DIGESTIVE ENZYME ACTIVITIES DURING PEJERREY (*Odontesthes bonariensis*) ONTOGENY

Daniela Irina Pérez-Sirkin[1,2], Mikhail Solovyev[3,4], Tomás Horacio Delgadin[1,2], Javier E. Herdman[5], Leandro A. Miranda[5], Gustavo Manuel Somoza[5], Paula Gabriela Vissio[1,2], Enric Gisbert[6*]

[1]Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.

[2]Instituto de Biodiversidad y Biología Experimental y Aplicada (IBBEA), CONICET-UBA, Buenos Aires, Argentina.

[3]Institute of Systematics and Ecology of Animals Siberian Branch of Russian Academy of Sciences, 11 Frunze St., Novosibirsk 630091, Russia

[4]Tomsk State University, 36 Lenin Ave., Tomsk 634050, Russia

[5]Instituto Tecnológico de Chascomús (CONICET-UNSAM), Chascomús, Argentina.

[6]Institut de Recerca i Tecnologia Agroalimentaries, Centre de Sant Carles de la Ràpita (IRTA-SCR), Crta. Poble Nou km 5.5, 43540 Sant Carles de la Ràpita, Spain

*Corresponding author: Enric Gisbert. E-mail: enric.gisbert@irta.cat

Short running title: Digestive enzyme ontogeny in pejerrey

Abstract

The first step for assessing and refining the nutritional requirements during the larval and early juvenile stages of a fish is the study of the ontogeny of digestive system functionality. The combination of these studies with the ecological and anatomical knowledge of the species of interest establishes the base for facing one of the major aquaculture challenges: promoting larvae growth and survival. Thus, this study is focused on describing the changes in the activity of the main digestive (pancreatic and intestinal) enzymes during larval development of the agastric South American pejerrey (*Odontesthes bonariensis*). Digestive enzymes for protein, lipid and carbohydrate hydrolysis were present from the first-week post-hatching (6.85 ± 0.07 mm total length, TL). Changes in the activity of trypsin, chymotrypsin, and total alkaline proteases indicated that the exocrine pancreas in pejerrey achieved its functional development at 2 weeks post-hatching (9.22 ± 0.17 mm TL). Interestingly, α-amylase and maltase total activities progressively increased over development, suggesting that gradual incorporation of dietary carbohydrates in a feeding protocol may have a protein-sparing effect, as well as a cheap and fast way to obtain energy for pejerrey's growth and development. The analysis of intestinal enzymes revealed that the typical shift between intracellular and luminal protein digestion that occurs during larval development in gastric species does not take place in pejerrey, indicating that in agastric species intracellular protein digestion plays a major role in comparison to luminal digestion during larval development. Contrary to gastric species, our results suggest that the alkaline phosphatase to leucine-alanine peptidase ratio for evaluating gut maturation in agastric species is not recommended, and other parameters should be measured when evaluating the maturation process in fish larvae from this group of species.

Keywords: digestive enzymes, enzyme activity, larvae, ontogeny, pejerrey.

1. Introduction

Regardless of the globalization of the aquaculture industry that has resulted in a few dozens of finfish species cultured worldwide (FAO, 2018), the culture of local species is a must in order to diversify this sector, promote local biodiversity, and preserve cultural heritage and traditions. Among the large list of candidate finfish species for aquaculture diversification, the pejerrey, *Odontesthes bonariensis* (Valenciennes 1835), is a South American euryhaline fish that it is highly appreciated for the quality of its flesh, as well as a game fish (Somoza et al., 2008). In particular, pejerrey muscle contains high levels of polyunsaturated fatty acids (PUFA) compared to saturated ones, as well as a greater proportion of n-3 PUFA with respect to n-6 PUFA, a feature that is typical of marine species, but rare in freshwater fish (Kopprio et al., 2015). Despite of these characteristics, the commercial aquaculture of this species has not been fully developed in any South American country. Beyond socio-cultural and economic reasons, other major biological barriers that may explain this issue are the low performance in terms of survival and growth, and high levels of skeletal malformations at early life stages (Berasain et al., 2000; Miranda et al., 2006; Gómez-Requeni et al., 2013; Bertucci et al., 2018). One of the main reasons explaining the above-mentioned list of biological drawbacks that limit pejerrey aquaculture is the absence of a feeding protocol for early life stages based on experimental studies. Such information would allow the standardization of larval rearing procedures resulting in an improvement of survival, growth, and skeletal quality, guaranteeing fry availability for proper aquaculture development. Despite the vast knowledge of pejerrey biology and ecology (Somoza et al., 2008; Strüssmann et al., 2010; Miranda et al., 2013; Elisio et al., 2014; 2015; Hughes et al., 2017), studies on the first stages of development are limited to sex determination-differentiation and morphological descriptions of eggs and larvae (Strüssmann et al., 1996; Miranda et al., 2003; Chalde et al., 2011; Fernandino et al., 2011; González et al., 2015; Zhang et al., 2018). Until now, studies about the ontogeny of the digestive system that could help to adapt a feeding protocol to morphophysiological changes of the digestive system during development are limited to a report that described the ontogeny of four digestive pancreatic enzymes (trypsin, lipase, alkaline and acid phosphatases) in two Atherinopsids, including the pejerrey (Toledo Cuevas et al., 2011). For this reason, further insight into a wider repertoire of digestive enzymes is needed in order to get a deeper knowledge of the digestive capacities of this agastric species.

Digestion is a key process in animal metabolism since it determines the availability of nutrients needed for all biological functions. Thus, digestive physiology studies are an important issue when considering the culture of a determined species (Gisbert et al., 2013; Castro-Ruiz et al., 2019). Assessing the functionality of the digestive system along ontogeny is a valuable tool for inferring the digestion capacity of larvae; and thereby, designing an adequate feeding protocol to meet larval nutritional requirements, as well as promoting larval growth and survival (Gisbert et al., 2013). In this sense, the biochemical analysis of the digestive enzyme activities is an easy and reliable method for providing valuable information on the digestive physiology of fish larvae and their nutritional condition (Bolasina et al., 2006).

Thereby, the aim of this study was to describe changes in the activity of the main digestive (pancreatic and intestinal) enzymes in pejerrey. The information obtained will allow improving actual feeding practices during the first stages of development, which will have a positive impact on the improvement of actual rearing practices, as well as on larval quality.

