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Digestive and metabolic profile at the biochemical level of juvenile flounder *Paralichthys orbignyanus* (Valenciennes, 1839) (Pleuronectiformes: Paralichthyidae)

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Abstract; The flounder *Paralichthys orbignyanus* that spans from Rio de Janeiro (Brazil) to San Matías Gulf, Argentina has an important ecological role and a great potential use for aquaculture. However, studies on its digestive physiology (i.e. occurrence of digestive enzymes) as well on energy reserves content in metabolic tissues are lacking. We determined the occurrence and characteristics of amylase, maltase, sucrase, lipase and aminopeptidase-N in digestive tract of juveniles as an index of the ability to digest dietary glycogenic substrates from different sources, lipids and for final protein digestion. Glycogen, triglycerides and protein content in different tissues were also analyzed to identify storage sites. Presence and distribution in the digestive tract of the enzymes amylase, maltase, sucrase, lipase and N-aminopeptidase, as well as glycogen, triglycerides and protein suggest that juveniles of *P. orbignyanus* exhibit an adequate digestive battery to potentially perform complete hydrolysis of various dietary substrates and capacity for storage and/or utilization of energy reserves in liver and muscle. Moreover, our results show that juveniles of *P. orbignyanus* could accept a diet with high carbohydrates content, which could represent and advantage for its potential application in fish farming due to the possibility to use cheaper diets.

Keywords: flatfishes, digestive enzymes, energy reserves

Resumen: **Perfil digestivo y metabólico a nivel bioquímico de juveniles del lenguado *Paralichthys orbignyanus* (Valenciennes, 1839) (Pleuronectiformes: Paralichthyidae).** El lenguado *Paralichthys orbignyanus* el cual se distribuye desde Rio de Janeiro (Brasil) hasta el Golfo de San Matías, Argentina, desempeña un importante rol ecológico y es de gran potencial para acuicultura. Sin embargo, faltan estudios sobre fisiología digestiva (ej. existencia de enzimas digestivas clave), y sobre sitios de reserva de energía. Determinamos la existencia y características de amilasa, maltasa, sacarasa, lipasa y N-aminopeptidasa (APN) en tracto digestivo de juveniles como índice de capacidad de digerir sustratos dietarios glucogénicos y lipídicos y para digestión final de proteínas y el contenido de glucógeno, triglicéridos y proteínas en diferentes tejidos para identificar sitios de almacenamiento. La existencia, características y la distribución en tracto digestivo de las actividades de amilasa, maltasa, sacarasa, lipasa y APN y el contenido de glucógeno, triglicéridos y proteínas sugiere que juveniles de *P. orbignyanus* exhiben una adecuada batería digestiva para la hidrólisis completa de varios sustratos dietarios y capacidad para almacenamiento y/o utilización de reservas de energía en hígado y músculo. Los resultados sugieren además que juveniles de *P. orbignyanus*

soportarían una dieta con alto contenido de carbohidratos, lo que podría representar una ventaja para su posible aplicación en la producción comercial debido al menor costo de la dietas.

Palabras clave: lenguado, enzimas digestivas, reservas de energía.

Introduction

Flatfishes that are critical components of benthic communities in shelf, deep sea, small sea, riverine and estuarine ecosystems worldwide are ecologically and economically important (Link *et al.* 2015; Munroe 2015). In this context, *Paralichthyidae* flatfishes have a main ecological role and are important commercial fisheries throughout the Atlantic, from the deep Arctic to the coasts of southern Africa and South America (Díaz de Astarloa 2002, Magnone *et al.* 2014 Munroe 2015, Ruiz-Jarabo *et al.* 2015, Walsh *et al.* 2015). In Argentine waters species of the flatfishes *Paralichthys* are of great commercial value due to the top quality of their flesh (Diaz de Astarloa 1994, Rivera-Prisco *et al.* 2001). However, nothing is known about the digestive and metabolic profile of this species. In this context, in spite of the inherent physiological and ecological importance and potential application, studies about digestive enzymes in flounder *Paralichthys orbignyanus* are lacking**.**

The ability to balance the acquisition, storage and use of energy is critical for survival, growth, and maintenance of animals (Secor 2001, Karasov & Douglas 2013). In this context, to know the digestive characteristics (i.e. the presence and levels of specific digestive enzyme activities in the digestive tract) constitutes an important clue to evaluate the performance of an individual and the nature of the dietary components which can be potentially used in metabolic processes. Digestive enzymes are a link between digestion and absorption. Therefore, it is clear the importance to determine the occurrence in the digestive tract of key digestive enzymes as well as their biochemical characteristics to establish the ability of an animal to digest and utilize various dietary nutrients (Sunde *et al.* 2004, del Valle & López Mañanes 2011, 2012, Karasov & Douglas 2013, Sanz *et al.* 2015). This is particular true in cultured species where the knowledge of the digestive profile is a useful tool for the selection of adequate feed ingredients (Lan & Pan 1993, Murashita *et al.* 2013, Pujante *et al.* 2016). Little is known about physiological and anatomical characteristics of the digestive system of *P. orbignyanus*. To our knowledge the work of Campos *et al* (2010) associating food intake with neuropeptide Y expression levels is the only one available about some aspect of digestive physiology in this specie. In order to increase the knowledge of different aspects of the biology of *P. orbignyanus* and to estimate the potential digestive capacity for different dietary substrates and storage of energy substrates, in this work we determined the presence and the biochemical characteristics of carbohydrases, lipase and aminopeptidase-N in the digestive tract and the content of energy reserves in different tissues of juveniles. α-amylases (α-1,4 glucan-4-gluconohydrolase), have a central physiological importance in carbohydrate metabolism due to their role in the initial steps of the digestion of key glycogenic dietary and/or storage substrates (Xie *et al.* 2014, Janecek *et al.* 2014, Singh *et al.*2014, Tiwari *et al.* 2015, Peng *et al.* 2015, Date *et al.* 2015). Maltase has a main role in carbohydrates digestion since participates both in the initial steps by assisting to α- amylase and in the final steps to yield glucose (Lin *et al.* 2012, 2014, 2016, Dhital *et al.* 2013, Hooton *et al.* 2015). The occurrence of disaccharidases such as maltase and sucrase in the gastro-intestinal tract would further allow the potential utilization of dietary specific glycogenic disaccharides (i.e. maltase, sucrose) as glucose sources (Pavasovic *et al.* 2007, Pinoni *et al.* 2011, 2013). Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) have a central physiological importance in all animals due to their role in the digestion of lipids into fatty acids for absorption and in the hydrolysis of triglycerides storages (Casas-Godoy *et al.* 2012, Karasov & Douglas 2013, Chang & Leung 2014, Michiels *et al.* 2015a). L-alanine aminopeptidase-N or aminopeptidase-N (APN) (E.C. 3.4.11.2) which is , a membrane-bound exopeptidase (Hooper 1994, Sanderink *et al.* 1988, Luciani *et al.* 1998, Mentlein 2004, Wong *et al.* 2012, Chen *et al.* 2013, Hooton *et al.* 2015) plays a main role in the final steps of digestion of dietary proteins by producing di-/tri-peptides and single amino acids (Alpers 1987, Mentlein 2004, Goodman 2010, Fairweather *et al.* 2012). In this way, APN is commonly used as an indicator of the capacity to digest proteins (Ramirez-Otarola *et al.* 2011, del Valle & López Mañanes 2011, Michiels *et al.* 2015b). On the other hand, the levels and types of energy reserves (i.e. type of energy reserve mainly stored and the organs and/or tissues of storage) are an expression of the metabolic characteristics and

