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Differential modulation of digestive enzymes and energy reserves at different times after feeding in juveniles of the marine estuarine-dependent flatfish *Paralichthys orbignyanus* (Valenciennes, 1839)

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Abstract

Integrative works on the responses of digestive enzymes and energy reserves in conjunction with morphological traits at distinct postprandial times in marine estuarine-dependent flatfishes of ecological and economic importance, such as *Paralichthys orbignyanus* are lacking. We determined total weight (TW), hepatosomatic index (HSI), digestive enzymes activities in the intestine and energy reserves concentration in liver and muscle at 0, 24, 72 and 360h after feeding in juveniles of *P. orbignyanus*. Amylase activity decreased at 72h (about 30%). Maltase, sucrase and lipase activities

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peak increased at 24h (67, 600 and 35%). Trypsin and APN activities at 24 and 72 h, respectively were lower than t = 0 (53 and 30%). A peak increase of glycogen and triglycerides concentration in liver (24h) (86 and 89 %) occurred. In muscle, glycogen and triglycerides concentrations were unchanged at 24h and higher at72 and 360h (100 and 60%). No changes in TW, HSI, free glucose in liver and muscle, and protein in liver were found. The protein concentration in muscle peak increased at 24 and 360 h after feeding (60%). The results indicate a distinct and specific response of central components of carbohydrate, lipid and protein metabolism that could constitute adjustments at the biochemical level upon periods of irregular feeding and even of long-term food privation inside coastal lagoon or estuaries. The distinct responses of digestive enzyme in intestine, and energy reserves in liver and muscle suggest the differential modulation of tissue-specific anabolic and catabolic pathways that would allow the maintenance of physical condition.

Keywords: Digestive tract, energy storage, estuarine-dependent flatfish- Paralichthys orbignyanus, post-feeding

Abbreviations

MCh Mar Chiquita Coastal Lagoon

TL Total length

ST Standard length

TM Total mass

HSI Hepatosomatic index

AMS Amylase

MAL Maltase

SUC Sucrase

LIP Lipase

TRY Trypsin

APN N-Aminopeptidase

GLY Glycogen

GLU Free glucose

TAG Triglycerides

PROT Protein

Introduction

Fish require different sources of energy to maintain physical condition and fundamental processes, such as growth, metabolism and reproduction (Karasov and Douglas 2013; Steimberg 2018). However and in spite, that is largely known that the intestine is the main site of digestion and absorption of nutrients in fish , understanding about biochemical specifics of digestion is still sparse for most species including marine estuarine-dependent flatfishes (Small *et al.,*2022). Moreover, integrative works on postprandial temporal dynamics of key digestive enzymes and energy reserves along with morphological traits in marine estuarine-dependent flatfishes inhabiting coastal lagoons are lacking.

The degree to which digestive and metabolic characteristics permit or limit changes in feeding conditions is a crucial topic in studies of digestive and nutrition physiology (Karasov *et al.,*2011; Volkoff and Ronnestad, 2020; Nespolo *et al.,*2022). In this context, digestive enzymes in the gastrointestinal tract have a main physiological role as being a link between ingestion, absorption and assimilation of nutrients (Karasov and del Rio, 2007; Karasov *et al*., 2011; Karasov and Douglas,2013). The occurrence of specific digestive enzymes (carbohydrases, lipases and endo and ectoproteases) is usually linked to the nature of the dietary substrates potentially utilized in metabolic processes (Karasov & Douglas, 2013; Sanz *et al.,* 2015; Steimberg, 2018). Therefore, changes in the activity of specific digestive enzymes could lead to modifications in the capacity for digestion of the corresponding dietary substrate (carbohydrates, lipids or protein) and, in turn, to modified availability of resulting metabolites (Karasov and Douglas 2013; Steimberg, 2018).

Due to environmental factors or behavioral patterns that limit access to food, various fishes are exposed to differential times of feeding restriction (Secor and Carey 2016; Ersminger *et al.,* 2021). In many animals, upon temporal shifts in feeding conditions, the modulation of the activity of digestive enzymes and of the concentration of energy reserves is a key response leading to digestive and metabolic adjustments (Karasov *et al.,*2011; Lálles, 2020; Albanesi *et al.,*2022). The determination of the activity of digestive enzymes at distinct times after feeding permits to establish the postprandial temporal dynamics of central component of carbohydrates, proteins and lipids metabolism and also to evaluate the potential effects of variable periods of food restriction on digestive parameters (Caruso *et al.,*2014; Pujante *et al.,*2018). The concomitant determination of the concentration of energy reserves in storage sites and morphological traits permits a further integrative understanding of the possible adjustments in digestive/metabolic parameters and body condition (Caruso *et al.,*2014; Pujante *et al.,*2018; Albanesi *et al.,* 2021b; 2022). Although, food restriction can generally have negative effects on fish metabolism and physiology (Shan et al. 2008; Choi et al. 2012; Yang et al. 2019), in various species it results beneficial in response to multiple stresses (Lu *et al.,*2019; Ensminger *et al.,*2021). To know the temporal dynamics and adjustments of digestive enzymes and energy reserves at different times post-feeding is also important for the case of fish under aquaculture since it can represent an important tool to minimize costs of maintenance. Flatfish of the family Paralichthyidae have an important trophic role and constitute a remarkable economical resource along the Southwest Atlantic (Díaz de Astarloa *et al.,*2002;Walsh *et al.,*2014; Ruiz-Jarabo *et al.,*2015). *Paralichthys orbignyanus* (Valenciennes, 1839) is an estuarine-dependent marine species, distributed from Rio de Janeiro (Brazil) to the Gulf of São Matías (Argentina) with great potential for aquaculture (Sampaio *et al.,*2008; Radonic and Macchi, 2009; Bolasina, 2011). *Paralichthys orbignyanus* utilizes estuaries or coastal lagoons for nursery, feeding, and shelter, but reproduces offshore (Cousseau *et al.,*2001; Alarco *et al.,*2010). Mar Chiquita Coastal Lagoon (MCh) (Buenos Aires Province, Argentina; 37°32′–37°45′ S 57°19'–57°26′ W) is a World Biosphere Reserve (UNESCO). Inside MCh, juveniles of *P. orbignyanus* can bear variable intervals