2. Material and methods

2.1. Larval and early juvenile rearing

Fertilized eggs were obtained from natural spawnings from a broodstock maintained at the INTECH aquatic facilities (Argentina) during December 2017. After hatching, larvae were transferred to three 130 L aerated blue tanks (initial density = 7 larvae L^{-1}) and maintained until 9 weeks post-hatching (wph) under natural photoperiod, and a salinity equivalent to 15 g L^{-1} (NaCl equivalents). Water temperature was kept at 24.0 ± 1.0 ºC, a mixed-sex producing temperature for this species (Yamamoto et al., 2014).

During the experimental period, and from 2 days post-hatching (dph), larvae were fed 5 times a day exclusively with *Artemia* sp. nauplii (average proximate composition = 52 % protein, 19% lipids, 15% carbohydrates; Léger et al., 1987) until 4 wph, when 2 of those meals were replaced with a commercial feed (TetraMin® = 47% crude protein; 10% crude fat). From the 6th wph until the end of the experiment, larvae were fed twice a day with *Artemia* and three times a day with the above-mentioned commercial feed.

2.2. Sampling

All fish samplings were performed before the first feeding in the morning in order to avoid the potential effect of exogenous enzymes from live prey on larval digestive activities. Pejerrey larvae were randomly collected at 1, 2, 3, 4, 5, 7 and 9 wph, that corresponded to 7 14, 21, 28, 35, 49 and 63 dph. At each sampling point, the number of specimens sampled varied in order to get a minimum wet weight (WW) of 200 mg for analytical purposes. Once removed from the rearing tank, fish were anesthetized with benzocaine (50 ppm in water) and after measuring their body weight, they were frozen at -80 ºC and then lyophilized for shipping purposes. There were less than 6 months between sampling time and enzyme activity measurements in order to avoid the potential loss of enzyme activities (Solovyev and Gisbert, 2016).

2.3. Enzymatic assays

The preparation of fish extracts from analytical purposes was conducted at the Centre of Sant Carles de la Ràpita of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain). For enzymatic analyses, the whole body of larvae aged 1-week was processed because larvae were too small to be dissected. At older ages, lyophilized pejerrey specimens were dissected in a prechilled glass plate maintained at 0–4 ºC and their digestive system (abdominal region) was removed and processed for the assays.

Lyophilized samples were homogenized in 30 volumes (v/w) of Tris-Mannitol buffer (50 mM Mannitol, 2 mM Tris-HCl; pH 7.5) for 30 s (Ultra-Turrax T25, Germany); then, 100 µL of 0.1M $CaCl_2$ was added to the homogenate and then, samples were sonicated (Vibra-cell©, Sonics, Germany) for 1 minute. During the homogenizing process, samples were kept on ice (0-4 °C) for reducing the loss of enzymatic activity (Solovyev and Gisbert, 2016). An aliquot of each homogenate was stored at -80 °C until their analysis for determining activities of pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and α-amylase) and intestinal cytosolic (leucine-alanine peptidase) enzymes. Prior to enzyme analysis, extracts were centrifuged (3,300 *g* for 3 min at 4 °C) to reduce tissue and cell debris. Processed samples were analyzed within the first three weeks after their homogenization in order to prevent a loss of activity of pancreatic digestive enzymes (Solovyev and Gisbert, 2016).

The remaining homogenate was processed for intestinal brush border purification according to Gisbert et al. (2018) in order to properly determine alkaline phosphatase, maltase, and aminopeptidase N activities. Briefly, homogenates were centrifuged (9,000 *g* for 10 min at 4 °C), their precipitate discarded, and then the supernatant centrifuged once again (24,000 *g* for 30 min at 4 °C). The pellet, containing the brush border (BB) of enterocytes, was re-suspended in 1 mL of buffer (0.1 M KCl, 5 mM Tris-Hepes, 1 mM DTT; pH 7.5) and stored at -80 °C until analysis (Crane et al., 1979).

The determination of the activity of pancreatic and intestinal digestive enzymes was conducted using standard spectrophotometric methods as described in Gisbert et al. (2009). As pejerrey is an agastric fish, pepsin activity was not considered in this study. The activity of all digestive enzymes considered in the present study were measured at 30 °C. Regarding pancreatic enzymes, trypsin activity was assayed using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate in 50mM Tris-HCl, 20mM $CaCl_2$ + 1.5 mM NaCl buffer (pH 8.2) and changes in absorbance measured at λ = 407 nm (Holm et al., 1988). One unit of trypsin (U) was defined as 1 μmol BAPNA hydrolyzed per min^{-1} mL^{-1} of homogenate. Chymotrypsin activity was measured at λ = 405 nm using SAPNA (N-Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide) as substrate in 50mM Tris-HCl, 20mM $CaCl_2$ + 1.5 mM NaCl buffer (pH 8.2). Chymotrypsin activity (U) corresponded to the 1 μmol SAPNA hydrolyzed per min^{-1} mL^{-1} of homogenate (Worthington, 1991). The activity of total alkaline proteases was determined after 2 h of incubation using 0.5 % (w/v) azocasein as substrate in 50mM Tris-HCl, 20mM $CaCl_2$ + 1.5 mM NaCl buffer (pH 8.5). The reaction was stopped with trichloroacetic acid (20% w/v), the extract centrifuged (10,000 *g*, 5 min at 4 °C) and the absorbance of the supernatant read at room temperature and measured at λ = 366 nm. One unit of total alkaline proteases per mL (U) was defined as 1 μM azocasein hydrolyzed per min^{-1} mL^{-1} of homogenate (García-Careño and Haard, 1993). The α-amylase activity was measured at λ = 580 nm using soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate (0.3 % m/v) (Métais and Bieth, 1968); and its activity (U) was defined as the mg of starch hydrolyzed during 30 min per mL^{-1} of homogenate. Bile salt-activated lipase activity was measured using pNPM (p-nitrophenyl myristate) as substrate dissolved in 100mM Tris-HCl, 20mM $CaCl_2$ buffer (pH 8), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. Lipase activity (U) was defined as the µmol of substrate hydrolyzed per min^{-1} mL^{-1} of homogenate measured at $\lambda = 405$ nm (Iijima et al., 1998).