adjustments (i.e. carbohydrates, lipid and/or protein utilization and or synthesis) of an animal (del Valle *et al.* 2006, Sánchez-Paz *et al.* 2006, 2007). In this context, we determined amylase, maltase, sucrase, lipase and aminopeptidase-N as an index of the potential ability of juveniles of *P. orbignyanus* to digest glycogenic substrates from different sources, dietary lipids and for the final protein digestion, respectively. In addition, glycogen, triglycerides and protein content were determined in liver and muscle to identify potential storage sites for these energy substrates.

Materials and methods

Animal collection and maintenance: Cultured juvenile fish (mean length: 118 mm, mean body weight: 18.24g) were maintained in aquaria containing 26 L of sea water (28 psu) continuously aerated and filtered. A regime of 12 h light/12 h dark was applied and the temperature was kept at 22±2°C. The water was continuously filtered by means of an Atman filter (HF-0400). Juvenile fish were daily fed with a balanced food (28% carbohydrates, 44 % proteins, 6% lipids) (5% of body weight g individual^{-1}) (Bolasina *et al.* 2011), and starved 24 h prior to the sacrifice.

Sample procedures: Fish were weighed and cold-anaesthetized by putting them on ice for about 8 min. The stomach, small and large intestine, liver and muscle were individually excised.

The stomach, small and large intestine and liver were separately homogenized in 50 mM Tris/HCl pH 7.4 (4 ml g tissue^{-1}) (CAT homogenizer 9 120, tool T10) on ice. The same procedure was followed for the body muscle although 8 ml g tissue^{-1} was used.

Biochemical assays: Amylase activity was determined using the method described by Biesiot & Capuzzo (1990) with some modifications as we previously described (Asaro *et al.* 2011). Amylase activity was determined in a reaction medium containing 15 mg ml^{-1} starch in 50 mM phosphate buffer (pH 7.4) at 30 °C. The reaction was initiated by the addition of an aliquot of the corresponding sample (linearity zone on activity vs. protein concentration plot). After 15 minutes, the reaction was stopped by the addition of 1.5 ml of dinitrosalicylic acid reagent (DNS) (Miller 1959), and after a further incubation for 10 min at 100 °C, assay tubes were immediately cooled in ice.

The amount of released maltose was determined by reading the absorbance at 540 nm. Amylase activity was expressed as µg maltose min^{-1} mg protein^{-1}. To study the effect of pH, temperature and starch concentration on amylase activity, the procedure was the same as described above except that the activity was determined in the presence of varying pH (5.2-8.0) (50 mM phosphate buffer), temperature (4-45°C) and starch concentration (0.06-17.88 mg ml^{-1}) in the reaction mixture. Maltase and sucrase activities were assayed by measuring the glucose released from the hydrolysis of the corresponding substrate (maltose and sucrose, respectively) (del Valle & Lopez Mañanes 2008, 2011). The reaction was initiated by adding an aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) to a reaction mixture containing 42 mM of the corresponding substrate (sucrose or maltose) in 0.1 M maleate-NaOH buffer (pH 6.4) at 37°C (Asaro *et al.* 2011). After incubation for 10 min, the reaction was stopped by the addition of 1.5 ml of the combined enzyme color glucose reagent solution (oxidate glucose 10 kU L, peroxidase 1 kU, l,4-aminophenazone 0.5 mmol L^{-1}, phosphates pH 7.0100 mmol L^{-1}, hydroxybenzoate12 mmol L^{-1}) (Wiener Lab AA Kit cod. 1400101). 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. The disaccharides activities were expressed as µg glucose min^{-1} mg protein^{-1}. To study the effect of pH, temperature and maltose and sucrose concentration on maltase and sucrase activity, respectively, the procedure was the same as described above except that the activity was determined in the presence of varying pH **(**maltase: 3.5-8.0, sucrase 5.2-8.0) (0.1 mM maleate buffer), temperature (4-45°C) and substrate concentration (0.56-42 mM) in the reaction mixture.

Lipase activity was determined by measuring p-nitrophenylpalmitate (pNPP) hydrolysis (Markweg *et al.* 1995) with some modifications (Michiels *et al.* 2013). The reaction was initiated by the addition of pNPP (final concentration 0.7 mM) to a reaction *mixture* containing a suitable aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) in 50 mM Tris-HCl buffer (pH 8.5)/4 µl Tween-80. The incubation was carried out at 37°C for 15 min. The reaction was stopped by addition of 500 µl of 0.2% TCA (w/v). The amount of p-nitrophenol (pNP) released was determined by reading the absorbance at 410 nm (Metrolab 330). Samples were incubated as described above but at varying pH (6.0-9.0) (50 mM phosphate buffer, pH 6.0, 50 mM Tris-HCl buffer,

pH 7.2-9.0), temperature (4-45°C) and pNPP concentrations (0.017-0.9 mM) of the reaction mixture for determining the effect of pH, temperature and pNPP concentration on lipase activity, respectively.

