; Pelusio et al., 2023). The findings of this study align with previous research on herbivorous species, such as Nile tilapia, which has a reported gastric evacuation rate of 7.15 hours (Uscanga et al., 2010). In contrast, carnivorous fish like European seabass and gilthead sea bream require over 17 hours for complete intestinal emptying (Busti et al., 2022; Nikolopoulou et al., 2011). These differences can be attributed to variations in stomach structure and dietary preferences among species (Busti et al., 2022; Day et al., 2011; Uscanga et al., 2010).

Buddington et al. (1987) reported that herbivorous and carnivorous fish actually require similar protein levels in their diets. Herbivorous fish achieve adequate protein intake from their typically low-protein diets by consuming larger amounts of food continuously and utilising the entire intestine for nutrient absorption and enzyme production. Carnivorous fish, however, obtain sufficient protein directly from their diet but require more time for nutrient absorption in the intestines. This may explain the faster gut evacuation observed in herbivores compared to carnivores (Day et al., 2011; Klumpp and Nichols, 1983). Another factor contributing to these differences in digestion could be the absence of a true gastric stomach in some herbivores, which limits acidic digestion. Grey mullets, for instance, possess a unique gizzard-like stomach that traps food particles for mechanical digestion, compensating for the lack of acid digestion, and facilitating subsequent enzymatic breakdown in

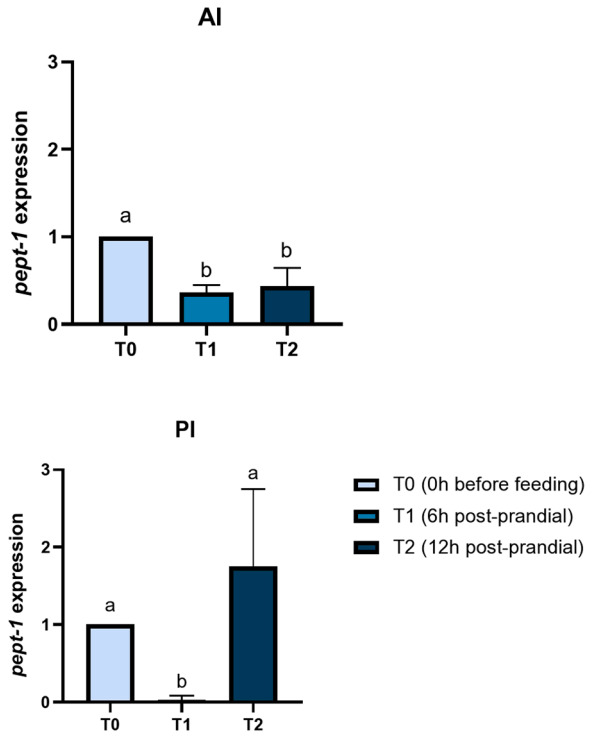

Fig. 4. Relative gene expression profiles of AI (anterior intestine) and PI (posterior intestine) of mugil (*Mugil cephalus*) at T0 (0 h before feeding), T1 (6 h post-prandial) and T2 (12 h post-prandial). Gene expression in samples collected at times T1 and T2 was normalized relating to the samples collected at time zero (T0). For each sample, the gene expression was normalized to the *ef1a* and expressed as 2-ΔΔCt. Different letters indicate significant difference ($P \leq 0.05$) between time. Non-parametric tests have been carried out on both results.

the gut (Bertini et al., 2023; Crosetti and Blaber, 2016; Lobel, 1981; Payne, 1978). The gizzard's role in digestion is well-documented in poultry, where it aids in reducing particle size and enhancing nutrient breakdown in the anterior intestine (Svihus, 2011). These traits may provide insights into the unique digestive processes of grey mullet.

α-Amylase plays a key role for carbohydrate digestion (Azzout-Marniche et al., 2019; Yang et al., 2023). In grey mullet, starch digestion is notably well-developed compared to that of carnivorous fish, reflecting its dietary needs and evolutionary adaptation to a more plant-based diet (Hidalgo et al., 1999; Solovyev et al., 2014). In this study, α-amylase activity was significantly higher at T1 (6 hours post-prandial) than at T0, supporting its effective role in breaking down complex carbohydrates (Cockson and Bourn, 1973). While α-amylase is present in both herbivorous and carnivorous fish, herbivores generally exhibit a higher α-amylase/protease ratio, aligned with their plant-based diet (Solovyev et al., 2014). In this experiment, α-amylase activity ranged from 12 to 15 U mg^{-1} protein, compared to 0.4–1.2 U mg^{-1} protein for total alkaline proteases, resulting in a high α-amylase/protease ratio. A similar pattern was observed in Nile tilapia, where intestinal α-amylase activity reached 5.6 U g^{-1} protein versus 3.6 U g^{-1} protein for total alkaline proteases, also reflecting a high α-amylase/protease ratio (Magouz et al., 2020).