Regarding intestinal enzymes, alkaline phosphatase (AP) activity was quantified using PNPP (4-nitrophenyl phosphate) as substrate in 30 mM Na_2CO_3 buffer (pH 8). One unit (U) was defined as 1 µg nitrophenol released per min^{-1} mL^{-1} of BB homogenate and measured at $\lambda = 407$ nm (Bessey et al., 1946). Aminopeptidase N (AN) activity was determined according to Maroux et al. (1973) using sodium phosphate buffer 80 mM (pH 7.0) and L-leucine p-nitroanilide as substrate (in 0.1 mM DMSO). One unit of enzyme activity (U) was defined as 1 µg nitroanilide released per min^{-1} mL^{-1} of BB homogenate measured at $\lambda = 410$ nm. Maltase (MAL) activity was determined using D(+)-maltose as substrate in 100 mM sodium maleate buffer (pH 6.0) (Dahkqvist, 1970); one unit of maltase (U) was defined as µmol of glucose liberated min^{-1} mL^{-1} of homogenate at $\lambda = 420$ nm. The activity of the cytosolic intestinal digestive enzyme leucine-alanine peptidase (LAP) was quantified using the dipeptide leucine-alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0); one unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min^{-1} mL^{-1} of homogenate at $\lambda = 530$ nm (Nicholson and Kim, 1975). The index of intestinal maturation was calculated as the ratio of alkaline phosphatase to leucine-alanine peptidase (Cahu and Zambonino, 1995).

All enzymatic activities were measured using a microplate scanning spectrophotometer (Synergy HT, Bio-Tech, Germany) and expressed as specific (mU mg $protein^{-1}$) and total (mU $individual^{-1}$) enzyme activities. Soluble protein in enzyme extracts was quantified by the Bradford technique (1976) using bovine serum albumin as a standard. All the assays were made in triplicate (methodological replicates).

2.3. *Statistical analyses*

The activity of digestive enzymes was presented as mean ± standard error of the mean (SEM). Total and specific enzyme activity data were analyzed through non-parametric tests as normality and homocedasticity assumptions were not met even after ln(*x*) and square root (*x*) transformations. These analyses were performed under R environment with the nparLD package by using a longitudinal data model named LD-F1 and the ANOVA type statistic provided by the package (Noguchi et al., 2012), considering weeks as a within factor (F1). Whenever LD-F1 tests were significant, multiple post hoc comparisons were run between all weeks. All *P* values were adjusted by the Benjamini and Hochberg (1995) method to control the false discovery rate (FDR). Wet body weight and total length were transformed to ln(*x*) to meet normality and homocedasticity assumptions. For WW, the Greenhouse-Geisser correction was applied since sphericity assumption was not met. Transformed data were analyzed in IBM® SPSS® Statistics 23 software by repeated-measures ANOVA with week as a within factor followed by post hoc comparisons applying the Sidak correction to the *P* values. Adjusted *P* values lower than 0.05 were considered statistically significant.

3. Results

3.1. Larval growth analysis

Growth results of pejerrey in terms of WW and TL under the current feeding protocol applied during the first 9 weeks of life are shown in Figure 1. During the first feeding phase (food item: *Artemia* nauplii), a clear increase in WW and TL was observed between the 1st and 2nd wph (WW = 1.60 ± 0.10 mg *vs.* 5.63 ± 0.67 mg; TL = 6.85 ± 0.20 mm *vs.* 9.22 ± 0.50 mm; $P < 0.05$), which slightly continued to increase during the following weeks, especially in TL. After the inclusion of the compound feed in the feeding protocol, somatic growth sharply increased between the 4th and 5th wph (WW =

6.78 ± 0.47 mg *vs.* 31.38 ± 4.32 mg; TL = 10.62 ± 0.33 mm *vs.* 17.41 ± 0.89 mm; $P < 0.05$). In addition, an increase in WW and TL could be observed between weeks 7^{th} and 9^{th} (WW = 33.37 ± 5.75 mg *vs.* 54.92 ± 5.83 mg; TL = 17.34 ± 1.06 mm *vs.* 21.19 ± 0.84 mm). A high variability in the WW due to an increase in fish size dispersion was observed from the 5^{th} week onwards.

3.2. Pancreatic enzymes

Changes in the specific and total activities of the main pancreatic enzymes (trypsin, chymotrypsin, total alkaline proteases, α-amylase and bile-salt activated lipase) are shown in Figure 2. The activity of all assayed pancreatic enzymes was detected from the first week of development. Total and specific activities of pancreatic enzymes showed slightly differences between them, except of α-amylase. Trypsin total activity progressively increased from the 1^{st} to the 7^{th} wph, increasing from 0.02 ± 2.0 $*10^{-3}$ to 0.59 ± 0.01 mU individual^{-1} ($P < 0.05$) and then slightly decreased (0.49 ± 0.02 mU individual^{-1}; $P < 0.05$). Its total activity presented an increase greater than 2.5-fold between different week intervals (1 - 2, 4 - 5, and 5 - 7 wph). Trypsin specific activity slightly decreased from the 1^{st} to the 5^{th} wph, decreasing from 17.76 ± 1.59 mU to 13.55 ± 1.41 mg protein^{-1} ($P < 0.05$), but it significantly increased at the 7 wph, more than 2.5 times (57.18 ± 0. 72 mU mg protein^{-1}; $P < 0.05$) and then, it decreased at the 9^{th} wph (23.84 ± 0.89 mU mg protein^{-1}; $P < 0.05$). Chymotrypsin total and specific activities progressively increased until they reached their maximum levels at the 7^{th} wph (total activity = 17.68 ± 0.56 mU individual^{-1}; specific activity = 1724.78 ± 33.72 mU mg protein^{-1}), whereas both sharply decreased at the 9^{th} wph (total activity = 9.39 ± 0.15 mU individual^{-1}; specific activity = 454.01 ± 14.39 mU mg protein^{-1}). As it was expected, the activity of total alkaline proteases presented a similar profile than that of trypsin and chymotrypsin.

Total and specific activities of bile salt-activated lipase progressively increased until they reached their maximum levels at the 7th wph (total activity = 6.38 ± 0.44 mU individual^{-1}; specific activity = 552.60 ± 40.12 mU mg protein^{-1}), whereas they sharply decreased at 9th wph (total activity = 4.53 ± 0.38 mU individual^{-1}; specific activity = 194.03 ± 18.75 mU mg protein^{-1}). Interestingly, total activity values displayed an increase greater than 2.5-fold during the transition to the compound feed. In the case of α-amylase, its total activity increased from the 1st wph (0.26 ± 0.04 mU individual^{-1}) until the end of the trail (8.53 ± 0.25 mU individual^{-1}) with the exception of the 3rd and 4th wph where not statistically significant differences were found between them. The main change in activity was recorded between the 5th and the 7th wph, changes that were related to the change in the feeding protocol. In addition, α-amylase specific activity oscillated during all the experimental period, showing the highest activity values at the 7th wph (500.58 ± 12.49 mU mg protein^{-1}; $P < 0.05$).