The APN activity was determined by using L-alanine-p-nitroanilide (L-Ala pNA) as substrate (Roncari & Zuber, 1969) as we previously described (del Valle & López Mañanes, 2008, 2011, Michiels *et al*. 2015a) with some modifications. In the standard assay, the reaction was initiated by adding the substrate (final concentration 0.41 mM) to a reaction mixture containing an adequate aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) in 0.08 mM Tris buffer pH 7.6. After incubation for 15 min, the reaction was stopped by the addition of 0.5 mL of cold acetic acid 2 M and absorbance was determined at 384 nm. To study the effect of pH and temperature on APN activity, the procedure was the same as described above except that the activity was determined in the presence of varying pH (range 6.0-9.0) **(**50 mM phosphate buffer, pH 6.0, 50 mM Tris-HCl buffer pH 7.4-9.0**)** and temperature (4-45°C) of the reaction mixture. To study the effect of L-Ala-pNA concentration on APN activity, the procedure was the same as described above except that the activity was determined in the presence of varying L-Ala-pNA concentrations (0.04-0.4 mM) in the reaction mixture.

The determination of enzyme activities was always performed on fresh samples. The ratios of amylase and APN, amylase and lipase, lipase and APN (referred to as A/APN, A/L and L/APN) in the small intestine were determined. These ratios can be interpreted as the index of relative investments into carbohydrate, protein and lipid digesting enzymes. Proteins were assayed according to Bradford (1976). Bovine serum albumin was used as standard.

Glycogen was determined as glucose equivalent, after hydrolysis, according to Schmitt & Santos (1993). The corresponding sample was boiled for 4 min and then incubated in acetate buffer (pH 4.8) (1:2) in the absence and in the presence of 0.2 mg $ml^{-1}$of α-amyloglucosidase for 2.5 h at 55°C (Pinoni *et al.* 2011, 2013). After incubation, samples were centrifuged at 3,000 rpm for 30 min (IEC-Centra 7R, refrigerated). Glucose was quantified in the supernatant using a commercial kit for measuring glycemia (Wiener Lab AA). Released glucose from glycogen was determined as the difference between the assays with and without α-amyloglucosidase. Results are presented as mg glucose g $tissue^{-1}$. Free glucose content was determined from assay in the absence of α-amyloglucosidase.

Triglycerides (TG) were measured by the colorimetric method of glycerol phosphate oxidase with a commercial Kit (TAG Wiener-Lab AA cod. 861110001) as previously described (Pinoni *et al.* 2011, 2013. Michiels *et al.*2015b). The sample was incubated with this reactant for 5 min at 37°C. The amount of released glycerol was determined by reading the absorbance at 505 nm of the colored quinone complex.

Statistical analyses: The results of the effect of different substrate concentrations on the enzymatic activities were analyzed by a nonlinear regression analysis (GraphPad Prism4.0 software). The curve that appears is the one that best fit to the experimental data according to estimation by GraphPad Prism 4.0 software, showing adjustment to Michaelis-Menten model. Km value (Michaelis–Menten constant) was estimated from this curve (GraphPad Prism 4.0 software). Statistical analysis was determined using the Sigma 3.0 program for Windows, which automatically performs previous test of equality of variances and normality. Analysis of variance (one-way ANOVA) or t-test were used to estimate the statistical significance of the differences and $P<0.05$ was considered significant. ANOVA (Student-Newman-Keuls) was used to identify differences.

Results

Digestive enzymes activities in the digestive tract of juveniles of P. orbignyanus: effect of pH, temperature and substrate: Initially, amylase, maltase, sucrase, lipase and APN activities were detected in the small intestine and partially characterized.

Amylase activity was determined within the range of pH 5.2-8.0. Amylase activity appeared to be similar within the range of pH studied (Fig. 1a). Figure 1b shows the effect of temperature (4–45 °C) on amylase activity. The activity increased from 4°C to 37°C. At 45°C amylase activity was similar to that at 37°C (Fig. 1b). The effect of starch concentrations on amylase activity is shown in Figure 1c. Amylase activity exhibited Michaelis-Menten kinetics (apparent Km= 0.054 mM).

Maltase activity was determined within the range of pH 3.5-8.0. At pH values of 3.5 and 5.2, the activity was about 73 and 97% of the corresponding activity at pH 6.4. (Fig.1d). At pH

8.0, maltase activity was about 40% lower than that at pH 6.4. Maltase activity was maintained over a wide range of temperature (4-45°C) (Fig. 1e) and exhibited Michaelis-Menten kinetics (apparent Km=3.30) (Fig. 1f).

Sucrase activity was determined within the range of pH 5.2-8.0, appearing to be maintained all over this pH range (Fig 1g). Sucrase activity was maximal at 37°C whereas at lower (4-20°C) and higher (45°C) temperatures, the activity was about 50-60% of maximal activity (Fig. 1h). Sucrase activity also showed Michaelis-Menten kinetics (apparent Km=1.36) (Fig. 1I).