In rabbitfish (*Siganus canaliculatus*), another herbivorous species, when fed a diet with raw fish inclusions, amylase values ranged from 80 to 85 U mg^{-1} , while protease values were also in the same range, from 80 to 85 U mg^{-1} , resulting in an equal amylase/protease ratio in the middle intestine (Xie et al., 2018). However, when the same species was fed a diet with *Enteromorpha prolifera* macroalgae inclusions, amylase values increased to a range of 130–150 U mg^{-1} , while protease values ranged from 62 to 67 U mg^{-1} (Xie et al., 2018), leading to a higher amylase/protease ratio. The higher amylase/protease ratio in fish fed macroalgae compared to those fed raw fish suggests that the increased fiber content in the macroalgae diet may inhibit protease activity (Horie et al., 1995; Xie et al., 2018). Similarly, Chan et al. (2004) observed contrasting levels of amylase activity in two herbivorous species (37 mU mg^{-1} protein for monkeyface prickleback, *Cebidichthys violaceus* and 115 mU mg^{-1} protein for rock prickleback, *Xiphister mucosus*), as well as in two carnivorous species (25 mU mg^{-1} protein for high cockscomb, *Anoplarchus purpurescens* and 101 mU mg^{-1} protein for black prickleback, *Xiphister artropurpureus*). These differences in amylase activity are likely not primarily due to variations in feeding habits, but rather to the phylogeny of the species, as suggested by previous studies (Chan et al., 2004; German et al., 2004; MacKinlay and Shearer, 1996). In carnivorous species such as Asian seabass, amylase activity ranged from 2.1 to 2.4 U mg^{-1} protein, while total alkaline proteases ranged from 100 to 115 U mg^{-1} protein (Raffic et al., 2020), resulting in a lower amylase/-protease ratio. Surprisingly, in gilthead sea bream, amylase activity was 4.49 U mg^{-1} protein, and total alkaline proteases were 0.56 U mg^{-1} protein (Parma et al., 2020), leading to a higher amylase/protease ratio. While direct comparisons between studies and methods across species are challenging, these findings support the hypothesis that grey mullet have a greater capacity for carbohydrate digestion compared to carnivorous species (German et al., 2004; Gisbert et al., 2018; Falcón--Hidalgo et al., 2011; Xie et al., 2018). The amylase/protease ratio for herbivores (Xie et al., 2018) typically ranges from 1 to 3, for omnivores (Magouz et al., 2020) it is around 8, and for carnivores (Raffic et al., 2020) it is less than 0.005. In this study, the average amylase/protease ratio for grey mullet is approximately 16. Therefore, it can be concluded that grey mullet is more closely aligned with omnivorous fish than with herbivorous or carnivorous species.

Lipids play a vital role in fish growth by providing energy, essential fatty acids, and fat-soluble nutrients (Kim et al., 2012). In this study, bile salt-activated lipase activity was highest at 12 hours (T2), being twice as high as at 0 hours (T0) and 6 hours (T1), which is when feeding was presumably completed. The delayed activation of lipase is likely due to the time required for chyme to pass through the stomach and for bile salts to be secreted, triggering lipase activation. This pattern aligns with previous research on permit (*Trachinotus falcatus*) (Lazado et al., 2017), where lipase activity also peaked at 12 hours, possibly reflecting lipid metabolism rhythms and the circadian clock (Lazado et al., 2017).

Brush border enzymes are responsible for the further hydrolysis of peptides in the intestine (Matias et al., 2023). To our knowledge, this is the first study to examine these enzymes in juvenile and ongrowing stages of flathead grey mullet and other Mugilidae species. Leucine-alanine peptidase activity significantly increased from T0 (0 hours) to T2 (12 hours), peaking at 12 hours. This increase is likely due to the key role this cytosolic enzyme plays in the final stage of protein digestion (Matsui et al., 2007; Wu et al., 2008). While cross-species comparisons are challenging, Lazado et al. (2017) reported a peak in leucine-alanine peptidase activity at 18 hours post-prandial in permit. Unlike constant enzyme levels, these activity peaks enhance proteolytic efficiency (Lazado et al., 2017) and may play a role in conserving bioenergetic resources.

Aminopeptidase is a proteolytic enzyme that removes amino acids from the ends of proteins and peptides, facilitating their absorption by intestinal cells (Taylor, 1993). It also plays a crucial role in protein turnover (Chen et al., 2011; Gonzales and Robert-Baudouy, 1996). Similar to proteases, aminopeptidase activity was highest in the anterior intestine at 6 hours post-prandial (T1), aligning with the progression of protein digestion initiated by alkaline proteases in this region.

Alkaline phosphatase, an enzyme located in the intestinal brush border, facilitates fatty acid absorption through enterocyte membranes (Buchet et al., 2013; Lallès, 2019). Nutrient absorption primarily occurs in the anterior intestine, with minimal absorption in the posterior section (Bakke et al., 2010; Pérez-Jiménez et al., 2009; Pujante et al., 2015; Pujante et al., 2017). In the present study, no significant differences were observed in alkaline phosphatase activity between the anterior and posterior sections. As a marker of intestinal integrity, alkaline phosphatase is associated with reduced gastrointestinal inflammation (Lallès, 2020; Messina et al., 2019). Therefore, the stable enzyme activity observed in this study suggests a healthy intestinal condition (Guardiola et al., 2018).

Pept1 is a key co-transporter responsible for the absorption of oligopeptides (Li et al., 2021). The uptake of di- and tri-peptides via Pept1 is influenced by enzymes such as trypsin, chymotrypsin, and alkaline proteases (Terova et al., 2013; Solovyev et al., 2023). The expression pattern of *pept1* at T0, T1, and T2 post-prandial intervals underscores its critical role in protein digestion. At T1 (6 hours post-prandial), the higher expression of *pept1* in the anterior intestine aligns with the peak in protease activity, suggesting a coordinated process for nutrient absorption (Ogihara et al., 1996; Ostaszewska et al., 2010). Gilannejad et al. (2021) observed a similar increase in *pept1* expression in gilthead sea bream after feeding, with upregulation persisting for over 20 hours. This indicates ongoing peptide absorption, suggesting that *pept1* upregulation helps replenish peptides in preparation for the next digestion cycle. In line with this, *pept1* expression is expected to be higher in the presence of food and lower in the absence of food. In the current study, *pept1* expression was higher in the anterior intestine at T1, as digestion had just begun and food had already reached this region, while it had not yet arrived in the posterior intestine. This is in contrast to the pattern observed in mammals and birds (Verri et al., 2011). The results of this study also align with findings on alkaline phosphatase activity in the anterior intestine. Given that *pept1* is involved in absorbing di- and tri-peptides, which are products of protein digestion (Chen et al., 2005; Ostaszewska et al., 2010), there may be a correlation, or possibly a complementary function, between *pept1* and alkaline phosphatase. Alkaline phosphatase could help create a favourable environment for peptide breakdown, thereby facilitating their absorption through *pept1* in the anterior intestine (Ogihara et al., 1996). Additionally, the higher *pept1* expression in the anterior intestine at T1 (6 hours post-prandial) corresponds with an increase in trypsin and chymotrypsin activity, likely initiating peptide breakdown to optimize absorption. This is