of food availability and feeding limitation. Like other fishes that feed on mobile, difficult to catch or irregularly available prey, juveniles of *P. orbignyanus* can feed intermittently with long episodes between meals (Day *et al.,* 2014). This constitutes a challenge, with potential effects for the survival of the species resource. Therefore, the objective of this study was to determine at distinct post-feeding times: a) the total weight and hepatosomatic index as parameters of physical conditions; b) amylase, maltase, sucrase, lipase, trypsin and APN activities in the intestine and c) glycogen, free glucose, triglycerides and protein concentration in storage organs in juveniles of *P. orbignyanus*. We hypothesized that juveniles of estuarine-dependent flatfish exhibit differential adjustments at distinct post-feeding times in digestive and metabolic characteristics at the biochemical level to face irregular intervals of food restriction and to maintain body condition with the following predictions; (a) juveniles of *P. orbignyanus* should maintain the total weight and hepatosomatic index over different times after feeding (b) juveniles of *P. orbignyanus* should exhibit a differential and specific modulation of amylase, membrane-bound disaccharidases, lipase, trypsin and APN in the intestine at different times after feeding (c) juveniles of *P. orbignyanus* should exhibit a temporal increase in the concentration or glycogen and triglycerides in key storage organs at short-term after feeding followed by a further mobilization of reserves

Materials and Methods

Fish stock, holding conditions and husbandry routine

Juveniles (mean length: 8.70 ± 0.50 cm, mean body weight: 9.37± 0.18g) (INIDEP, Mar del Plata, Argentina) were maintained for 20 days, in six fiberglass circular tanks (100 L, 28 psu, pH 8.1, 22 ± 2°C, 12 h light/12 h dark) (Albanesi et al 2021b). Flounders were fed *ad libitum* 6 days a week with a balanced diet (Núcleo Alimentos MDQ SRL 41.8% carbohydrates, 46% proteins, 2.8% of lipids). Everyday, excess food and feces were withdrawn. Digestive, metabolic and morphological parameters were determined at once (time 0, t0) and at 24, 72, 360 h post-feeding. Individuals were placed on ice until loss of consciousness (10 min) and after wards processed (del Valle *et al.,* 2016;

Albanesi *et al.,*2021a, b). Total length (TL), standard length (SL) and total weight (TW) of each flatfish were registered (Albanesi *et al.,*2021b). At once, for each fish intestine, liver and muscle were immediately removed. The small intestine was externally washed with homogenation buffer. All the tissues were weighed and placed on ice. Hepatosomatic index (HSI %) was determined as: (liver weight/total weight) x 100. The intestine and liver (4 ml of tissue g^{-1}) and muscle (8 ml of tissue g^{-1}) were mixed with homogenizing medium (50 mM Tris-HCl, pH 7.4) and homogenized on ice in a motor-driven hand-operated homogenizer (CAT homogenizer, T10 tool)(Albanesi *et al.,*2021b).
This study was approved (RD 2022–105, FCEyN) and conducted following the regulations and statements of Ethics Committee CICUAL (OCA 1499/12; FCEyN, UNMdP, Argentina).

Biochemical assays

The α-amylase activity was determined colorimetrically by measuring the amount of maltose released (Biesiot and Capuzzo, 1990) with modifications (Albanesi *et al.,*2021b). The reaction started with the addition of the corresponding sample to a mixture of the reaction containing the substrate starch (15 mg mL^{-1}) in 50 mM phosphate buffer pH 7.4. at 30°C. After 15 minutes of incubation, the reaction was stopped by adding 300ul of the dinitrosalicylic acid reagent (DNS) (Miller, 1959) and placed at 100 C during 10 min. After cooling on ice followed by 5 min at room temperature, the released maltose was determined reading absorbance at 540 nm.

Maltase and sucrase activities were assayed by a colorimetric method to determine the glucose released from maltose and sucrose, respectively (Albanesi et al.,2021 b). The reaction was initiated by adding the sample to a reaction mixture containing the corresponding substrate (42 mM) in 0.1 M malate buffer; pH 6.4 and incubated during 10 min at 30 °C. The reaction was stopped by addition of 1.5 mL of a glycemia kit (glucose oxidase 3,000U/L, peroxidase 400U/L, 4–1.225mM aminophenazone in 0.92M Tris, 2.75mM phenol, pH 7.4) (Glycemia kit Wiener Lab Glycemia AA) After 5 min at 37 °C., the amount of released glucose was determined by reading the absorbance at 505 nm of the colored quinone complex.

Lipase activity was determined measuring p-nitrophenol (pNP) liberated from the sustrate p-nitrophenyl-palmitate (pNPP) (Albanesi *et al.,*2021 b). The reaction was initiated by the addition of pNPP (final concentration 0.85 mM) to a reaction mixture containing the corresponding sample in 50 mM Tris-HCl buffer pH 8.5 at 37 °C. After incubation during 5 min, the assay was arrested with 0.5 ml of TCA 0.1% (w / v). p-nitrophenol was quantified by absorbance at 410 nm.

Trypsin activity was determined using N-α-benzoyl-DL-arginine-4-nitroanilide (BAPNA)(1.23mM) as substrate (Albanesi *et al.,*2021b). The reaction was initiated by the addition of BAPNA (final concentration 1.23 mM) to a reaction mixture containing the sample in 50 mM Tris -HCl buffer; pH 9.0, 400 mM Cl_2Ca at 45°C. After 15 min, the assay was arrested with 250 µl of 0.1 M KOH and absorbance determined at 405 nm.

APN activity was determined by using L-alanine-p-nitroanilide (L-Ala) (Albanesi *et al.,*2021b). The reaction was initiated by the addition of L- Ala (final concentration 0.33 mM) to a reaction mixture containing the sample in 50mM Tris-HCl buffer; pH 7.4 at 45°C. After 15 min, the reaction was arrested with 0.2 ml of cold 2 M acetic acid and absorbance was measured at 384 nm.