3.3. Intestinal enzymes

Changes in the specific and total activities of brush border (alkaline phosphatase, maltase and aminopeptidase N) and cytosolic (leucine-alanine peptidase) intestinal enzymes are shown in Figure 3. The activity of all intestinal enzymes was detected from the first week of development. Total activities of BB enzymes showed a similar profile: they progressively increased from the 1st until the 9th wph, when they reached their maximum activities (AN = $3.2*10^{-3} \pm 3.55*10^{-4}$ to 0.25 ± 0.01 mU individual^{-1}; AP = from = $0.04 \pm 9.88*10^{-4}$ to 1.53 ± 0.02 mU individual^{-1}; MAL = $7.85*10^{-4} \pm 1.72*10^{-4}$ to 0.23 ± 0.01 mU individual^{-1}; $P < 0.05$). Interestingly, immediately after the administration of the compound feed that occurred between the 4th and the 5th wph, no differences in total activity were found in AP either MAL ($P > 0.05$). Only MAL total activity presented an increase greater than 2.5 times between several post-hatching weeks (1^{st} - 2^{nd}, 3^{th} -. 4^{th}, and 7^{th} - 9^{th} wph). Aminopeptidase N specific activity showed no differences between the first 3 weeks and then increased until reaching maximum activity levels at the 7^{th} and the 9^{th} wph (168.63 and 175.98 mU mg protein^{-1}, respectively, $P > 0.05$). Alkaline phosphatase specific activity was more stable during development, showing no differences throughout the 1^{st} and the 5^{th} wph, whereas an increase in activity was detected between the 7^{th} and the 9^{th} wph (1092.59 and 1093.7 mU mg protein^{-1}, respectively, $P > 0.05$). Maltase specific activity gradually increased until reaching its highest activity values at the 9^{th} wph (5.40 ± 0.32 mU mg protein^{-1}). Interestingly, the greatest increase was observed between the 3^{rd} and 4^{th} weeks, before the administration of the compound diet, whereas no differences in activity were found immediately after the inclusion of the compound feed (4^{th} - 5^{th} wph, $P > 0.05$). On the other hand, total and specific activities of leucine-alanine peptidase oscillated during the first 4 wph, and then both reached their maximum values at the 7^{th} week (total activity = 1750 ± 190 mU individual^{-1}; specific activity = 428,720 ± 58,710 mU mg protein^{-1}), and decreased between the 7^{th} and the 9^{th} wph (total activity = 1260 ± 210 mU individual^{-1}; specific activity = 177,620 ± 38,950 mU mg protein^{-1}; $P < 0.05$).

The ratio of alkaline phosphatase to leucine-alanine peptidase was calculated as an indicator of the shift between intracellular and luminal protein digestion (Figure 4). A variable profile (AP/LAP) was obtained, without a marked change or trend towards luminal digestion.

4. Discussion

The ontogeny of the digestive tract has been deeply studied in many fish species, particularly marine ones, with the purpose of improving larval culture techniques and weaning processes (Rønnestad et al., 2013). Considering the difficulties in assessing the nutritional requirements of fish larvae due to their small size and reduced acceptance of microdiets, the knowledge about the ontogeny of the activity of the main digestive enzymes allow to infer the digestion capacity of larvae; and thereby, designing an adequate feeding protocol to meet larval nutritional requirements, as well as promoting their growth and survival (Gisbert et al., 2013). Despite of this and the interest on the potential culture of Atheriniformes, few studies have been performed on the ontogeny of the digestive system in this group of fishes (Horn et al., 2006; Toledo-Cuevas et al., 2011). These studies concluded that despite this taxon having simple digestive tracts, as they are an agastric group of species, they present different feeding habits, which make them very interesting for comparative studies (Horn et al., 2006). Thus, the present study is the first description of the ontogenic changes of nine digestive enzymes during the larval development of the pejerrey, an interesting species for the South American aquaculture, which is not commercially exploited so far. Different authors have proposed that the larvae-juvenile transition in pejerrey could externally be visualized by the reabsorption of a fin fold between the anus and the anal fin, a change reported from 42 to 56 dph at 24 ºC (20.1 to 23 mm TL; Chalde et al. 2011). Considering the former authors, data on digestive enzyme activities reported in the present study corresponded to the larval stage of pejerrey; showing that, for this species, there is a good match between external morphological indicators and physiological ones.

Digestive enzymes for protein, lipids, and carbohydrate hydrolysis were detected from the first week post-hatching (6.85 mm TL). In particular, trypsin total activity presented three major changes in activity during the study, although these changes differed in their magnitude. The first one was comprised from the 1st to the 5th wph, and it was characterized by an increase in total activity of 562%; similarly, the second one (Δ = 439 %) occurred between the 5th and the 7th wph, whereas trypsin total activity decreased between the 7th and the 9th wph (Δ = -16 %). The two above-mentioned increments in activity may be explained by the progressive fast larval growth observed between hatching and the 4th wph, as well as to the changes in the feeding protocol (a swift between a diiet based only on *Artemia* nauplii to a co-feeding regime based on live prey and the inert diet). Changes in enzyme activities during the first stages of development resulting in an enhancement of larval proteolytic capacities are generally associated with the development of the exocrine pancreas, as well as changes in the diet (Rønnestad et al., 2013). Regarding the decrease in trypsin total activity observed between the 7th and 9th wph (Δ = -16 %), this may be attributed to the adaptation of larvae to the compound feed, as well as to the full maturation of the exocrine pancreas (Rønnestad et al., 2013). Concerning the reduction in trypsin specific activity during the first weeks of life, these activity changes did not correspond to a real reduction in the proteolytic digestive larval performance, as values in total activity might indicate; but rather to an increase of larval growth and the presence of protein from other non-digestive tissues (trunk musculature attached to the abdominal cavity). This result is a common feature when dealing with small larvae (Zambonino-Infante et al., 2008), whereas at larger sizes (>5 wph) the above-mentioned artifact was not detected.