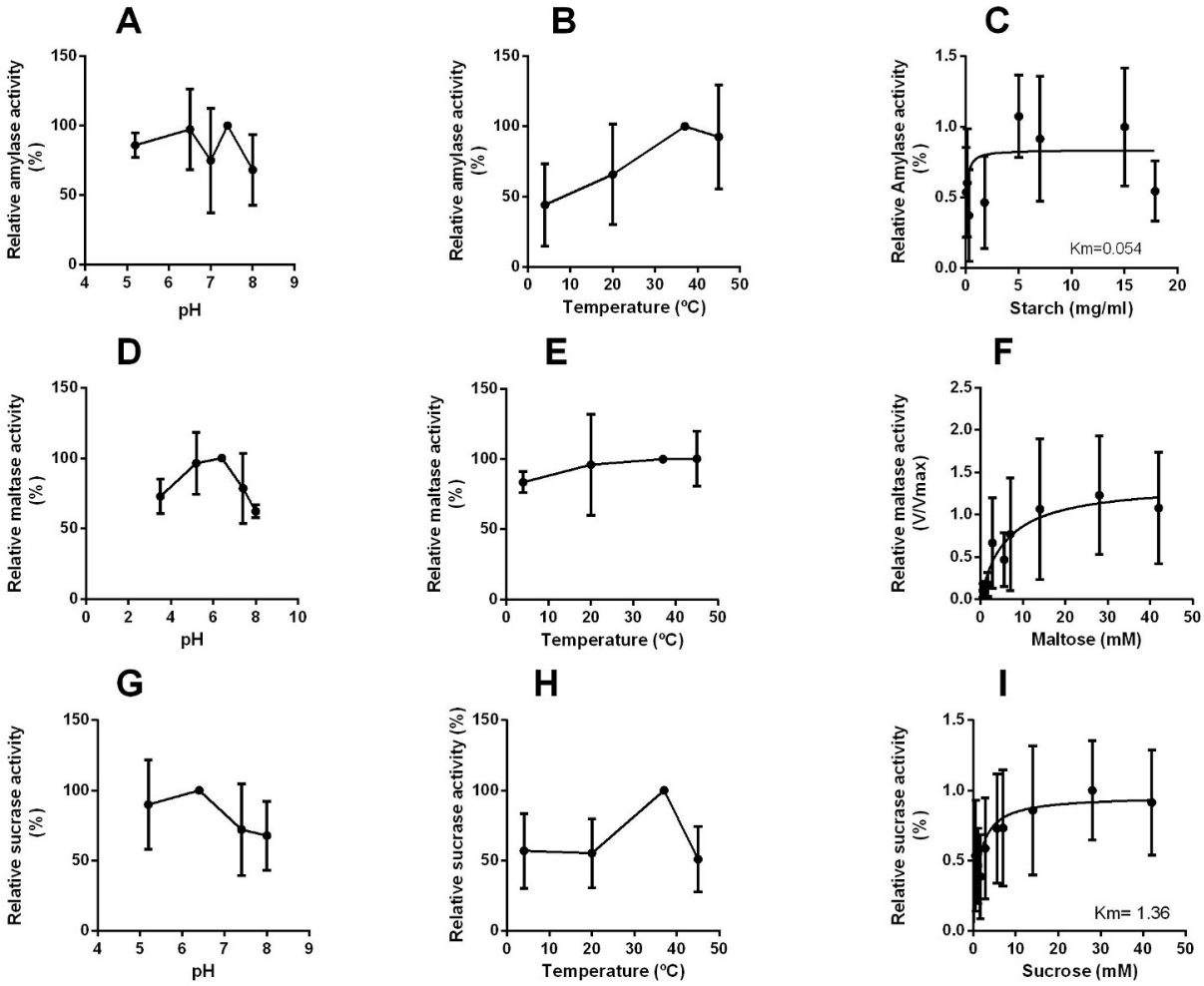

Figure 1.- Partial characterization of carbohydrases (amylase, maltase and sucrase) in the small intestine of *Paralichthys orbignyanus* (A) Effect of pH (5.2–8.0) on amylase activity. The values of amylase activity are expressed in relation to the activity at pH 7.4 (B) Effect of temperature (4-45°C) on amylase activity. The values of amylase activity are expressed in relation to the activity at 37°C. (C) Effect of starch concentration on amylase activity. The activity was measured at 37°C and at pH 7.4. The curves are the ones which best fit the experimental data (GraphPad Prism 2.01). The values of activity are expressed in relation to the corresponding activity in the presence of 15 mg ml^{-1} starch (100%) D) Effect of pH (3.5–8.0) on maltase activity. The values of maltase activity are expressed in relation to the activity at pH 6.4 (100%,) (E) Effect of temperature (4-45°C) on maltase activity. The values of maltase activity are expressed as relation to the activity at 37°C (100%). (F) Effect of maltose concentration on maltase activity. The activity was measured at 37°C and at pH 6.4. The curves are the ones which best fit the experimental data (GraphPad Prism 2.01). The values of activity are expressed in relation to the corresponding activity in the presence of 28 mM maltose. (G) Effect of pH (5.2–8.0) on sucrase activity. The values of sucrase activity are expressed in relation to the activity at pH 6.4 (H) Effect of temperature (4-45°C) on sucrase activity. The values of sucrase activity are expressed in relation to the activity at 37°C. (I) Effect of sucrose concentrations on sucrase activity. The activity was measured at 37°C and at pH 6.4. The values of activity are expressed in relation to the corresponding activity in the presence of 28 mM sucrose (100%). Data are the mean ±SE of 5 individuals.

Lipase activity was determined within the range of pH 6.0-9.0. Lipase activity was similar at pH 6.0 and 7.4 and increased about 20 and 40% at pH 8.5 and 9.0, respectively (Fig. 2A). Figure 2B shows the effect of temperature on lipase activity. Lipase activity was maximal at 37°C. At 4 and 20°C the activity was about 64% lower than the activity at 37°C whereas at 45°C, decreased about 73% (Fig. 2B). The effect of pNPP concentrations on lipase activity is shown in Figure 2C. Lipase activity in the small intestine exhibited Michaelis-Menten kinetics (apparent Km=1.16 mM).

Figure 3A shows the effect of pH on APN activity in the small intestine. APN activity appeared to enhance from pH 6.0 to 7.4- 8.0. The activity further increased at pH 9.0 (about 30 % in relation to the value at pH 7.4-8.0). The effect of temperature on APN activity is shown in Figure 3B. APN activity appeared to increase with enhancement of temperatures from 4°C to 37-45°C. APN activity showed a Michaelis-Menten kinetics (apparent Km= 0.22) (Fig. 3C).