Glycogen in the liver and in the muscle was determined by hydrolysis of α-amyloglucosidase (Sigma Chemicals) (Albanesi et al. 2021b). Samples were boiled for 4 minutes and incubated in acetate buffer pH 4.8 in the presence and absence of 0.2 mg ml -1 of α-amyloglucosidase during 2.5 h at 55 °C. After incubation and centrifugation at 3000 rpm for 15 minutes, glucose was quantified in the supernatant by using a commercial kit for enzyme glycemia (Wiener Lab AA) as described above for maltase and sucrase activities. Glucose resulting from glycogen hydrolysis was quantified as the difference between the values with and without α—amyloglucosidase.

Triglycerides were measured by the colorimetric method of glycerol phosphate oxidase using commercial kit (TAG Wiener-Lab AA). After incubating the sample during 5 min at 37 °C, the released glycerol was quantified by absorbance at 505 nm of the formed quinone complex (Albanesi *et al.,*2021b).

Protein concentration was measured according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Statistical analysis was performed utilizing the Sigma 3.0 program for Windows. Previous test of equality of variances and normality is automatically done by this program. One-way ANOVA was utilized to establish the statistical significance of the differences. ANOVA (Student-Newman-Keuls) was used to identify differences and $p<0.05$ was taken as significant. (Albanesi *et al.,* 2021b, 2022).

3 Results

Morphological traits of juveniles of *Paralichthys orbignyanus* at distinct times post-feeding

No differences were found in the total weight and hepatosomatic index of juveniles at different times post-feeding (Table 1).

Digestive enzyme activities of juveniles of *Paralichthys orbignyanus* at distinct times post-feeding

Amylase activity in the intestine did not change at 24h post-feeding. The activity diminished at 72h (around 30%) and it maintained constant until 360h. (Figure 1a). Maltase and sucrase activities were higher at 24h after feeding compared to the activity at t=0 (about 67% and 600% respectively). At 72-360h after feeding, maltase and sucrase activities decreased to similar values than t=0 (Figure 1b-c).

Lipase activity increased at 24 h (about 35%), whereas at 72 - 360 h it was similar to t=0 (Figure 2a). At 24h post-feeding, trypsin activity was lower (about 53%). No changes occurred at longer times after feeding (t=72 -360 h) (Figure 2b). APN activity was unchanged at 24 h, it was lower (about 30%) at 72h than t = 0 and remained constant until 360 h (Figure 2c).

Energy reserves of juveniles of *Paralichthys orbignyanus* at distinct times post-feeding

In liver, glycogen concentration was higher at 24h post-feeding. At 72-360h it diminished until being equal to t=0 (Figure 3a). Free glucose concentration remained unvariable at any time after feeding (Figure 3b). In muscle, no changes occurred in glycogen concentration at 24 h. At 72h glycogen concentration was higher (about 100%) and maintained constant until 360 h (Figure 3c). Free glucose concentration did not vary at any time after feeding (Figure 3d).

In liver, triglyceride concentration increased at 24 h (around 83%). At 72 -360 h it was similar to t = 0 (Figure 4a). Protein concentration was similar to t=0 at any time of experimental period (Figure 4b). In muscle, triglycerides concentration did not vary at 24-72 h post-feeding. At 360 h it increased (about 60%) with respect to t = 0 (Figure 4c). Protein concentration was higher at 24 h after feeding (around 60%), decreased at 72 to similar values than t=0 and further increased (%) at 360 h (Figure 4d).

Discussion

Juveniles of the flounder *P. orbignyanus* constitute an adequate model to study the temporal postprandial dynamics at the biochemical level of central digestive and metabolic parameters of the carbohydrates, protein and lipid metabolism in an estuarine-dependent flatfish. Inside estuaries or coastal lagoons, such as MCH, juveniles of *P. orbignyanus* can face irregular periods of food availability and food restriction due to the characteristics of the prey (mobile, difficult to catch with irregular availability) (Day *et al.,*2014). The intestine exhibits a battery of specific digestive enzymes to carry out a complete breakdown of main dietary items (del Valle *et al.,*2016; Albanesi *et al.,*2021b). Furthermore, *P. orbignyanus* has great potential for aquaculture due to its broad tolerance to key environmental factors and flesh quality (Sampaio *et al.,*2008, Radonic and Macchi, 2009, Magnone *et al.,*2015, Radonic *et al.,*2017, 2018). To the best of our knowledge no studies occur about the determination of digestive enzymes activities and energy reserves in estuarine dependent flatfishes at different times after feeding.

In fish, glucose homeostasis is crucial to sustain central functions and responses to different stresses (Polakof *et al.,* 2012; Gominho-Rosa *et al.,*2015; Steimberg, 2018; Small, 2022). Carbohydrases such as amylase and membrane- bound disaccharidases are crucial for the initial and last stages, respectively, of main glycogenic dietary substrates and then, in the provision of circulating glucose (Grosell *et al.,*2011; Ballantyne, 2014; Steimberg, 2018; Small, 2022). The modulation of the activity of specific digestive enzymes in the intestine can change the ability for the digestion of the corresponding dietary item. This is commonly used to establish which nutrients are implicated in adjustments to intrinsic and extrinsic variables (Karasov and Douglas, 2013; Pujante *et al.,*2016; Karasov and Caviedes-Vidal, 2020). The lower amylase activity at 72 h post-ingestion and the maintenance at similar levels up to 360 h, suggests a decline in the amylolytic ability and, as a result, for first stages of glycogenic carbohydrate digestion of juveniles of *P. orbignyanus*. A post-feeding decrease of amylase activity, occurs in various fishes such as pre-juveniles of the mullet *Mugil liza* (Valenciennes, 1836) from MCh (Albanesi *et al.,*2022), juveniles of fresh- and brackish-wate*r Rutilus rutilus* (Linnaeus, 1758) (Abolfathi *et al.,*2012), and adults of the marine Red porgy *Pagrus pagrus* (Linnaeus, 1758) (Caruso *et al.,*2014) and the fresh-water Siamese fighting fish *Bette splendens* (Regan, 1910)(Hahor *et al.,* 2023) although, with distinct timing and extent of decrease. In this context, it has been pointed out that the determination of digestive enzymes of fishes requires the use of a species-specific protocol (Solovyev et al. 2023). This can also applied to the study of postprandial dynamics and the effect of irregular periods of feed restriction on the activity of digestive enzymes which appeared to be qualitative and quantitative different depending habitat and dietary habit (Abolfathi et al., 2012; Caruso et al., 2014; Imsland et al., 2020 ; Albanesi et al., 2022).