The activity of other proteolytic pancreatic enzymes like chymotrypsin followed the same activity profiles as trypsin. These results are of special importance since chymotrypsin is activated by trypsin through the cleavage of the bond between arginine and isoleucine, causing structural modifications and formation of the substrate-binding site (Appel, 1986). Thus, the parallel activity profiles of both proteases indicated a tight regulation of alkaline proteolytic activities in pejerrey larvae, which is of special relevance considering that this is an agastric species. Considering these results and the increment in total alkaline protease activities observed at the 2nd wph, we may conclude that the exocrine pancreas in pejerrey under presented rearing conditions achieved its functional development at the 2nd wph when larvae measured 9.22 mm in TL.

Regarding α-amylase, specific activity values showed a saw-type profile with minimal values observed at the 2nd and the 5th wph, and maximal values at the 7th wph; changes that seemed to be correlated to shifts in the feeding protocol. However, when considering α-amylase total activity values, this glucosidase showed a progressive increase over development, which contrasts to the zooplanktonic feeding habits of this species (Zagarese, 1991). In this sense, it has been generally considered that the feeding habits of fish species are well correlated to their digestive physiology, *i.e.*, α-amylase activity is higher in herbivorous and omnivorous fish compared to carnivores (Hidalgo et al. 1999; Solovyev et al., 2016). Thus, the high levels of α-amylase at late stages of development in this species may be an adaptation for proper digestion of carbohydrate content in zooplanktonic preys [0.3 – 29.0% in dry weight; Ventura, (2006)]. Additionally, the α-amylase activity in carnivorous species is typically high at early stages and decreases during development as the pancreatic function develops (Cahu and Zambonino-Infante, 2001), which contrasts with our data on pejerrey as a planktivorous fish. Although the early increase in α-amylase may be genetically programmed, its progressive increase with larval development may be dietary induced. Thus, changes in the activity of this glucosidase during the first weeks of larval rearing may be attributed to the carbohydrate content (10 – 15%) of *Artemia* nauplii that may stimulate the synthesis of this enzyme (Léger et al., 1987; Ma et al., 2005; Castro-Ruiz et al, 2019), whereas the dramatic increase observed after the 5th wph may be attributed to the shift to the inert diet due to its content in corn starch and algal ingredients (data provided by manufacturer). Although this species is considered carnivorous, some authors described it as an opportunistic zooplanktivorous, since plant elements have also been found in their gut contents (Scasso and Campos, 1999; Cassemiro et al., 2003). Moreover, the high α-amylase found in this agastric species may be a strategy for improving dietary protein digestion, since the increased α-amylase activity from the high levels of carbohydrate may have exposed more protein substrate leading to increased proteolytic activity.

Bile salt-activated lipase is a key enzyme for fat digestion, especially for hydrolyzing triacylglycerides, in addition to phospholipids, esters of cholesterol, and lipid-soluble vitamins. Its activity is modulated by lipid composition (Morais et al., 2004; Zambonino-Infante et al., 2008). Under present experimental conditions, total and specific lipase activities progressively increased during the first 5 wph, whereas after the transition to the inert compound diet at the 7^{th} wph, a sharp increase in activity was recorded. This increase may be attributed to the further maturation of the exocrine pancreas function rather than dietary fat levels of the compound diet (13% dry weight) that were lower than those of live prey (19%; Léger et al., 1987). Thus, the above-mentioned changes in lipase activity may be also related to the fatty acid profile of the diet (Morais et al., 2004), although the understanding of the underlying mechanisms controlling lipase secretion and regulation are still incomplete. The decrease in lipase activity observed at the 9^{th} wph may be related to the adaptation of larvae to the formulated diet during this period of co-feeding, although they might be either indicative of changes in the nutritional requirements, which were reflected in a reduction in larval growth rate (Castro-Ruiz et al., 2019).

The present results related to the ontogenic changes in activity of pancreatic and intestinal enzymes in pejerrey and their nutritional regulation may be considered as the first step for assessing and refining the nutritional requirements during the larval stages of this agastric species. For instance, the progressive increase of carbohydrate hydrolases during the larval period (from hatching until 21 mm TL) may suggest that the gradual incorporation of dietary carbohydrates in a feeding protocol may have a protein-sparing effect, as well as a cheap and fast way to obtain energy for proper development and growth of pejerrey. Regarding dietary lipids, Gómez-Requeni et al. (2013) reported that an increase of lipids in formulated diets increased growth and survival performance in this species; thus, considering the bile salt-activated lipase showed an important increase just before the introduction of the compound diet at the 3rd wph when larvae measured 10.5 mm TL, the incorporation of enriched *Artemia* metanauplii during the first meals of this species could be a beneficial strategy to improve larval growth and development, since actual feeding practices just consider the use of *Artemia* nauplii and un-enriched metanauplii.

When considering total activity values of the three assayed intestinal brush border enzymes, all of them showed a steady increase during the studied period, indicating the development of the intestinal mucosa. In gastric species, it is generally considered that gut maturation correlates with changes in activity between those enzymes anchored into the enterocyte's brush border (*i.e.*, alkaline phosphatase, aminopeptidase N and maltase) and peptidases from the cytosol (leucine-alanine peptidase). The above-mentioned changes are also linked to changes in the mode of digestion, shifting from an alkaline proteolytic digestion, based on pancreatic alkaline proteases in combination with intestinal cytosolic peptidases (intracellular digestion) to an acid (luminal) digestion in which pepsin takes a major role in protein digestion (Zambonino-Infante et al., 2008). Although this pattern in the changes in the mode of digestion has been deeply studied and characterized among a large variety of freshwater and marine gastric species (Rønnestad et al., 2013), little is known about agastric ones. Present data on pejerrey indicated that there is not a shift in the ratio of brush border to cytosolic intestinal enzymes as described in gastric species, indicating that in agastric species intracellular protein digestion plays a major role in comparison to luminal digestion during larval development. As already mentioned, there is another study where the activity of some digestive enzymes was evaluated in larvae of pejerrey fed under a different feeding protocol (Toledo-Cuevas et al., 2011). Unlike our results, the former authors reported a rise in the activities of brush border enzymes in correlation with the decrease in cytosolic activities. These different conclusions could be explained by differences in the extraction protocols performed for intestinal brush border enzyme purification (Gisbert et al., 2018) or by different feeding protocols (Rønnestad et al., 2013).