No amylase, lipase or APN activities were detected in the stomach (data not shown). Amylase specific activity was similar in the small and large intestine (Fig 4A). Maltase specific activity in the small intestine was higher (about 340%) than in the large intestine (t=5.05 p=0.0004) (Fig. 4B). No differences in sucrase specific activity were found between the small and large intestine (Fig 4C). Lipase activity was not detected in the large intestine (Fig. 4D). APN specific activity in the small intestine was about two fold higher than the activity in the large intestine (t=3.66, p<0.011) (Fig. 4E).

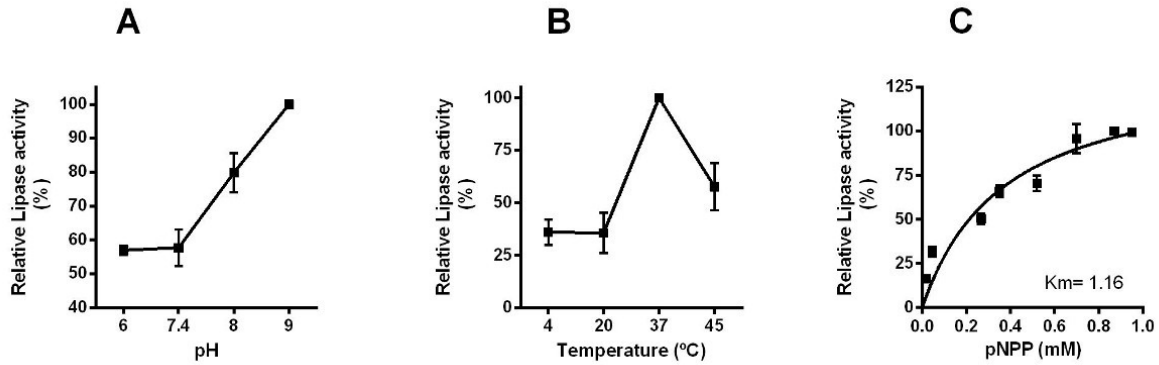

Figure 2.-(A) Effect of pH (6.0-9.0) on lipase activity in the small intestine of *Paralichthys orbignyanus*. The lipase activity values are expressed in relation to the specific activity at pH 8.5. (B) Effect of temperature (4-45°C) on lipase activity small intestine of *Paralichthys orbignyanus.* The activity is expressed in relation to the specific activity at 37°C, (C) Effect of pNPP concentration (0.017-0.9 mM) on lipase activity in small intestine of *Paralichthys orbignyanus*. The values of activity are expressed in relation to the corresponding activity in the presence of 0.87 mM pNPP Data are the mean ±SE of 5 individuals.

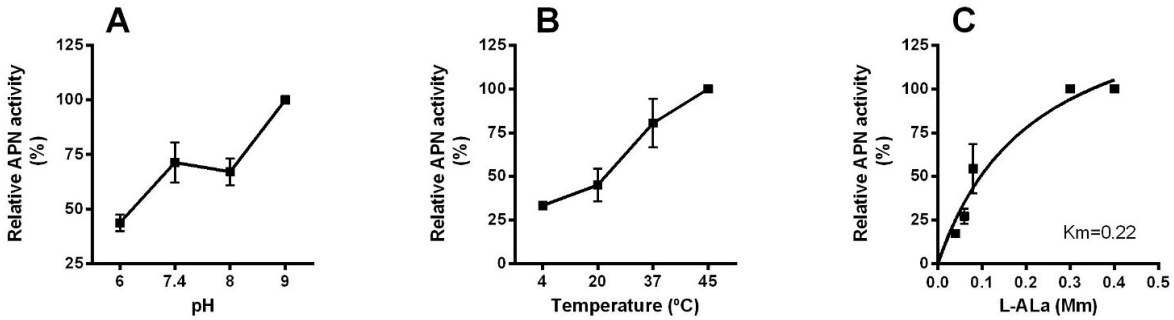

Figure 3.- (A) Effect of pH (6.6-9.0) on APN activity in thesmall intestine of *Paralichthys orbignyanus*. The APN activity values are expressed in relation to the specific activity at pH 7.6 (B) Effect of temperature (4-45°C) on the APN activity in small intestine of *Paralichthys orbignyanus*. The activity is expressed in relation to the activity at 37°C (C) Effect of L-Ala pNA concentration (0.04-0.4 mM) on APN activity in small intestine of *Paralichthys orbignyanus*. The values of activity are expressed in relation to the corresponding activity in the presence of 0.4 mM. Data are the mean ±SE of 5 individuals.

No amylase, lipase or APN activities were detected in the stomach (data not shown). Amylase specific activity was similar in the small and large intestine (Fig 4A). Maltase specific activity in the small intestine was higher (about 340%) than in the large intestine (t=5.05 p=0.0004) (Fig. 4B). No differences in sucrase specific activity were found between the small and large intestine (Fig 4C). Lipase activity was not detected in the large intestine (Fig. 4D). APN specific activity in the small intestine was about two fold higher than the activity in the large intestine (t=3.66, p<0.011) (Fig. 4E).