consistent with findings in gilthead sea bream juveniles, where *pept1* expression peaks after feeding, indicating its active role in nutrient absorption during digestion (Gilannejad et al., 2021). Comparative studies with other aquaculture species underscore the importance of studying *pept1* for optimizing diet formulations. For example, in rainbow trout, higher *pept1* expression is linked to improved protein digestibility, highlighting the importance of synchronizing feeding schedules with peak absorption times for more sustainable growth (Ostaszewska et al., 2010). Further research suggests that *pept1* expression may vary depending on the protein source in fish diets, as seen in species such as grass carp (Liu et al., 2013), Atlantic cod (Bakke et al., 2010), and turbot juvenile (Wei et al., 2016). Additionally, *pept1* gene expression is influenced not only by protein sources but also by hormonal factors. Research has demonstrated that hormones such as leptin, cholecystokinin, and gastrin can regulate intestinal transport processes, affecting *pept1* expression and function. This hormonal regulation indicates a complex interaction between dietary and endocrine signals in controlling nutrient absorption efficiency (Bakke et al., 2010; Ostaszewska et al., 2010).

5. Conclusion

This study provides valuable insights into the digestive processes of juvenile flathead grey mullet, focusing on the enzymatic activities and gene expression involved in nutrient digestion and absorption. The observed increase in total alkaline protease and trypsin activity at 6 hours post-feeding suggests a peak in protein digestion and highlights the role of these proteases in activating other pancreatic enzymes, which may align with the grey mullet's fast gut-evacuation rate. The α-amylase results underscore its importance as a key enzyme in carbohydrate digestion, particularly in comparison to carnivorous species, with peak activity observed at 6 hours post-prandial (T1). This finding further supports the species' omnivorous feeding habits, emphasizing its enhanced ability to break down complex starches derived from plant sources. Additionally, this study reveals a significant increase in Pept1 gene expression in the anterior intestine during active digestion (T1), with a shift in gene expression observed between the anterior and posterior intestines at later stages (T2). This suggests dynamic regulation of nutrient absorption, potentially influenced by complementary functions with enzymes such as alkaline phosphatase, trypsin, and chymotrypsin. Overall the findings emphasize the importance of aligning feeding practices with the specific digestive adaptations of *Mugil cephalus*. By synchronizing feeding schedules with peak digestive enzyme activity and Pept1 gene expression, nutrient uptake can be optimized, reducing feed waste and enhancing growth efficiency. This approach not only improves fish health and welfare but also promotes faster achievement of market size, which is particularly advantageous in *Mugil cephalus* aquaculture, where fish of a specific size are required for roe (bottarga) production.

Funding

This research was undertaken as a part of the FEAMP 2014–2020 project, action 3 A. Pilot project for the application of techniques and methods aimed at the enhancement and diversification of fish products from Valliculture financed by Emilia Romagna region. Analyses of digestive enzymes conducted at IRTA were supported by the ACUISOST project funded by the Ministerio de Agricultura, Pesca y Alimentación from the Spanish Government and EU Next Generation funds (PRTR).

Part of the research was carried out under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 – Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – Next Generation EU. Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP J33C22001190001 (Department of Veterinary Medical Sciences, Alma Mater Studiorum, University of Bologna).

CRediT authorship contribution statement

Silvia Natale: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Andrea Bertini:** Methodology, Investigation, Writing – review & editing. **Enric Gisbert:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing. **Karl B. Andree:** Methodology, Investigation, Writing – review & editing. **Elisa Benini:** Methodology, Writing – review & editing. **Dario Vallainc:** Methodology, Writing – review & editing. **Pier Paolo Gatta:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing. **Alessio Bonaldo:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing. **Luca Parma:** Conceptualization, Methodology, Investigation, Supervision, Writing – review & editing.

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.

References

Azzout-Marniche, D., Chaumontet, C., Piedcoq, J., Khodorova, N., Fromentin, G., Tomé, D., Gaudichon, C., Even, P.C., 2019. High Pancreatic Amylase Expression Promotes Adiposity in Obesity-Prone Carbohydrate-Sensitive Rats. J. Nutr. 149, 270–279. https://doi.org/10.1093/jn/nxy262.

Bakke, S., Jordal, A.-E.O., Gómez-Requeni, P., Verri, T., Kousoulaki, K., Aksnes, A., Rønnestad, I., 2010. Dietary protein hydrolysates and free amino acids affect the spatial expression of peptide transporter PepT1 in the digestive tract of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 156, 48–55. https://doi.org/10.1016/j.cbpb.2010.02.002.

Bertini, A., Natale, S., Gisbert, E., Andrée, K.B., Concu, D., Dondi, F., De Cesare, A., Indio, V., Gatta, P.P., Bonaldo, A., Parma, L., 2023. Exploring the application of Corynebacterium glutamicum single cell protein in the diet of flathead grey mullet (*Mugil cephalus*): effects on growth performance, digestive enzymes activity and gut microbiota. Front. Mar. Sci. 10. https://doi.org/10.3389/fmars.2023.1172505.

Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J. Biol. Chem. 164, 321–329. https://doi.org/10.1016/S0021-9258(18)43072-4.

Bezerra, R.S., Lins, E.J.F., Alencar, R.B., Paiva, P.M.G., Chaves, M.E.C., Coelho, L.C.B.B., Carvalho, L.B., 2005. Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*). Process Biochem 40, 1829–1834. https://doi.org/10.1016/j.procbio.2004.06.066.