Little is known about biochemical characteristics and postprandial temporal dynamics of membrane-bound disaccharidases in intestine of fishes (Small, 2022; Albanesi *et al.,*2022). In animals, sucrase activity in intestine corresponds to the sucrase–isomaltase complex while maltase activity can correspond to this complex and or to be accounted by sucrase-independent maltase–glucoamylase complex (Burke, 2019; Karasov and Caviedes Vidal, 2020). The similar qualitative postprandial

temporal modulation of maltase and sucrase activities in intestine of juvenile of *P. orbignyanus* point out that both activities could correspond to a unique complex (i.e sucrase-isomaltase complex). However, due to the marked difference in the extent of responses between both activities at 24 h after-ingestion, we cannot discard that maltase activity of juvenile of *P. orbignyanus* could also correlate with the sucrase-independent maltase–glucoamylase complex. However, further biochemical characterization is required to prove this idea. In the intestine of the marine estuarine-dependent mullet *M. liza* sucrase and maltase activities correspond to the sucrase-isomaltase and sucrase-independent maltase–glucoamylase complex, respectively (Albanesi *et al.,*2021a).The peaked increase in maltase and sucrase activities in the intestine of juveniles of *P. orbignyanus* at 24 h post-feeding suggests a temporary modulation of these activities which could result in an increase in ability for last stages of dietary carbohydrates hydrolysis. This in turn, could result in a major provision of available glucose. In several fish species, including juvenile of *P. orbignyanus*, the liver is central in the maintenance of glucose homeostasis (Tseng and Hwang, 2008; Polakof et al.,2012; Chen et al.,2018; Albanesi *et al.,*2021a, b). The increase in glycogen concentration in the liver at 24 h post-ingestion in juvenile of *P. orbignyanus* point out this organ as a site of postprandial glycogen reserve building. The increased activity of digestive enzymes can result in an enhanced accessibility of specific metabolites for construction and keeping of energy reserves in specific organs (Debnath *et al.,*2007; Talukdar et al.,2020). The concomitant enhancement of liver glycogen concentration and in maltase and sucrase activity in the intestine in juvenile of *P. orbignyanus* suggests coordinated postprandial adjustments in key components of carbohydrate metabolism. In fishes, the liver is a main source of metabolites to be used by other tissues such as muscle (Roques *et al.,*2020). The flux of glucose from the liver can be modified by modulation of synthesis and degradation routes pathways (Polakof *et al.,*2012; Nebo *et al.,*2018; Roques *et al.,*2020). In the liver, in juveniles of *P. orbignyanus*, the further decrease in glycogen at 72 h post-ingestion point out its regulation (eg. enhanced catabolism and utilization). This could represent a biochemical adjustment to provide glucose as substrate for other organs. Glucose requirements are mainly covered by mobilization of

glycogen stores and or by de novo synthesis of glucose by gluconeogenic pathways in the liver (Polakof *et al.,*2012). In various fishes, enzymes participating in glycogen building and gluconeogenic routes are regulated at distinct levels (expression, amount or activity) after feeding (Conde-Sieira and Soegas, 2017; Yang *et al.,*2019). In hepatocytes of the marine Japanese flounder (*Paralichthys olivaceus;* Temminck and Schlegel, 1846), the serine-threonine kinase (protein kinase B) AKT2 is involved in the glucose metabolism pathways (Gu *et al.,*2022). However, nothing is known to our knowledge in estuarine-dependent flatfishes. The increased glycogen in muscle of juveniles of *P. orbignyanus* along with the lowering in liver at 72 h post-ingestion, supports that also in estuarine-dependent flatfish, the liver would be a fount of glucose for energy reserve building in muscle. In detritivore estuarine-dependent fish, such as *M. liza*, a similar modulation of liver and muscle glycogen concentration was observed in shorter times of feeding restriction (Albanesi *et al.,*2022). The maintenance of free glucose levels in muscle at different times after feeding without decrease in glycogen concentration suggests the use of circulating glucose for the construction of this glycogen reserve in juvenile of *P. orbignyanus*. In various fishes, skeletal muscle is a site of glucose entrance through a specific membrane-transporter which is under fine control (Marin-Juez *et al.,*2013; Yang *et al.,*2021).

In fishes, lipids constitute a major source of energy for supporting key physiological processes, swimming and migration (Sandre *et al.,* 2017; Small, 2022). The peaked increase at 24 h in lipase activity in intestine of *P. orbignyanus* along with that in maltase and sucrase activities point out also a temporal enhancement in digestive capacity for dietary lipids which would conduct to a higher availability of resulting metabolites. The increase in triglyceride concentration 24 h post-ingestion indicates that liver is also a site of postprandial synthesis and storage of this energy reserve. The further decrease at 72 and 360 h after feeding in triglycerides concentration in the liver suggests a mobilization of this reserve. In fishes, triglyceride anabolic route is assumed to be mostly equal to mammals (Tocher, 2003; Karasov and Douglas, 2013; Steimberg 2018; Small, 2022). Excess fatty acids can be transported from the liver via lipoproteins, accumulated, and stored as triglycerides in

reserve organs such as muscle (Grosell *et al.,* 2011; Ballantyne, 2014). The enhanced triglycerides concentration in muscle at 360 h post-ingestion concomitant with its decrease in liver, supports the idea that liver is a source of metabolites for lipogenesis in muscle of juveniles of *P. orbignyanus*. In juveniles of the marine fish red seabream *Pagrus major* (Temminck & Schlegel, 1843) lipid content increases in muscle after food deprivation for 5 days which is linked to a reduction in lipid amount in liver. Moreover, this variation in muscle lipid quantity is related to an increase in the expression of key enzymes of lipogenesis (Kaneko *et al.,*2016).