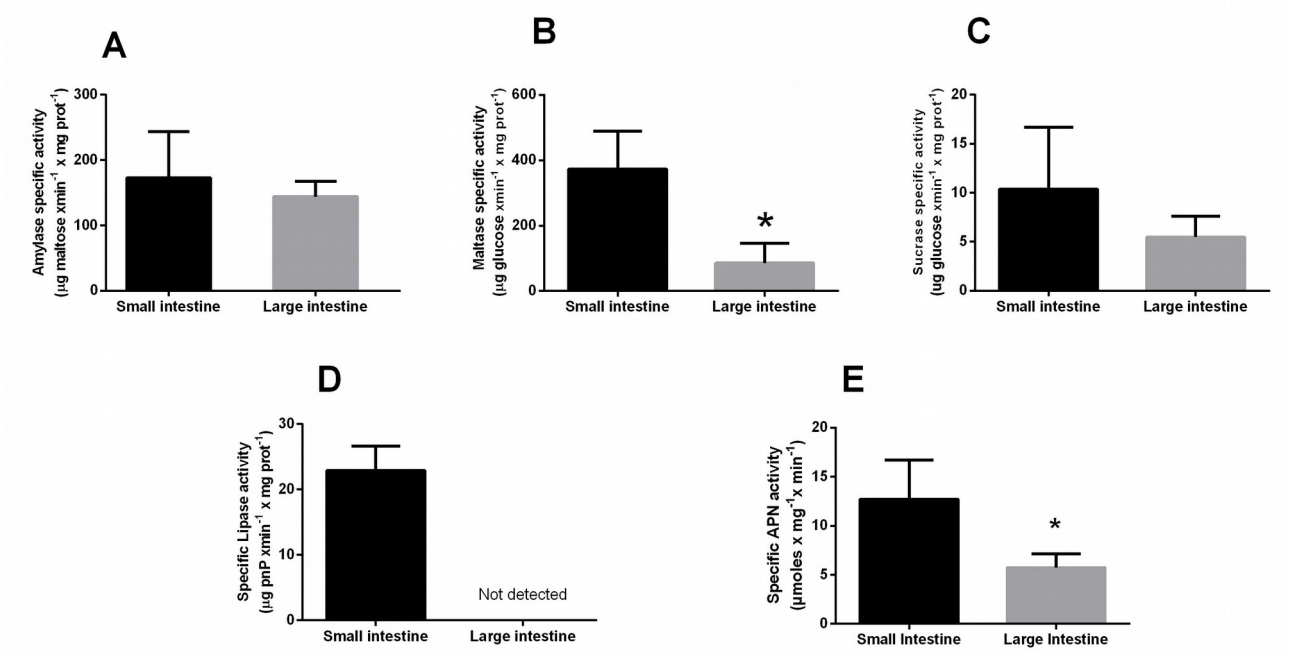

Figure 4.- Amylase (A), maltase (B), sucrase (C), lipase (D) and APN (E) specific activity in different parts of the intestine of *Paralichthys orbignyanus*. Data are the mean ± SE of five individuals. *indicates significant differences between different parts of the intestine ($p < 0.05$).

The ratios A/APN, A/L and L/APN in the small intestine are shown in Table I.

Table I- Ratios of amylase and APN, amylase and lipase (A/APN and A/L) and lipase and APN (L/APN).

A/APN	A/L	L/APN
13.59	7.52	1.81

Energy reserves in juveniles of P. orbignyanus: Glycogen was detected in the muscle and liver. Glycogen concentration was similar in both tissues (p>0.05) (Fig 5A). Free glucose concentration in liver was about four fold higher than that in muscle (t= 2.74 p=0.018) (Fig 5B). Triglyceride concentration in liver was higher than in the muscle (t=4.98, p=0.0011) (Fig 5C). Both liver and muscle exhibited similar protein concentration (p>0.05) (Fig 5D).

Discussion

The flounder *Paralichthys orbignyanus* has an important ecological role in SW Atlantic coast as well as a high potential use for aquaculture (Sampaio *et al.* 2001, 2007, 2008, Bambill et al. 2006, Radonic & Macchi 2009, Bolasina 2011). In this context, it is clear that the knowledge about its digestive battery at the biochemical level is of great importance not only, for a further understanding of its ecophysiology but also, to potentially improve its commercial use. Thus, the determination of enzyme activities in the digestive tract, provides essential information for future development and/or utilization of appropriate diets, particularly for juveniles. The results of this study show the presence of amylase, maltase, sucrase, lipase and aminopeptidase-N (APN) activities in the intestine of juveniles of *P. orbignyanus*. Therefore, this species exhibits a digestive battery capable of to potentially digesting different kinds of glycogenic carbohydrates (i.e., starch, glycogen, dextrin,