Bkhairia, I., Ktari, N., Younes, I., Kammoun, M., Nasri, M., Ghorbel, S., 2015. Golden Grey Mullet (*Liza aurata*) Alkaline Proteases: Biochemical Characterization, Application in the Shrimp Wastes Deproteinization, Laundry Commercial Detergents, and Preparation of Antioxidant Protein Hydrolysate. J. Aquat. Food Prod. Technol. 24, 597–613. https://doi.org/10.1080/10498850.2013.796582.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Buchet, R., Millán, J.L., Magne, D., 2013. Multisystemic functions of alkaline phosphatases. Methods Mol. Biol. Clifton NJ 1053, 27–51. https://doi.org/10.1007/978-1-62703-562-0_3.

Buddington, R.K., Chen, J.W., Diamond, J., 1987. Genetic and phenotypic adaptation of intestinal nutrient transport to diet in fish. J. Physiol. 393, 261–281. https://doi.org/10.1113/jphysiol.1987.sp016823.

Busti, S., Bonaldo, A., Diana, A., Perfetti, S., Viroli, C., Fontanillas, R., Eriksen, T.B., Gatta, P.P., Parma, L., 2022. The incidence of different pellet size on growth, gut evacuation, feed digestibility and feed waste in gilthead sea bream (*Sparus aurata*). Aquaculture 555, 738204. https://doi.org/10.1016/j.aquaculture.2022.738204.

Busti, S., Bonaldo, A., Dondi, F., Cavallini, D., Yúfera, M., Gilannejad, N., Moyano, F.J., Gatta, P.P., Parma, L., 2020. Effects of different feeding frequencies on growth, feed utilisation, digestive enzyme activities and plasma biochemistry of gilthead sea bream (*Sparus aurata*) fed with different fishmeal and fish oil dietary levels. Aquaculture 529, 735616. https://doi.org/10.1016/j.aquaculture.2020.735616.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time

PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.
Cahu, C.L., Infante, J.L.Z., 1994. Early weaning of sea bass (*Dicentrarchus labrax*) larvae with a compound diet: Effect on digestive enzymes. Comp. Biochem. Physiol. A Physiol. 109, 213–222. https://doi.org/10.1016/0300-9629(94)90123-6.
Cardona, L., 2006. Habitat selection by grey mullets (Osteichthyes: Mugilidae) in Mediterranean estuaries: the role of salinity. Sci. Mar. 70, 443–455. https://doi.org/10.3989/scimar.2006.70n3443.
Castro-Ruiz, D., Andree, K.B., Blondeau-Bidet, E., Fernández-Méndez, C., García-Dávila, C., Gisbert, E., Darias, M.J., 2021. Isolation, identification, and gene expression analysis of the main digestive enzymes during ontogeny of the Neotropical catfish *Pseudoplatystoma punctifer* (Castelnau, 1855). Aquaculture 543, 737031. https://doi.org/10.1016/j.aquaculture.2021.737031.
Chan, A.S., Horn, M.H., Dickson, K.A., Gawlicka, A., 2004. Digestive enzyme activities in carnivores and herbivores: comparisons among four closely related prickleback fishes (Teleostei: Stichaeidae) from a California rocky intertidal habitat. J. Fish. Biol. 65, 848–858. https://doi.org/10.1111/j.0022-1112.2004.00495.x.
Chen, S.-H., Cao, M.-J., Huang, J.-Z., Wu, G.-P., 2011. Identification of a puromycin-sensitive aminopeptidase from zebrafish (*Danio rerio*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 159, 10–17. https://doi.org/10.1016/j.cbpb.2011.01.005.
Chen, H., Pan, Y., Wong, E.A., Webb Jr, K.E., 2005. Dietary protein level and stage of development affect expression of an intestinal peptide transporter (cPepT1) in chickens. J. Nutr. 135, 193–198. https://doi.org/10.1093/jn/135.2.193.
Cockson, A., Bourn, D., 1973. Protease and Amylase in the digestive tract of Barbus paludinosus. Hydrobiologia 43, 357–363. https://doi.org/10.1007/BF00015356.
Crane, R.K., Boge, G., Rigal, A., 1979. Isolation of brush border membranes in vesicular form from the intestinal spiral valve of the small dogfish (*Scyliorhinus canicula*). Biochim. Biophys. Acta BBA - Biomembr. 554, 264–267. https://doi.org/10.1016/0005-2736(79)90024-5.
Crosetti, D., Blaber, S.J.M., 2016. Biology, ecology and culture of grey mullets (Mugilidae). CRC Press.
D'Souza, V.M., Buckley, D.J., Buckley, A.R., Pauletti, G.M., 2003. Extracellular Glucose Concentration Alters Functional Activity of the Intestinal Oligopeptide Transporter (PepT-1) in Caco-2 Cells. J. Pharm. Sci. 92, 594–603. https://doi.org/10.1002/jps.10325.
Dankwa, H., Jr, J., Yankson, K., 2009. Food and Feeding Habits of Grey Mullets (Pisces: Mugilidae) in Two Estuaries in Ghana. West Afr. J. Appl. Ecol. 8. https://doi.org/10.4314/wajae.v8i1.45786.
Day, R.D., German, D.P., Manjakasy, J.M., Farr, I., Hansen, M.J., Tibbetts, I.R., 2011. Enzymatic digestion in stomachless fishes: how a simple gut accommodates both herbivory and carnivory. J. Comp. Physiol. [B 181, 603–613. https://doi.org/10.1007/s00360-010-0546-y.
De, D., Ghoshal, Kundu, J., 2012. Effect of Feeding Different Levels of Protein on Growth Performance, Feed Utilization and Digestive Enzyme of Grey Mullet (*Mugil cephalus L*). Anim. Nutr. Feed Technol. 12, 179–186.
Decker, L.A., 1977. Worthington enzyme manual. Worthington Biochemical Corp.
El-Sharkawy, E.A., El-Razek, I.M.A., Amer, A.A., Soliman, A.A., Shukry, M., Gewaily, M. S., Téllez-Isaías, G., Kari, Z.A., Dawood, M.A.O., 2023. Effects of sodium butyrate on the growth performance, digestive enzyme activity, intestinal health, and immune responses of Thinlip Grey Mullet (*Liza ramada*) juveniles. Aquac. Rep. 30, 101530. https://doi.org/10.1016/j.aqrep.2023.101530.
Falcón-Hidalgo, B., Forrellat-Barrios, A., Farnés, O.C., Hernández, K.U., 2011. Digestive enzymes of two freshwater fishes (*Limia vittata* and *Gambusia punctata*) with different dietary preferences at three developmental stages. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 158, 136–141. https://doi.org/10.1016/j.cbpb.2010.10.009.
Fernandez, I., Moyano, F.J., Dıaz, M., Martınez, T., 2001. Characterization of a-amylase activity in five species of Mediterranean sparid fishes (Sparidae, Teleostei). https://doi.org/10.1016/S0022-0981(01)00228-3.
García-Carreño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*pacifAstacus astacus*) extracts. J. Food Biochem. 17, 97–113. https://doi.org/10.1111/j.1745-4514.1993.tb00864.x.
German, D.P., Horn, M.H., Gawlicka, A., 2004. Digestive Enzyme Activities in Herbivorous and Carnivorous Prickleback Fishes (Teleostei: Stichaeidae): Ontogenetic, Dietary, and Phylogenetic Effects. Physiol. Biochem. Zool. 77, 789–804. https://doi.org/10.1086/422228.
Gilannejad, N., Moyano, F.J., Martínez-Rodríguez, G., Yúfera, M., 2021. The digestive function of gilthead seabream juveniles in relation to feeding frequency. Aquaculture 531, 735867. https://doi.org/10.1016/j.aquaculture.2020.735867.
Gisbert, E., Nolasco, H., Solovyev, M., 2018. Towards the standardization of brush border purification and intestinal alkaline phosphatase quantification in fish with notes on other digestive enzymes. Aquaculture 487, 102–108. https://doi.org/10.1016/j.aquaculture.2018.01.004.
Glencross, B., Blyth, D., Irvin, S., Bourne, N., Campet, M., Boisot, P., Wade, N.M., 2016. An evaluation of the complete replacement of both fishmeal and fish oil in diets for juvenile Asian seabass, *Lates calcarifer*. Aquaculture 451, 298–309. https://doi.org/10.1016/j.aquaculture.2015.09.012.
Gonzales, T., Robert-Baudouy, J., 1996. Bacterial aminopeptidases: Properties and functions.
Guardiola, F.A., Saraiva-Fraga, M., Cuesta, A., Esteban, M.A., 2018. Changes in natural haemolytic complement activity induced by stress in gilthead seabream (*Sparus aurata L.*). Fish. Shellfish Immunol. 78, 317–321. https://doi.org/10.1016/j.fsi.2018.04.056.
Hickling, C.F., 1970. A Contribution to the Natural History of the English Grey Mullets [Pisces, Mugilidae. J. Mar. Biol. Assoc. U. K. 50, 609–633. https://doi.org/10.1017/S0025315400004914.
Hidalgo, M.C., Urea, E., Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture 170, 267–283. https://doi.org/10.1016/S0044-8486(98)00413-X.
Holm, H., Hanssen, L.E., Krogdahl, A., Florholmen, J., 1988. High and low inhibitor soybean meals affect human duodenal proteinase activity differently: In vivo comparison with bovine serum albumin. J. Nutr. 118, 515–520. https://doi.org/10.1093/jn/118.4.515.
Horie, Y., Sugase, K., Horie, K., 1995. Physiological differences of soluble and insoluble dietary fibre fractions of brown algae and mushrooms in pepsin activity in vitro and protein digestibility. Asia Pac. J. Clin. Nutr. 4, 251–255.
Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish. Physiol. Biochem. 18, 59–69. https://doi.org/10.1023/A:1007725513389.
Kim, D.-K., Kim, K.-D., Seo, J.-Y., Lee, S.-M., 2012. Effects of Dietary Lipid Source and Level on Growth Performance, Blood Parameters and Flesh Quality of Sub-adult Olive Flounder (*Paralichthys olivaceus*). Asian-Australas. J. Anim. Sci. 25, 869–879. https://doi.org/10.5713/ajas.2011.11470.
Klumpp, D., Nichols, P., 1983. Nutrition of the southern sea garfish *Hyporhamphus melanochir*: gut passage rate and daily consumption of two food types and assimilation of seagrass components. Mar. Ecol. Prog. Ser. 12, 207–216. https://doi.org/10.3354/meps012207.
Koven, W., Gisbert, E., Nixon, O., Solovyev, M.M., Gaon, A., Allon, G., Meiri-Ashkenazi, I., Tandler, A., Rosenfeld, H., 2019. The effect of algal turbidity on larval performance and the ontogeny of digestive enzymes in the grey mullet (*Mugil cephalus*). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 228, 71–80. https://doi.org/10.1016/j.cbpa.2018.11.005.
Lallès, J.-P., 2019. Biology, environmental and nutritional modulation of skin mucus alkaline phosphatase in fish: A review. Fish. Shellfish Immunol. 89, 179–186. https://doi.org/10.1016/j.fsi.2019.03.053.
Lallès, J.-P., 2020. Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation. Rev. Aquac. 12, 555–581. https://doi.org/10.1111/raq.12340.
Lazado, C.C., Pedersen, P.B., Nguyen, H.Q., Lund, I., 2017. Rhythmicity and plasticity of digestive physiology in a euryhaline teleost fish, permit (*Trachinotus falcatus*). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 212, 107–116. https://doi.org/10.1016/j.cbpa.2017.07.016.
Li, M., Liang, H., Xie, J., Chao, W., Zou, F., Ge, X., Ren, M., 2021. Diet supplemented with a novel Clostridium autoethanogenum protein have a positive effect on the growth performance, antioxidant status and immunity in juvenile Jian carp (*Cyprinus carpio var. Jian*). Aquac. Rep. 19, 100572. https://doi.org/10.1016/j.aqrep.2020.100572.
Liu, Z., Zhou, Y., Feng, J., Lu, S., Zhao, Q., Zhang, J., 2013. Characterization of oligopeptide transporter (PepT1) in grass carp (Ctenopharyngodon idella). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 164, 194–200. https://doi.org/10.1016/j.cbpb.2012.11.008.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San. Diego Calif. 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
Lobel, P.S., 1981. Trophic biology of herbivorous reef fishes: alimentary pH and digestive capabilities. J. Fish. Biol. 19, 365–397. https://doi.org/10.1111/j.1095-8649.1981.tb05842.x.
Loi, B., Papadakis, I.E., Leggieri, F., Giménez Papiol, G., Vallainc, D., 2020. Ontogeny of the digestive system and eye of reared flathead grey mullet, *Mugil cephalus* (Linnaeus, 1758), and evaluation of lipid deposition in the liver according to the feeding protocol. Aquaculture 526, 735386. https://doi.org/10.1016/j.aquaculture.2020.735386.
López-Olmeda, J.F., Noble, C., Sánchez-Vázquez, F.J., 2012. Does feeding time affect fish welfare. Fish. Physiol. Biochem. 38, 143–152. https://doi.org/10.1007/s10695-011-9523-y.
Luzzana, U., Valfrè, F., Mangiarotti, M., Domeneghini, C., Radaelli, G., Moretti, V., Scolari, M., 2005. Evaluation of different protein sources in fingerling grey mullet Mugil cephalus practical diets. Aquac. Int 13, 291–303. https://doi.org/10.1007/s10499-004-3099-9.
M. Yones, A.-M., K. I. El-Hammady, A., M. El-Kasheif, A., A El-Kasheif, M., 2019. Optimum contribution of dietary protein: energy ratio in the grey mullet (Mugil cephalus, linnaeus, 1758) diets. Egypt. J. Aquat. Biol. Fish. 23, 13–15. https://doi.org/10.21608/ejabf.2019.34025.
MacKinlay, D., Shearer, K., 1996. Feeding Ecology and Nutrition in Fish Symposium Proceedings.
Magouz, F.I., Dawood, M.A.O., Salem, M.F.I., El-Ghandour, M., Van Doan, H., Mohamed, A.A.I., 2020. The role of a digestive enhancer in improving the growth performance, digestive enzymes activity, and health condition of Nile tilapia (*Oreochromis niloticus*) reared under suboptimal temperature. Aquaculture 526, 735388. https://doi.org/10.1016/j.aquaculture.2020.735388.
Marais, J.F.K., 1980. Aspects of food intake, food selection, and alimentary canal morphology of Mugil cephalus (Linnaeus, 1958), Liza tricuspidens (Smith, 1935), L. Richardsoni (Smith, 1846), and L. Dumerili (Steindachner, 1869). J. Exp. Mar. Biol. Ecol. 44, 193–209. https://doi.org/10.1016/0022-0981(80)90152-5.
Marchi, A., Bonaldo, A., Di Biase, A., Cerri, R., Scicchitano, D., Nanetti, E., Candela, M., Picone, G., Capozzi, F., Dondi, F., Gatta, P.P., Parma, L., 2023. Towards a free wild-caught fishmeal, fish oil and soy protein in European sea bass diet using by-products from fishery and aquaculture. Aquaculture, 739571. https://doi.org/10.1016/j.aquaculture.2023.739571.
Maroux, S., Louvard, D., Barath, J., 1973. The aminopeptidase from hog intestinal brush border. Biochim. Biophys. Acta BBA - Enzymol. 321, 282–295. https://doi.org/10.1016/0005-2744(73)90083-1.