Adequate levels of dietary proteins are required to carry out normal growth and ensure fish homeostasis (Rahman *et al.,*2017; Small, 2022). Trypsin, an endoprotease, is the most important protease in the digestive tract of many fish playing a main role in the initial steps of dietary protein digestion (Steimberg, 2018; Small, 2022; Solovyev *et al.,* 2023). Aminopeptidase-N, a multifunctional membrane-bound ectoprotease, plays an essential role in the final steps of protein digestion (Fairweather *et al.* 2012; Steimberg, 2018; Small 2022). Therefore, the levels and modulation of the activity of trypsin and APN in the intestine is commonly used as indicator of the proteolytic digestive capacity and adjustments in this ability (Sissener *et al*., 2011; Ramirez-Otarola *et al*., 2011, 2018; Steimberg 2018; Small 2022). The lower trypsin activity in intestine of juveniles of *P. orbignyanus* at 24 h after feeding could lead to a decreased ability for initial protein digestion. However, since APN activity was unchanged, last steps of protein digestion and likely absorption of resulting amino acids would not be affected at this post-feeding time. In the intestine of mammals, APN can bind to specific transporters which influence the entrance of amino acids (Fairweather *et al.,*2012;). The digestive tract of some fishes exhibit high-capacity peptide transporters (Karasov and Douglas, 2013; Tian, 2015; Small, 2022; Zheng *et al.,* 2023). The further lowering of APN activity at 72 h post-feeding, suggests a specific temporal modulation of the different steps of protein digestion in intestine of juveniles of *P. orbignyanus*. In juveniles of the marine Meagre *Argyrosomus regius* (Asso, 1801), trypsin and APN in intestine also exhibit a different postprandial temporal dynamics (Matias *et al.*, 2023). Liver and muscle constitute sites of protein reserves in juvenile of

P.orbignyanus (Albanesi *et al.,*2021b). In several teleost fish, liver and muscle are sites of postprandial protein synthesis (Ballantyne, 2014; Mente *et al.,*2017). The increase in protein content in the liver at 72 h post-feeding points out the liver of *P.orbignyanus* juveniles as a site of postprandial protein synthesis. Furthermore, it suggests that the diminished trypsin and APN activities at 72 h after feeding which could lead to a lower availability of metabolites did not impact on protein reserves in this organ. In Salmonids, such as Atlantic salmon (Salmo salar; Linnaeus, 1758) and Arctic charr (Salvelinus alpinus; Linnaeus, 1758), food privation for short-term caused extensively rapid decreases in the protein and enzyme capacities (Krogdahl and Bakke-McKellep, 2005; Imsland *et al.,*2020). The degradation of proteins with different functions (structural; mechanical; enzymes) could lead to an impairment of crucial tissues and organs and then to disrupted homeostasis and at last to death (Secor and Carey, 2016). In various well nourished fish, intestinal tissue proteins degrade more intensely than other tissue proteins in the the first phases of food privation (Weatherley and Gill, 1981; Krogdahl and Bakke McKellep, 2005; Chan *et al.,*2008). This would not be happening in juveniles of *P.orbignyanus* which, furthermore, maintained the body mass throughout the experimental period. The maintenance of protein concentration in reserve organs would be an important element permitting survival over different times of food privation and for holding up body condition (Secor and Carey, 2016; Wilson *et al*., 2021). .

In brief, this work show a differential and specific modulation of central components of carbohydrate, lipid and protein metabolism in juveniles of *P. orbignyanus* (Figure 5) that could represent adjustments at the biochemical level to support periods of irregular feeding and even of long-term food privation inside coastal lagoon or estuaries. The distinct responses of carbohydrases, lipase and proteases in intestine, and of glycogen, protein and triglyceride concentration in liver and muscle suggest the differential modulation of tissue-specific anabolic and catabolic pathways that would allow the maintenance of physical condition. This study contributes to expand the comprehensive knowledge of marine estuarine-dependent flatfish, and particularly that of marked ecological, and economical importance.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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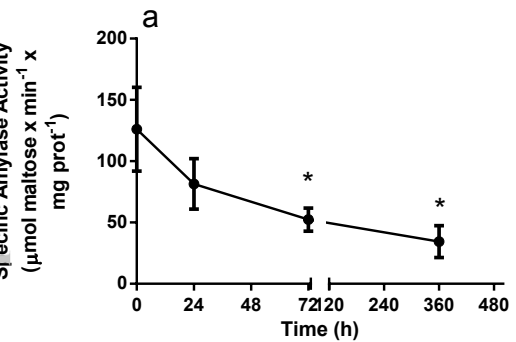
Figure. 1 Amylase (a), maltase (b) and sucrase (c) specific activities in the intestine of juveniles of *Paralichthys orbignyanus* at different times post-feeding. Asterisk (*) represents significantly different from t=0 (ANOVA, $p < 0.05$). Values are means ± SE for eight individuals.

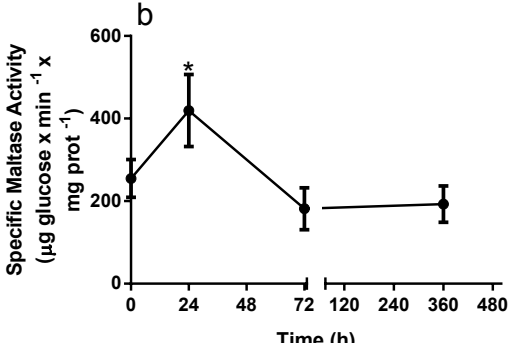
Figure. 2 Lipase (a), trypsin (b) and APN (c) specific activities in the intestine of juveniles of *Paralichthys orbignyanus* at different times post-feeding. Asterisk (*) represents significantly different from t=0 (ANOVA, $p < 0.05$). Values are means ± SE for eight individuals.