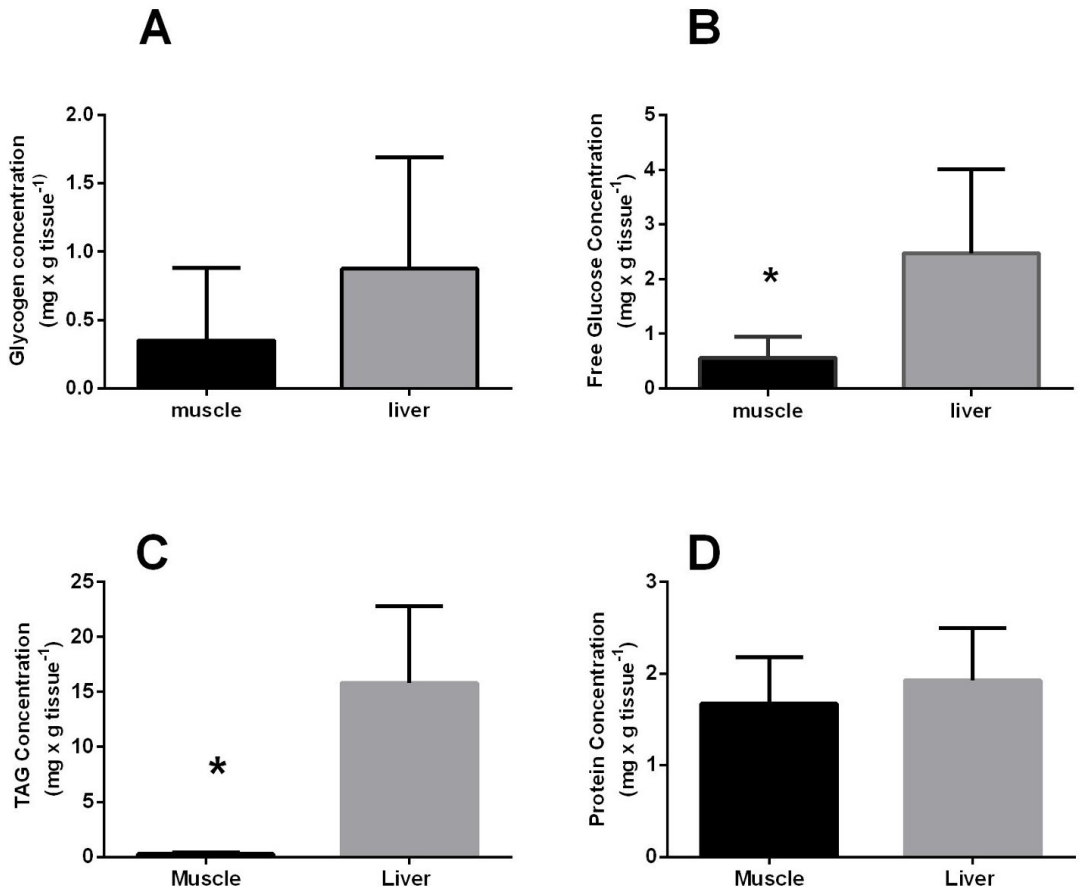

Figure 5.- Glycogen (A), free glucose (B), TAG (C), and protein (e) concentration in muscle and liver of *Paralichthys orbignyanus*. Data are the mean ± SE of five individuals. *indicates significant differences between muscle and liver ($p < 0.05$).

maltose and sucrose), lipids and proteins. In various animals, the occurrence in the digestive tract of specific digestive enzyme activities is related to the nature of the dietary components that could potentially be used in metabolic processes (del Valle & López Mañanes 2011, 2012, Pinoni *et al.* 2011, Ramirez-Otarola *et al.* 2011, Karasov & Douglas 2013). In fishes, digestive enzyme activities have been employed as a tool to understand the functioning of the digestive machinery at the biochemical level helping to better explain the utilization of various nutrients (Sanz *et al.* 2015). Fish appear to have a digestive enzyme apparatus qualitatively similar to that of other animals with very similar substrate specificities across taxonomic groups. However, the knowledge about biochemical characteristics of various digestive enzymes is still scarce and fragmentary, particularly in euryhaline flatfishes (Bakke *et al.* 2011).

Glucose homeostasis is essential for supporting the regular functions of various organs and in response to various environmental stresses (Polakof *et al.* 2011, 2012, La Fleur *et al.* 2014). The digestion of glycogenic carbohydrates is one of the main sources of glucose. In this context, in all animals, α-amylases have a central physiological importance due to their role in the initial steps of the digestion of key glycogenic substrates such as dietary starch and/or storage glycogen (Date *et al.* 2015, Gominho-Rosa *et al.* 2015, Tiwari *et al.* 2015). Maltase activity is the key enzyme for glycogenic carbohydrates digestion since participates both in the initial steps of hydrolysis by assisting to α- amylase and in the final steps to yield glucose (Dhital *et al.* 2013, Lin *et al.* 2016). The results of this work showing the presence of amylase and maltase activity in the small intestine of juveniles of *P. orbignyanus*, suggests the ability of these fishes to perform complete starch degradation and, furthermore, the potential to use dietary maltose and storage glycogen as glucose sources. Furthermore, the occurrence of sucrase

activity in the small intestine of juveniles of *P. orbignyanus* suggests the capacity for digestion and potential use of sucrose not only as a glycogenic substrate but also as a fructose source. The high activity of carbohydrases found in the intestine of juveniles of *P. orbignyanus* suggests the ability, as we mentioned above, for complete digestion of starch and/or glycogen. *P. orbignyanus* appears to exhibit a carnivore diet in the natural ambient. Various studies on α-amylase in fishes suggest that herbivorous and omnivorous fishes have higher α-amylase activities than carnivorous fishes (Fernandez *et al.* 2001, Chan *et al.* 2004, Drewe *et al.* 2004, Horn *et al.* 2006, Chaudhuri *et al.* 2012, Gominho-Rosa *et al.* 2015). However, amylase activity has been reported to occur in the gut of various carnivorous species (Chaudhuri *et al.* 2012, Karasov & Douglas 2013). Similarly, maltase activity appeared to be high in the gut of various species of omnivorous fishes (Gominho-Rosa *et al.* 2015), but it has also been found in the intestine of carnivorous fishes such as the trout, in which this activity is induced by feeding a high dextrin diet early in life (Karasov & Douglas, 2013). The maintenance of amylase, maltase and sucrase activity over a wide range of pH and temperature in juveniles of *P. orbignyanus* (Fig. 1) suggest that the capability for carbohydrates digestion would not be compromised under sudden changes in internal and/or external conditions. The tolerance to a wide range of temperatures is one of the characteristics of *P. orbignyanus* that supports its potential culture (Bianchini *et al*. 1996, Wasielesky *et al*. 1998). In low temperature an enhancement in plasma glucose levels occur after food intake suggesting the increase in digestion and absorption (Campos *et al.* 2011). The occurrence in digestive tract of carbohydrases active at different temperatures would then be important to sustain glucose homeostasis after food intake. In this context, we found that carbohydrases in the intestine of juveniles of *P. orbignyanus* are extremely tolerant to temperature changes and although speculative this could be related to the maintenance of adequate glucose availability over a wide range of temperatures. The Michaelis–Menten kinetics of amylase, maltase and sucrase activity in the small intestine of juveniles of *P. orbignyanus* in response to varying starch concentrations (Fig. 1C, F, I) is in agreement with that previously described for amylase activity in various animals (del Valle & López Mañanes 2008, 2011, 2012a, Asaro *et al.* 2011**).** The fact that no amylase activity was detected in the stomach, suggests that the digestion of glycogenic carbohydrates in juveniles of *P. orbignyanus* would start in the small intestine. Since amylase activity was similar in the small and large intestine (Fig 4A) carbohydrate digestion could be carried out throughout the intestine. However, the fact that maltase activity was found to be significantly lower in the large intestine (Fig 4B), indicates that the small intestine could be the main site capable of the total carbohydrates digestion and potential glucose absorption. In the intestine of fishes, glucose transporters such as sodium dependent glucose transporters SGLT1 and GLUT appear to contribute to the intestinal glucose absorption (Krogdahl *et al.* 2005, Castillo *et al.* 2009, Karasov & Douglas 2013). Whether this is the case of juveniles of *P. orbignyanus* remains to be investigated. No reports are available to our knowledge on the occurrence of sucrase activity in digestive tract of fishes. The occurrence of sucrase activity in intestine of juveniles of *P. orbignyanus* suggests also the potential use of sucrose as a fructose source. Fructose appears to be absorbed by the intestine of the some fishes (Bakke *et al.* 2011). In euryhaline flatfishes, carbohydrate metabolism plays a major role in energy supply for osmo- and iono-regulation, being the liver the major source for supplying of carbohydrate metabolites (Tseng & Hwang 2008). Our results show that in juveniles of *P. orbignyanus* both liver and muscle appear to be glycogen storage sites (Fig 5A). Furthermore, the high free glucose content in liver (Fig. 5B) suggests its role in the carbohydrates metabolism probably in the maintenance of an adequate and sustained glucose supply.