Masek, T., Vopalensky, V., Suchomelova, P., Pospisek, M., 2005. Denaturing RNA electrophoresis in TAE agarose gels. Anal. Biochem. 336, 46–50. https://doi.org/10.1016/j.ab.2004.09.010.

Masroor, W., Farcy, E., Blondeau-Bidet, E., Venn, A., Tambutté, E., Lorin-Nebel, C., 2019. Effect of salinity and temperature on the expression of genes involved in branchial ion transport processes in European sea bass. J. Therm. Biol. 85, 102422. https://doi.org/10.1016/j.jtherbio.2019.102422.

Matias, A.C., Ribeiro, L., Barata, M., Araújo, R.L., Pousão-Ferreira, P., 2023. Postprandial pattern of digestive enzymes and protein turnover in meagre (*Argyrosomus regius*) juveniles. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 265, 110828. https://doi.org/10.1016/j.cbpb.2023.110828.

Matsui, M., Fowler, J., Walling, L., 2007. Leucine aminopeptidases: Diversity in structure and function. Biol. Chem. 387, 1535–1544. https://doi.org/10.1515/BC.2006.191.

Messina, M., Bulfon, C., Beraldo, P., Tibaldi, E., Cardinaletti, G., 2019. Intestinal morpho-physiology and innate immune status of European sea bass (*Dicentrarchus labrax*) in response to diets including a blend of two marine microalgae, Tisochrysis lutea and Tetraselmis suecica. Aquaculture 500, 660–669. https://doi.org/10.1016/j.aquaculture.2018.09.054.

Métais, P., Bieth, J., 1968. Determination of alpha-amylase by a microtechnic. Ann. Biol. Clin. (Paris) 26, 133–142.

Ng, W.-K., Romano, N., 2013. A review of the nutrition and feeding management of farmed tilapia throughout the culture cycle. Rev. Aquac. 5, 220–254. https://doi.org/10.1111/raq.12014.