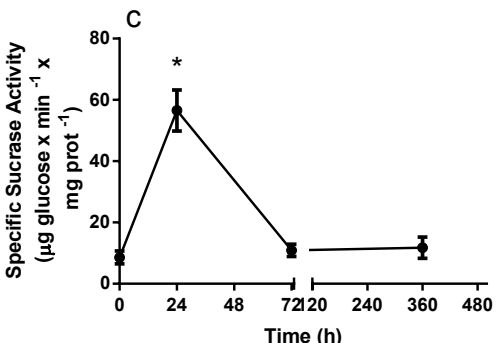
Figure. 3 Glycogen and free glucose concentration in liver and muscle of juveniles of *Paralichthys orbignyanus* at different times post-feeding. Glycogen (a) and free glucose (b) concentration in liver. Glycogen (c) and free glucose (d) concentration in muscle. Asterisk (*) represents significantly different from t=0 (ANOVA, $p < 0.05$). Values are means ± SE for eight individuals.

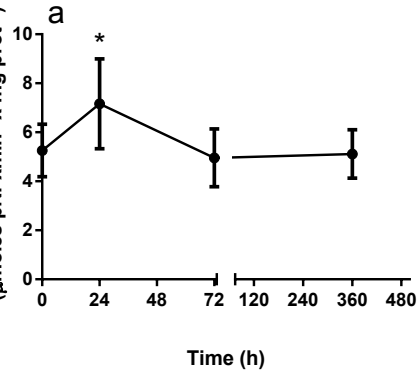
Figure. 4 Triglycerides (TAG) and protein concentration in liver and muscle of juveniles of *Paralichthys orbignyanus* at different times post-feeding. TAG (a) and protein (b) concentration in liver. TAG (c) and protein (d) concentration in muscle. Asterisk (*) represents significantly different from t=0 (ANOVA, $p < 0.05$). Values are means ± SE for eight individuals.

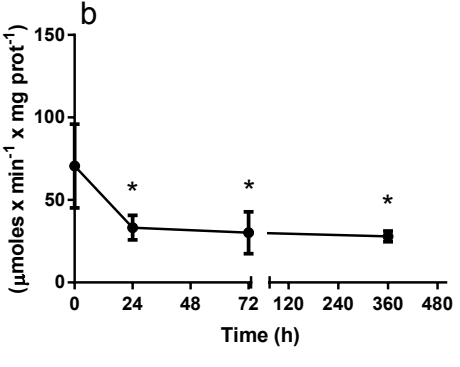
Figure. 5 Summary of morphological, digestive and metabolic parameters at different times after feeding of juveniles of *Paralichthys orbignyanus*. TW total weight, HSI hepatosomatic index, AMS amylase, MAL maltase, SUC sucrase, LIP lipase, TRY trypsin, APN N-aminopeptidase, GLY glycogen, GLU free glucose, TAG triglycerides, PROT proteins. Comparisons were made with respect to the corresponding parameter immediately after feeding (t = 0) as described in "Materials and methods" and in "Results". ↑ , ↓ , = indicate increase, decrease and unchanged, respectively.