When considering the activity of brush border enzymes, the pattern of maltase activity, with the highest values found at the 9th wph, differed to those observed in the other assayed brush border enzymes (alkaline phosphatase and aminopeptidase-N) that remained stable between the 7th and the 9th wph. These results may be due to the presence of corn starch in the compound diet, highlighting the importance of maltase in the digestion of starch-type carbohydrates. Thus, the pancreatic α-amylase would participate in the first stages of starch digestion, and its hydrolysis products (disaccharides like maltose) finally digested by maltase in the brush border of enterocytes. These results indicated that similarly to α-amylase, maltase activity is dietary regulated by starch levels as both enzymes participate in starch digestion (Koven et al., 2020). Regarding alkaline phosphatase, this enzyme has multiple functions at the intestinal level; for instance, this enzyme participates in the regulation of enterocyte luminal surface pH and barrier function, detoxification of pro-inflammatory microbial components, modulation of the gut microbiota and control of nutrient absorption (e.g. calcium, phosphorus, fatty acids) (Lallès, 2019). Thus, considering the increase and stabilization of alkaline phosphatase activity after the 7th wph (17.34 mm TL) and its important roles in the gut epithelium, we may conclude that intestinal villi of pejerrey may achieve is fully functionality and maturation at this stage of development (Zambonino-Infante and Cahu, 2001).

Conclusions

This is the first study in which a complete assessment of the activity of pancreatic and intestinal enzymes is conducted during the early ontogeny of pejerrey. The analysis of proteolytic pancreatic enzymes revealed that the exocrine pancreas in this agastric species achieved its functional development at 9.22 mm TL. Interestingly, the progressive increase in total activities of α-amylase and maltase suggested an opportunity for improving larval development as a fast energy source could be obtained by progressively increasing dietary carbohydrates. In addition, these results reflected a strategy of this species to improve protein digestion in the case of scarce zooplankton availability. The analysis of intestinal enzymes revealed that the typical shift between intracellular and luminal protein digestion that occurs during larval development in gastric species, did not take place in pejerrey, indicating that in agastric species intracellular protein digestion plays a major role in comparison to luminal digestion during larval development. Contrary to gastric species, our results indicated that the ratio of alkaline phosphatase to leucine-alanine peptidase for evaluating gut maturation in agastric species is not recommended, and other parameters should be measured when evaluating the maturation process in fish larvae from this group of species.

5. References

Appel, W., 1986. Chymotrypsin: molecular and catalytic properties. Clin. Biochem. 19, 317-322. https://doi.org/10.1016/S0009-9120(86)80002-9

Ball, J.R., Esler, D., Schmutz, J.A., 2007. Proximate composition, energetic value, and relative abundance of prey Wsh from the inshore eastern Bering Sea: implications for piscivorous predators. Polar Biol. 30, 699–708. https://doi.org/10.1007/s00300-006-0227-1

Benjamini, Y., and Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

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.

Berasain, G., Colautti, D., Velasco, C.A.M., 2000. Experiencias de cría de pejerrey. *Odontesthes bonariensis*, Revista de Icitiología 8, 1-7 (in Spanish).

Bertucci, J.I., Tovar, M.O., Unniappan, S., Navarro, J.C., Canosa, L.F., 2018. Effects of dietary sunflower oil on growth parameters, fatty acid profiles and expression of genes regulating growth and metabolism in the pejerrey (*Odontesthes bonariensis*) fry. Aquac. Nutr. 24, 748-757. https://doi.org/10.1111/anu.12603

Bolasina, S., Tagawa, M., Yamashita, Y., Tanaka, M., 2006. Effect of stocking density on growth, digestive enzyme activity and cortisol level in larvae and juveniles of Japanese flounder, *Paralichthys olivaceus*. Aquaculture 259, 432-443. https://doi.org/10.1016/j.aquaculture.2006.05.021

Bradford, M.M., 1976. A rapid and sensitive method for the quantification 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

Cahu, C.L., Zambonino Infante, J.L., 1995. Maturation of the pancreatic and intestinal digestive functions in sea bass *Dicentrarchus labrax*: effect of weaning with different protein sources. Fish Physiol. Biochem. 14, 431–437 https://doi.org/10.1007/BF00004343

Cahu, C., Zambonino Infante, J., 2001. Substitution of live food by formulated diets in marine fish larvae. Aquaculture 200, 161–180. https://doi.org/10.1016/S0044-8486(01)00699-8

Cassemiro, F.A.D.S., Hahn, N.S., Rangel, T.F.L.V.D., 2003. Diet and trophic ecomorphology of the silverside, *Odontesthes bonariensis*, of the Salto Caxias reservoir, rio Iguaçu, Paraná, Brazil. Neotrop. Ichthyol. 1, 127-131. http://dx.doi.org/10.1590/S1679-62252003000200007

Castro-Ruiz, D., Mozanzadeh, M.T., Fernández-Méndez, C., Andree, K.B., García-Dávila, C., Cahu, C., Gisbert, E., Darias, M.J., 2019. Ontogeny of the digestive enzyme activity of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Castelnau, 1855). Aquaculture 504, 210-218. https://doi.org/10.1016/j.aquaculture.2019.01.059

Chalde, T., Fernández, D.A., Cussac, V.E., Somoza, G.M., 2011. The effect of rearing temperature in larval development of pejerrey, *Odontesthes bonariensis*: morphological indicators of development. Neotrop. Ichthyol. 9, 747-756. http://dx.doi.org/10.1590/S1679-62252011005000040

Craig J.F., Kenley M.J., Talling J.F., 1978. Comparative estimations of the energy content of Wsh tissue from bomb calorimetry, wet oxidation and proximate analysis. Freshw. Biol. 8, 585–590. https://doi.org/10.1111/j.1365-2427.1978.tb01480.x

Dahkqvist, A., 1970. Assay of intestinal disaccharidase. Enzym. Biol. Clin 11, 52–66. https://doi.org/10.1159/000458348

Elisio, M., Chalde, T., Miranda, L.A., 2014. Seasonal changes and endocrine regulation of pejerrey (*Odontesthes bonariensis*) oogenesis in the wild. Comp. Biochem. Physiol. 175A, 102-109. https://doi.org/10.1016/j.cbpa.2014.05.020

Elisio, M., Chalde, T., Miranda, L.A., 2015. Seasonal changes and endocrine regulation of pejerrey (*Odontesthes bonariensis*) spermatogenesis in the wild. Gen. Comp. Endocrinol. 221, 236-243. https://doi.org/10.1016/j.ygcen.2015.01.011

FAO, 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome, Italy. Available at: http://www.fao.org/3/i9540en/i9540en.pdf

Fernandino, J.I., Popesku, J.T., Paul-Prasanth, B., Xiong, H., Hattori, R. S., Oura, M., Strüssmann C.A., Somoza G.M., Matsuda M., Nagahama Y, Trudeau, V.L., 2011. Analysis of sexually dimorphic expression of genes at early gonadogenesis of pejerrey *Odontesthes bonariensis* using a heterologous microarray. Sex. Dev., 5, 89-101. https://doi.org/10.1159/000324423

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

Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287, 381-387. https://doi.org/10.1016/j.aquaculture.2008.10.039

Gisbert, E., Morais, S., Moyano, F.J., 2103. Feeding and digestion. In: Quin, J.G. Larval Fish Aquaculture. Nova Science Publishers, New York, pp. 73-123.