Lipases are of central physiological importance in all animals due to their role in the digestion of lipids into fatty acids for absorption and in the hydrolysis of triglycerides storages. Although lipase activity has been found in the intestine of various species of fishes, it differs between fish species and knowledge about biochemical characteristics is still scarce and fragmentary (Bogevik *et al.* 2008, Wilson & Castro 2011, Pujante *et al.* 2016). The high lipase activity in the intestine of juveniles of *P. orbignyanus* suggests the ability to perform lipid degradation and its potential use for metabolicprocesses. Lipase activity response to pH in the intestine of juveniles of *P. orbignyanus* (Fig. 2A) suggests the occurrence of neutral lipase activity as shown in the larvae of *P. olivaeaus* (Bolasina *et al.* 2006). On the other hand, whether the maintenance of lipase activity over a wide range

of temperatures (Fig. 2B) could be related to the capacity of supporting adequate levels of lipids digestion under thermal acclimation requires further investigation. The fact that lipase activity was only detected in the small intestine (Fig. 4D) suggests this is the main site for dietary lipids degradation and potential absorption of products. Lipid absorption processes in fish appears mainly to occur in the proximal regions of the intestine (Denstadli *et al.* 2004, Hernandez-Blazquez *et al.* 2006, Bakke *et al.* 2011). However, the absorption of lipids in fish is still not well understood although it is presumed to occur as in mammals (Bakke *et al.* 2011). Lipid storage in the form of triglycerides is an evolutionary conserved process that exists in all organisms (Birsoy *et al.* 2013). Our results show that liver would be the site of triglycerides storage in juveniles of *P. orbignyanus* while the low triglycerides content in the muscle (Fig. 5C) suggests that this tissue could be a source for mobilization of these reserves. Since as we pointed out before, the digestion–absorption routes are unknown in *P. orbignyanus*, further experimental approach is needed to establish the possible interplay between activity of digestive enzymes in the intestine and metabolic pathways in the energy storage tissues. On the other hand, the differential triglyceride content in liver and muscle could also suggest the occurrence of tissue-specific differential pathways and/or of differential mechanisms of regulation and building of triglycerides reserves.

Proteins digestion and absorption of resulting products play a key role for providing an adequate availability of amino acids necessary for the building of new tissues and maintenance of key functions (Romano & Zheng 2012, Karasov & Douglas 2013). In this context, APN in the digestive tract has a main physiological role as being a membrane bound ectopeptidase involved in final steps of digestion of dietary proteins (Alpers 1987, Mentlein 2004, Goodman 2010, Fairweather *et al.* 2012). This appears to be also the case for various fishes (Bakke *et al.* 2010). The occurrence of APN activity in the intestine of juveniles of *P. orbignyanus* suggests the ability to perform extracellular total degradation of dietary proteins (Ramirez-Otarola *et al.* 2011, Fairweather *et al.* 2012). Recently, it has shown that APN expression in the gut of the grass carp *Ctenopharyngodon idellahas* can be modulated by different factors (Tang *et al.* 2016). In this context, it was suggested that a diet with lower protein stimulates APN expression in the gut of the grass carp and helps protein digestion (Tang et al. 2016). The fact that APN activity appears to be lower in the large intestine compared to the activity in the small intestine would indicate this tissue as the main site for dietary protein digestion in juveniles of *P. orbignyanus* (Fig. 4E). Amino acid and peptide transport is of central importance in the protein nutrition in animals (Karasov & Doouglas 2013, Chang & Leung, 2014). In mammalian intestine, APN and neutral amino acid transporters are able to form complexes, which alter the kinetic parameters of the transporters, and therefore influences amino acid absorption (Fairweather *et al.* 2012). Low-affinity/high-capacity peptide transporters have been characterized functionally in the digestive tract of some fishes (Verri *et al.* 2001, Karasov & Douglas 2013). Both liver and muscle appeared to be sites for protein reserves in juveniles of *P. orbygnianuys*, which could indicate a possible link between APN activity in the intestine and adequate absorption of amino acid (Fig. 5d). The structural features of mammal APN are characterized by multifunctional roles as those in metabolism of various peptides and interaction with other proteins (Chen *et al.* 2013). In this way, we cannot discard that the occurrence of APN in the intestine of juveniles of *P. orbygnianuys* can be linked to the functions proposed in mammal digestive tract. The A/APN A/L L/APN ratios (Table I) in juveniles of *P. orbignyanus* also suggest a high capability to digest carbohydrates and lipids. In this context, a better utilization of diets where carbohydrates and/or lipids partially replace protein, could lead to the manufacture of low-priced specific feeds (Sanz *et al.* 2015).

In summary, the occurrence and biochemical characteristics of amylase, maltase, sucrase, lipase and APN activities in digestive tract in juveniles of *P. orbignyanus* and the profile of energy reserves content, suggest that these individuals exhibit an adequate digestive battery to potentially perform complete hydrolysis of various dietary substrates and moreover, capacity to support a diet with high carbohydrates content. In this context, our results represent an important contribution not only to increase the scarce knowledge about biochemical physiology of *P. orbignyanus*, but also for its potential application in fish farming.

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