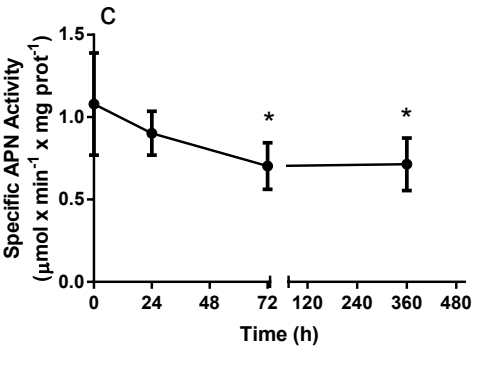




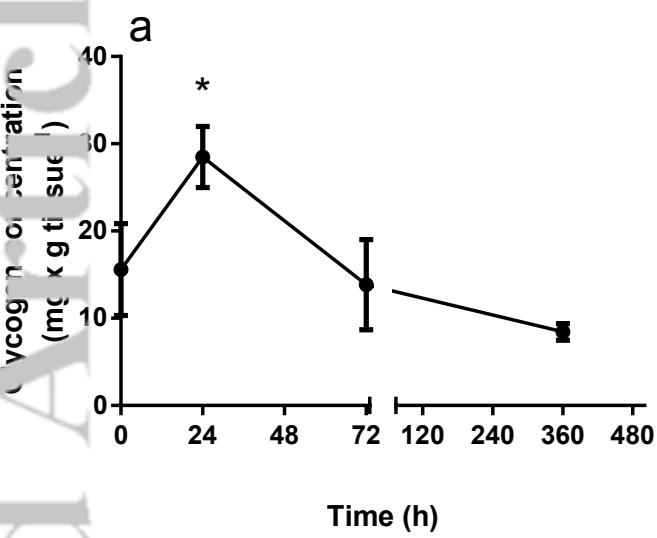


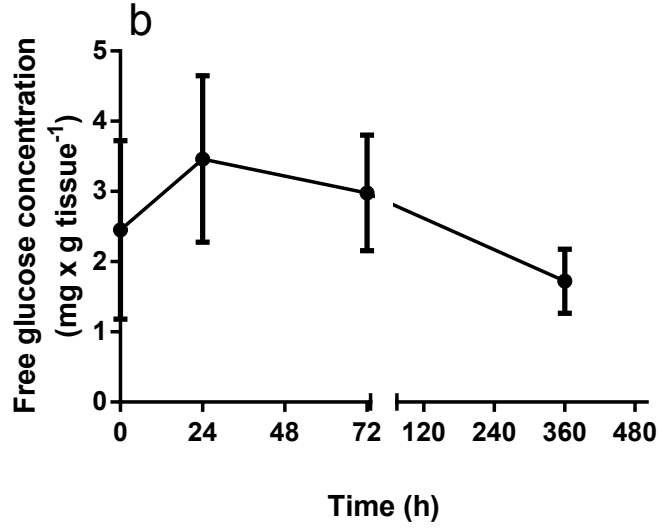




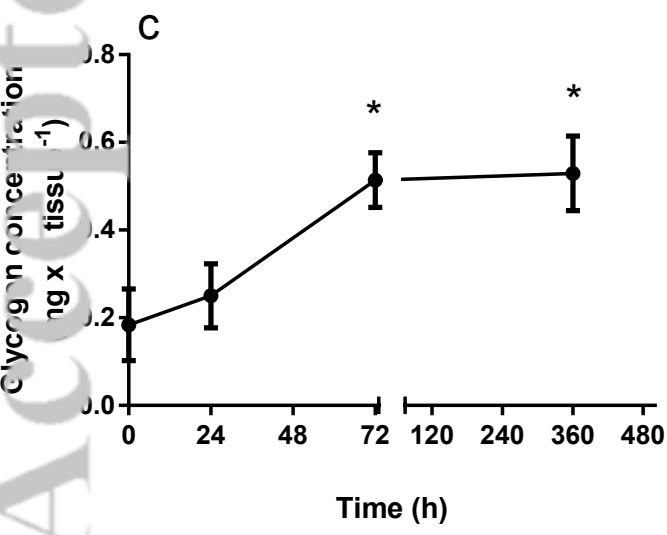


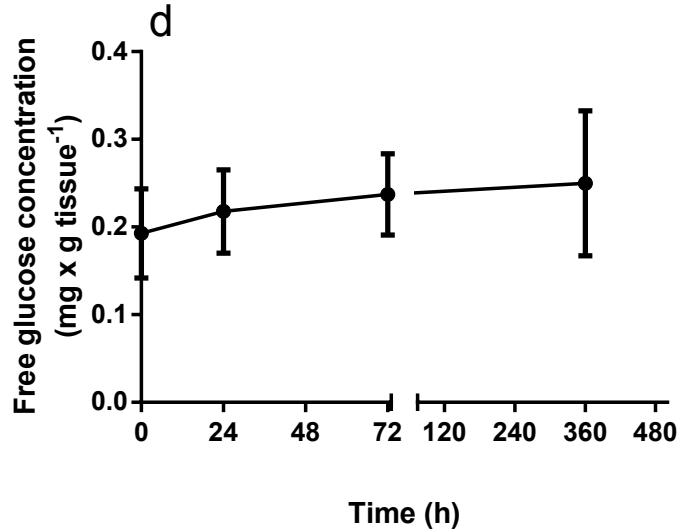
Liver

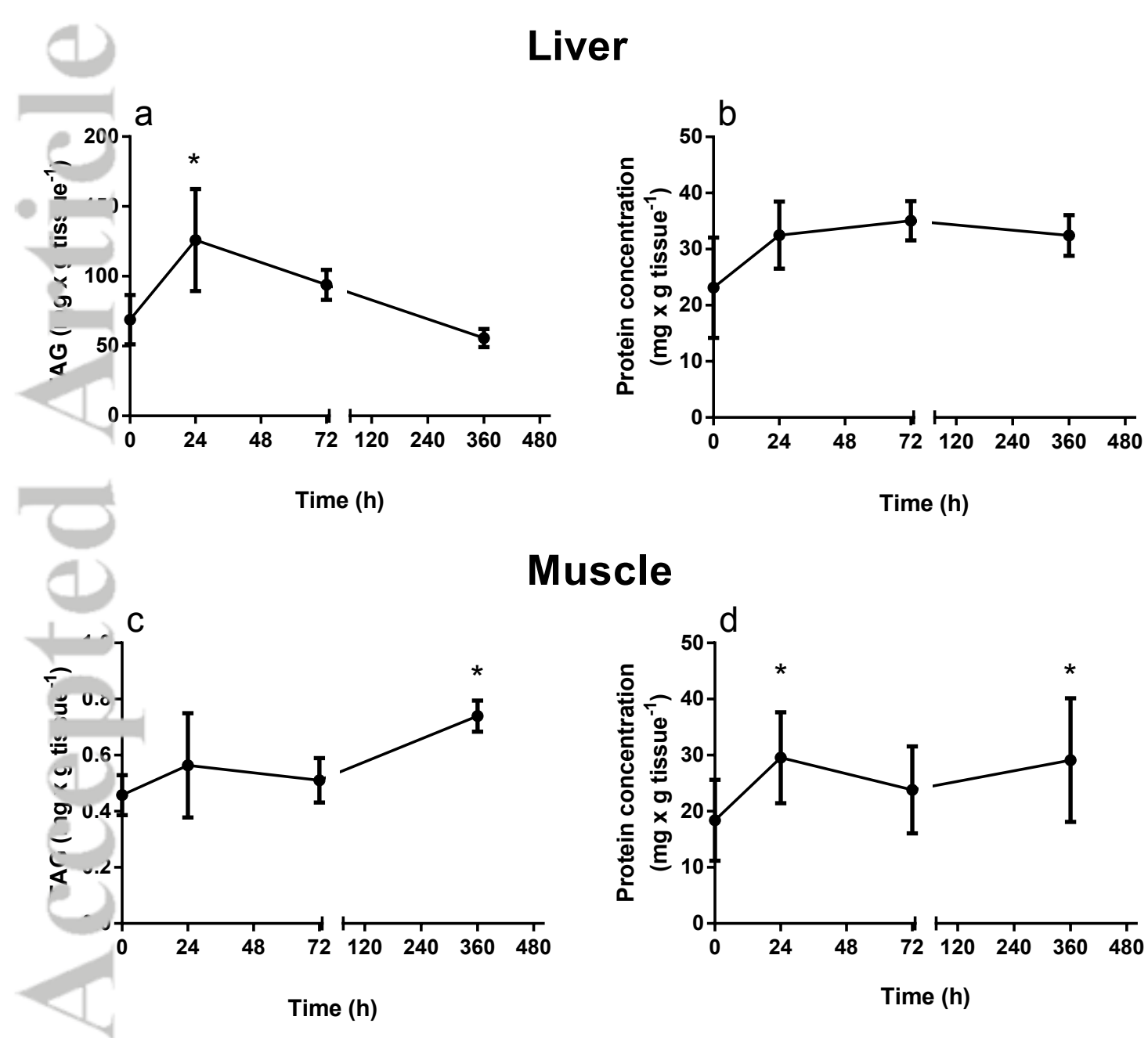




Muscle



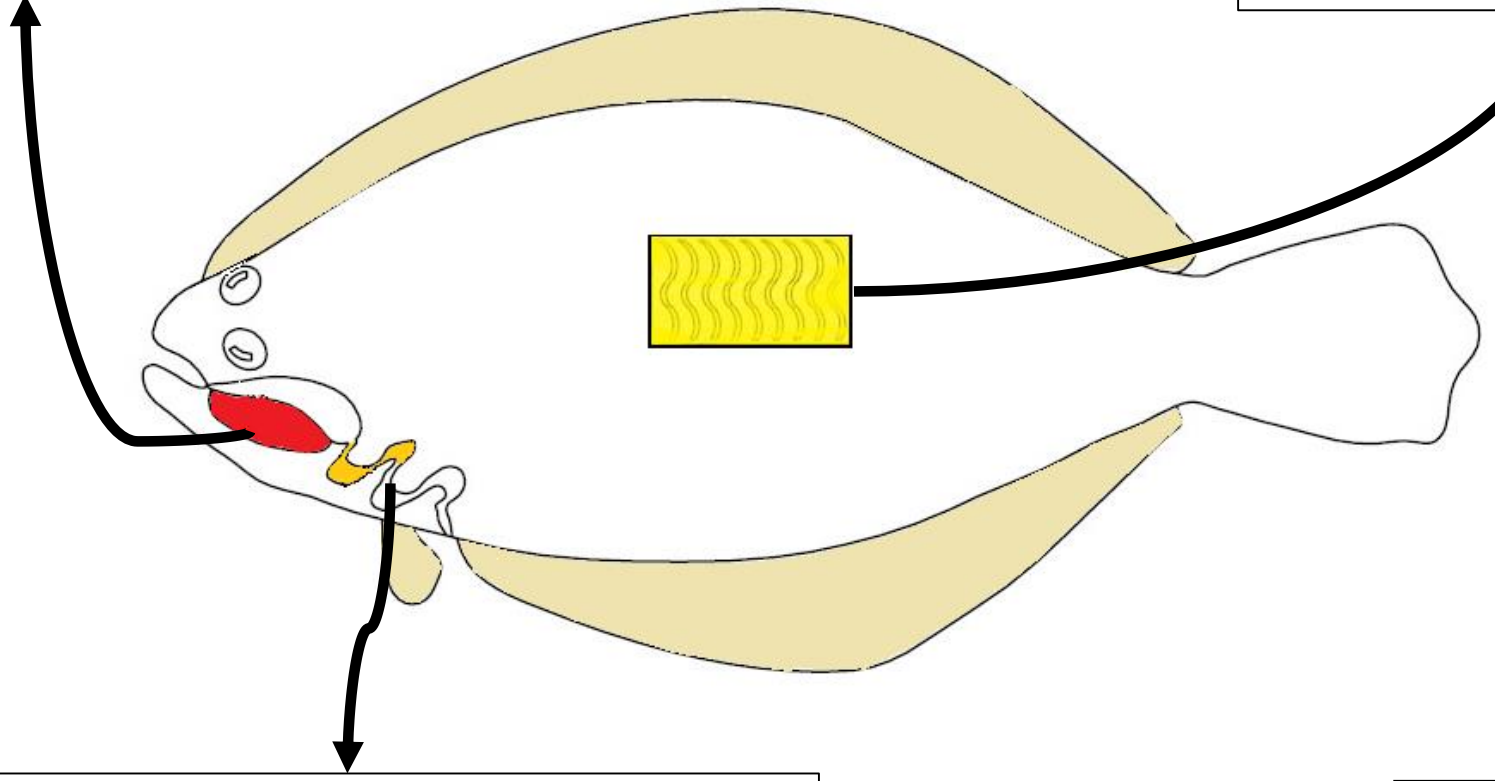




Responses at different times after feeding

LIVER
t24: GLY ⇧; GLU = ; TAG⇧; PROT = ; HSI=
t72: GLY = ; GLU = ; TAG =; PROT = ; HSI=
t360: GLY = ; GLU = ; TAG =; PROT = ; HSI=

MUSCLE
t24: GLY = ; GLU = ;TAG = ; PROT =
t72: GLY ⇧; GLU = ;TAG = ; PROT =
t360: GLY⇧ ; GLU = ;TAG ⇧; PROT =



TW
t24 =
t72=
t360=

INTESTINE
t24: AMS = ; MAL ⇧; SUC ⇧; LIP ⇧ ; TRY⇩ ; APN =
t72: AMS⇩ ; MAL = ; SUC = ; LIP = ; TRY⇩ ; APN ⇩
t360:AMS⇩ ; MAL = ; SUC = ; LIP = ; TRY⇩ ; APN ⇩

= similar to t=0
decrease respect to t=0
increase respect to t=0

TABLE 1. Total weight (TW) and hepatosomatic index (HSI) of juveniles and adults of *Paralichthys orbignyanus* at different times after feeding. Mean: M; standard deviation: (SD). Asterisk (*) represents significantly different from t=0 (ANOVA, $p < 0.05$). Values are means ± SE for eight individuals for juveniles

	TW (g)		**HSI (%)**	
	M	SD	M	SD
t=0	30.9	6.17	0.40	0.20
t=24	33.5	4.88	0.52	0.14
t=72	28.5	5.60	0.37	0.04
t=360	28.9	8.30	0.28	0.06