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

Gómez-Requeni, P., Bedolla-Cázares, F., Montecchia, C., Zorrilla, J., Villian, M., Toledo-Cuevas, E.M., Canosa, F., 2013. Effects of increasing the dietary lipid levels on the growth performance, body composition and digestive enzyme activities of the teleost pejerrey (*Odontesthes bonariensis*). Aquaculture 416–417, 15–22. https://doi.org/10.1016/j.aquaculture.2013.08.027

González, A., Fernandino, J.I., Somoza, G.M., 2015. Effects of 5α-dihydrotestosterone on expression of genes related to steroidogenesis and spermatogenesis during the sex determination and differentiation periods of the pejerrey, *Odontesthes bonariensis*. Comp Biochem. Physiol. 182A, 1-7. https://doi.org/10.1016/j.cbpa.2014.12.003

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, Å., & 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

Horn, M.H., Gawlicka, A.K., German, D.P., Logothetis, E.A., Cavanagh, J.W., Boyle, K.S., 2006. Structure and function of the stomachless digestive system in three related species of New World silverside fishes (Atherinopsidae) representing herbivory, omnivory, and carnivory. Mar. Biol. 149, 1237-1245. https://doi.org/10.1007/s00227-006-0281-9

Hughes, L.C., Somoza, G.M., Nguyen, B.N., Bernot, J.P., González-Castro, M., Díaz de Astarloa, J.M., Ortí, G., 2017. Transcriptomic differentiation underlying marine-to- *O. bonariensis* (Atheriniformes). Ecol. Evol. 7, 5258-5268. https://doi.org/10.1002/ece3.3133

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

Kopprio, G.A., Graeve, M., Kattner, G., Lara, R.J., 2015. Fatty acid composition of wild *Odontesthes bonariensis* (Valenciennes 1835) larvae: implications on lipid metabolism and trophic relationships. J. Appl. Ichthyol. 31, 752-755. https://doi.org/10.1111/jai.12786

Koven, W., Gisbert, E., Meiri-Ashkenazi, I., Nixon, O., Israeli, D., Tandler, A. Nolasco Soria, H, Solovyev, M. Rosenfeld, H., 2020. The effect of weaning diet type on grey mullet (*Mugil cephalus*) juvenile performance during the trophic shift from carnivory to omnivory. Aquaculture 518, 734848. https://doi.org/10.1016/j.aquaculture.2019.734848

Lallès, J.P., 2019. Intestinal alkaline phosphatase in the gastrointestinal tract of fish: biology, ontogeny, and environmental and nutritional modulation. Rev. Aquac. *in press* https://doi.org/10.1111/raq.12340

Léger, P., Bengston, D.A., Sorgeloos, P., 1987. The nutritional value of Artemia. In: Artemia research and its applications, Vol. 3. Ecology, culturing, use in aquaculture. Sorgeloos, P., Bengtson, D.A., Decleir, W. and Jaspers. E. (Eds). Universa Press, Wetteren, Belgium. 556 p.

Ma, H., Cahu, C., Zambonino Infante, J.L., Ya, H., Duana, Q., Le Gall, M.M., Ma, K., 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). Aquaculture 245, 239–248. https://doi.org/10.1016/j.aquaculture.2004.11.032

Maroux, S., Louvard, D., Baratti, J., 1973. The aminopeptidase from hog-intestinal brush border. Biochim. Biophys. Acta 321, 282–295. https://doi.org/10.1016/0005-2744(73)90083-1

Métais, P., Bieth, J., 1968. Détermination de l'α-amylase. Ann. Biol. Clin. 26, 133–142

Miranda, L.A., Chalde, T., Elisio, M., Strüssmann, C.A., 2013. Effects of global warming on fish reproductive endocrine axis, with special emphasis in pejerrey *Odontesthes bonariensis*. Gen. Comp. Endocrinol. 192, 45-54. https://doi.org/10.1016/j.ygcen.2013.02.034

Miranda, L.A., Berasain, G.E., Velasco, C.A.M., Shirojo, Y., Somoza, G.M., 2006. Natural spawning and intensive culture of pejerrey *Odontesthes bonariensis* juveniles. Biocell 30, 157-162.

Miranda, L.A., Strobl-Mazzulla, P.H., Strüssmann, C.A., Parhar, I., Somoza, G.M., 2003. Gonadotropin-releasing hormone neuronal development during the sensitive period of temperature sex determination in the pejerrey fish, *Odontesthes bonariensis*. Gen. Comp. Endocrinol. 132, 444-453. https://doi.org/10.1016/S0016-6480(03)00117-5

Morais, S., Narciso, L., Dores, E., Pousao-Ferreira, P., 2004. Lipid enrichment for Senegalese sole (*Solea senegalensis*) larvae: effect on larval growth, survival and fatty acid profile. Aquac. Int. 12, 281–298. https://doi.org/10.1023/B:AQUI.0000036184.13187.6b

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

Noguchi, K., Gel, Y.R., Brunner, E., and Konietschke, F., 2012. nparLD: An R Software Package for the Nonparametric Analysis of Longitudinal Data in Factorial Experiments. J. Stat. Soft. 50, 1-23. doi: 10.18637/jss.v050.i12.

Rønnestad, I., Yúfera, M., Ueberschär, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013. Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev. Aquac. 5, S59-S98. https://doi.org/10.1111/raq.12010

Scasso, F., & Campos, H., 1999. Comparison of two populations of silverside (*Odontesthes bonariensis*) in Eutrophic lakes of Central Chile. J. Freshw. Ecol. 14, 61-70. https://doi.org/10.1080/02705060.1999.9663655

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. doi: 10.1007/s10695-016-0226-2.