Nicholson, J.A., Kim, Y.S., 1975. A one-step l-amino acid oxidase assay for intestinal peptide hydrolase activity. Anal. Biochem. 63, 110–117. https://doi.org/10.1016/0003-2697(75)90194-3.

Nikolopoulou, D., Moutou, K.A., Fountoulaki, E., Venou, B., Adamidou, S., Alexis, M.N., 2011. Patterns of gastric evacuation, digesta characteristics and pH changes along the gastrointestinal tract of gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 158, 406–414. https://doi.org/10.1016/j.cbpa.2010.11.021.

Ogihara, H., Saito, H., Shin, B.-C., Terada, T., Takenoshita, S., Nagamachi, Y., Inui, K., Takata, K., 1996. Immuno-Localization of H+ /Peptide Cotransporter in Rat Digestive Tract. Biochem. Biophys. Res. Commun. 220, 848–852. https://doi.org/10.1006/bbrc.1996.0493.

Ostaszewska, T., Kamaszewski, M., Grochowski, P., Dabrowski, K., Verri, T., Aksakal, E., Szatkowska, I., Nowak, Z., Dobosz, S., 2010. The effect of peptide absorption on PepT1 gene expression and digestive system hormones in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 155, 107–114. https://doi.org/10.1016/j.cbpa.2009.10.017.

Parma, L., Pelusio, N.F., Gisbert, E., Esteban, M.A., D'Amico, F., Soverini, M., Candela, M., Dondi, F., Gatta, P.P., Bonaldo, A., 2020. Effects of rearing density on growth, digestive conditions, welfare indicators and gut bacterial community of gilthead sea bream (*Sparus aurata*, L. 1758) fed different fishmeal and fish oil dietary levels. Aquaculture 518, 734854. https://doi.org/10.1016/j.aquaculture.2019.734854.

Payne, A.I., 1978. Gut ph and digestive strategies in estuarine grey mullet (Mugilidae) and tilapia (Cichlidae). J. Fish. Biol. 13, 627–629. https://doi.org/10.1111/j.1095-8649.1978.tb03476.x.

Pedrosa, R.U., Mattos, B.O., Pereira, D.S.P., Rodrigues, M.L., Braga, L.G.T., Fortes-Silva, R., 2019. Effects of feeding strategies on growth, biochemical parameters and waste excretion of juvenile arapaima (*Arapaima gigas*) raised in recirculating aquaculture systems (RAS). Aquaculture 500, 562–568. https://doi.org/10.1016/j.aquaculture.2018.10.058.

Pelusio, N.F., Parma, L., Volpe, E., Ciulli, S., Errani, F., Natale, S., De Cesare, A., Indio, V., Carcano, P., Mordenti, O., Gatta, P.P., Bonaldo, A., 2023. Yeast-extracted nucleotides and nucleic acids as promising feed additives for European sea bass (Dicentrarchus labrax) juveniles. Front. Mar. Sci. 10. https://doi.org/10.3389/fmars.2023.1145660.

Pérez-Jiménez, A., Cardenete, G., Morales, A.E., García-Alcázar, A., Abellán, E., Hidalgo, M.C., 2009. Digestive enzymatic profile of *Dentex dentex* and response to different dietary formulations. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 154, 157–164. https://doi.org/10.1016/j.cbpa.2009.05.126.

Perez-Velazquez, M., Gatlin, D.M., González-Félix, M.L., García-Ortega, A., de Cruz, C.R., Juárez-Gómez, M.L., Chen, K., 2019. Effect of fishmeal and fish oil replacement by algal meals on biological performance and fatty acid profile of hybrid striped bass (*Morone crhysops* ♀ × *M. saxatilis* ♂). Aquaculture 507, 83–90. https://doi.org/10.1016/j.aquaculture.2019.04.011.

Pujante, I.M., Díaz-López, M., Mancera, J.M., Moyano, F.J., 2017. Characterization of digestive enzymes protease and alpha-amylase activities in the thick-lipped grey mullet (*Chelon labrosus*, Risso 1827). Aquac. Res. 48, 367–376. https://doi.org/10.1111/are.13038.

Pujante, I.M., Martos-Sitcha, J.A., Moyano, F.J., Ruiz-Jarabo, I., Martínez-Rodríguez, G., Mancera, J.M., 2015. Starving/re-feeding processes induce metabolic modifications in thick-lipped grey mullet (*Chelon labrosus*, Risso 1827). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 180, 57–67. https://doi.org/10.1016/j.cbpb.2014.10.005.

Quirós-Pozo, R., Ventura-Castellano, A., Ramírez-Bolaños, S., Roo-Filgueira, J., Robaina, L., 2021. Evaluation of Aloe vera by-product against cereals in feeds for golden mullet (*Liza aurata*). Aquac. Rep. 20, 100659. https://doi.org/10.1016/j.aqrep.2021.100659.

Raffic, S., Kondusamy, A., Praveena, E., Sambasivam, N., Balachandran, S., K, R., 2020. Effect of dietary prebiotic fructooligosaccharide on histology, digestive enzyme activity, biochemical and immunological parameters of Asian seabass (*Lates calcarifer*) juveniles. Indian J. Fish. https://doi.org/10.21077/ijf.2020.67.4.96657-10.

Ramos-Júdez, S., Duncan, N., 2022. Feeding habits and the influence of pellet diameter on the feeding responses of the flathead grey mullet (*Mugil cephalus*) in captivity. Anim. Feed Sci. Technol. 290, 115368. https://doi.org/10.1016/j.anifeedsci.2022.115368.

Rosas, V.T., Monserrat, J.M., Bessonart, M., Magnone, L., Romano, L.A., Tesser, M.B., 2019. Fish oil and meal replacement in mullet (*Mugil liza*) diet with Spirulina (*Arthrospira platensis*) and linseed oil. Comp. Biochem. Physiol. Toxicol. Pharmacol. CBP 218, 46–54. https://doi.org/10.1016/j.cbpc.2018.12.009.

Skalli, A., Zambonino-Infante, J.-L., Kotzamanis, Y., Fabregat, R., Gisbert, E., 2014. Peptide molecular weight distribution of soluble protein fraction affects growth performance and quality in European sea bass (icentrarchus labrax) larvae. Aquac. Nutr. 20, 118–131. https://doi.org/10.1111/anu.12058.

Solovyev, M., Gisbert, E., 2016. Influence of time, storage temperature and freeze/thaw cycles on the activity of digestive enzymes from gilthead sea bream (*Sparus aurata*). Fish. Physiol. Biochem. 42, 1383–1394. https://doi.org/10.1007/s10695-016-0226-2.

Solovyev, M., Gisbert, E., 2022. Feeding regimes affected the circadian rhythms of pancreatic digestive enzymes and somatic growth in flathead grey mullet (*Mugil cephalus*) fry. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 264, 111116. https://doi.org/10.1016/j.cbpa.2021.111116.

Solovyev, M., Kashinskaya, E., Gisbert, E., 2023. A meta-analysis for assessing the contributions of trypsin and chymotrypsin as the two major endoproteases in protein hydrolysis in fish intestine. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 278, 111372. https://doi.org/10.1016/j.cbpa.2023.111372.

Solovyev, M.M., Kashinskaya, E.N., Izvekova, G.I., Gisbert, E., Glupov, V.V., 2014. Feeding habits and ontogenic changes in digestive enzyme patterns in five freshwater teleosts. J. Fish. Biol. 85, 1395–1412. https://doi.org/10.1111/jfb.12489.

Svihus, B., 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. Worlds Poult. Sci. J. 67, 207–224. https://doi.org/10.1017/S0043933911000249.

Tacon, et al., 2011. pdf, n.d. Demand Supply Feed Ingred. Farm. F.

Talukdar, A., Deo, A.D., Sahu, N.P., Sardar, P., Aklakur, M., Prakash, S., Shamna, N., Kumar, S., 2020b. Effects of dietary protein on growth performance, nutrient utilization, digestive enzymes and physiological status of grey mullet, *Mugil cephalus* L. fingerlings reared in inland saline water. Aquac. Nutr. 26, 921–935. https://doi.org/10.1111/anu.13050.

Taylor, A., 1993. Aminopeptidases: structure and function. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 7, 290–298. https://doi.org/10.1096/fasebj.7.2.8440407.

Terova, G., Robaina, L., Izquierdo, M., Cattaneo, A., Molinari, S., Bernardini, G., Saroglia, M., 2013. PepT1 mRNA expression levels in sea bream (*Sparus aurata*) fed different plant protein sources. SpringerPlus 2, 17. https://doi.org/10.1186/2193-1801-2-17.

Tian, H.-Y., Zhang, D.-D., Li, X.-F., Zhang, C.-N., Qian, Y., Liu, W.-B., 2015. Optimum feeding frequency of juvenile blunt snout bream *Megalobrama amblycephala*. Aquaculture 437, 60–66. https://doi.org/10.1016/j.aquaculture.2014.11.032.

Uscanga, A., Moyano, F.J., Alvarez, C.A., 2010. Assessment of enzymatic efficiency on protein digestion in the tilapia *Oreochromis niloticus*. Fish. Physiol. Biochem. 36, 1079–1085. https://doi.org/10.1007/s10695-010-9385-8.

Vallainc, D., Concu, D., Papiol, G.G., Loi, B., Leggieri, F., Brundu, G., Chindris, A., Sanna, G., Fois, N., Antognarelli, F., Rossini, P., 2021. Producing flat-head grey mullet *Mugil cephalus* (Linnaeus, 1758) fries in captivity from sexually mature adults collected in Sardinian lagoons. Aquac. Rep. 21, 100844. https://doi.org/10.1016/j.aqrep.2021.100844.

Verri, T., Terova, G., Dabrowski, K., Saroglia, M., 2011. Peptide transport and animal growth: the fish paradigm. Biol. Lett. 7, 597–600. https://doi.org/10.1098/rsbl.2010.1164.

Wei, Y., Liang, M., Mu, Y., Zheng, K., Xu, H., 2016. The effect of ultrafiltered fish protein hydrolysate level on growth performance, protein digestibility and mRNA expression of PepT1 in juvenile turbot (Scophthalmus maximus L. Aquac. Nutr. 22, 1006–1017. https://doi.org/10.1111/anu.12319.

Willora, F.P., Vatsos, I.N., Mallioris, P., Bordignon, F., Keizer, S., Martınez-Llorens, S., Sørensen, M., Hagen, Ø., 2022. Replacement of fishmeal with plant protein in the diets of juvenile lumpfish (*Cyclopterus lumpus*, L. 1758): Effects on digestive enzymes and microscopic structure of the digestive tract. Aquaculture 561, 738601. https://doi.org/10.1016/j.aquaculture.2022.738601.

Wu, G.-P., Cao, M.-J., Chen, Y., Liu, B.-X., Su, W.-J., 2008. Leucine Aminopeptidase from Red Sea Bream (*Pagrus major*) Skeletal Muscle: Purification, Characterization, Cellular Location, and Tissue Distribution. J. Agric. Food Chem. 56, 9653–9660. https://doi.org/10.1021/jf801477r.

Xie, D., Xu, S., Wu, Q., Chen, F., Wang, S., You, C., Li, Y., 2018. Changes of visceral properties and digestive enzymes in the herbivorous marine teleost *Siganus canaliculatus* fed on different diets. Acta Oceanol. Sin. 37, 85–93. https://doi.org/10.1007/s13131-018-1165-9.

Yang, Y., Zhang, P., Huang, Z., Zhao, Z., 2023. Phenolics from Sterculia nobilis Smith pericarp by-products delay carbohydrate digestion by uncompetitively inhibiting α-glucosidase and α-amylase. LWT 173, 114339. https://doi.org/10.1016/j.lwt.2022.114339.