Solovyev, M.M., Kashinskaya, E.N., Izvekova, G.I., Gisbert, E., Glupov, V.V., 2016. 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

Somoza, G.M., Miranda, L.A., Berasain, G.E., Colautti, D., Remes Lenicov, M., Strüssmann, C.A., 2008. Historical aspects, current status and prospects of pejerrey aquaculture in South America. Aquac. Res. 39, 784-793. https://doi.org/10.1111/j.1365-2109.2008.01930.x

Strüssmann, C.A., Takashima, F., Toda, K., 1996. Sex differentiation and hormonal feminization in pejerrey *Odontesthes bonariensis*. Aquaculture 139, 31-45. https://doi.org/10.1016/0044-8486(95)01161-7

Strüssmann, C.A., Conover, D.A., Somoza, G.M., Miranda, L.A., 2010. Implications of climate change for the reproductive capacity and survival of New World silversides (family Atherinopsidae). J. Fish Biol. 77, 1818-1834. https://doi.org/10.1111/j.1095-8649.2010.02780.x

Toledo-Cuevas, E.M., Moyano López, F.J., Ramírez, D.T., Strüssmann, C.A., Álvarez-González, C.A., Martínez-Chávez, C.C., & Martínez- Palacios, C.A., 2011. Development of digestive biochemistry in the initial stages of three cultured Atherinopsids. Aquac. Res. 42, 776-786. https://doi.org/10.1111/j.1365-2109.2011.02853.x

Ventura, M. 2006. Linking biochemical and elemental composition in freshwater and marine crustacean zooplankton. Mar. Ecol. Prog. Ser. 327, 233–246. doi:10.3354/meps327233

Vigliano, F.A., Muños, L., Hernández, D., Cerutti, P., Bermúdez, R., Quiroga, M.I., 2011. An immunohistochemical study of the gut neuroendocrine system in juvenile pejerrey *Odontesthes bonariensis* (Valenciennes). J. Fish Biol. 78, 901–911. https://doi.org/10.1111/j.1095-8649.2011.02912.x

Worthington Biochemical Corporation, 1991. Worthington Enzyme Manual: Enzymes, Enzyme Reagents, Related Biochemical Worthington Biochemical Corp., Freehold, New Jersey.

Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R.S., Strüssmann, C.A., 2014. Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. PLoS ONE 9, e102574. https://doi.org/10.1371/journal.pone.0102574

Zambonio Infante, J.L., Cahu, C., 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comp. Biochem. Physiol. 130C, 477-487. https://doi.org/10.1016/S1532-0456(01)00274-5

Zambonino-Infante, J.L., Gisbert, E., Sarasquete, C., Navarro, I., Gutierrez, J., Cahu, C.L., 2008. Ontogeny and physiology of the digestive system of marine fish larvae. In: Cyrino, J.E.P., Bureau, D.P., Kapoor, B.G. (Eds.), Feeding and Digestive Functions of Fishes. Science Publishers, New Hamphire, pp. 281–348.

Zagarese, H.E., 1991. Planktivory by larval *Odontesthes bonariensis* (Atherinidae: Pisces) and its effects on zooplankton community structure. J. Plankton Res. 13, 549-560. https://doi.org/10.1093/plankt/13.3.549

Zhang, Y., Hattori, R.S., Sarida, M., García, E.L., Strüssmann, C.A., Yamamoto, Y., 2018. Expression profiles of amhy and major sex-related genes during gonadal sex differentiation and their relation with genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. Gen. Comp. Endocrinol. 265, 196-201. https://doi.org/10.1016/j.ygcen.2018.03.013

Acknowledgements

This work has been done within the framework of the network LARVAplus 'Strategies for the development and improvement of fish larvae production in Ibero-America' (117RT0521) funded by the Ibero-American Program of Science and Technology for Development (CYTED, Spain). This work has also been supported by Universidad de Buenos Aires (grant number: 20020160100110BA to PV), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 2014–2016:11220130100501CO to PV).

Author contributions

DIPS, LAM, GMS, PV, and EG contributed to the design of the work; JEH, LAM, and GMS contributed to the sampling; DIPS, MS, and EG contributed to the enzyme activity measurement and the interpretation of the results; THD contributed to the statistic analysis; DIPS, PV, and EG contributed to the writing of the manuscript. All authors approved the final version of the manuscript.

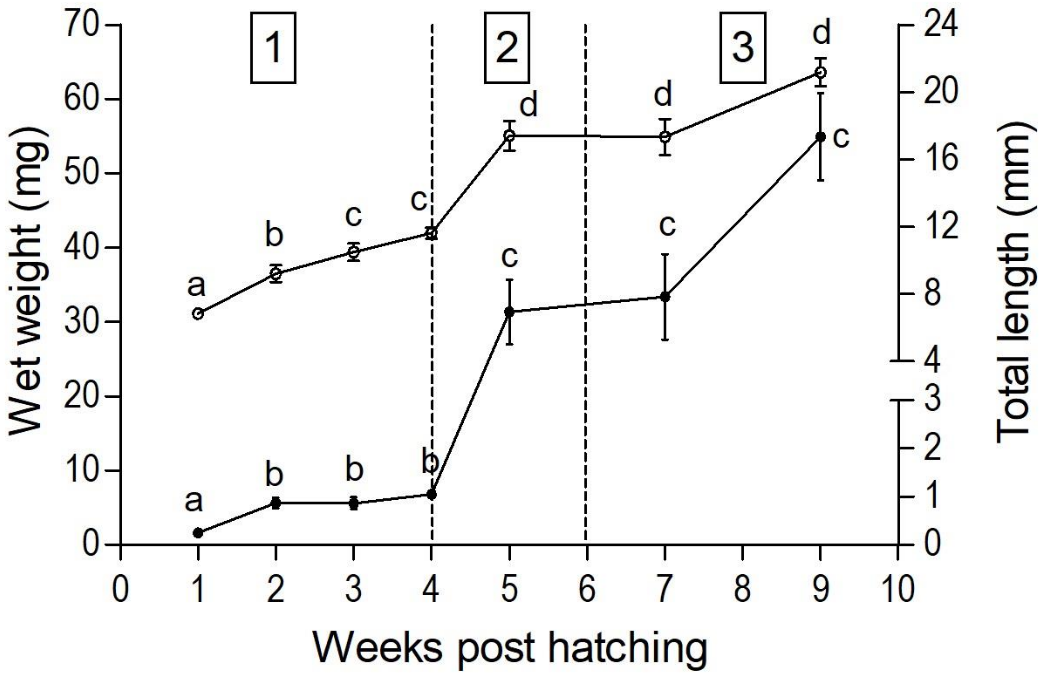

Figure 1. Larval and juvenile growth of pejerrey from 1 to 9 weeks post-hatching. Wet body weight (black circles) and total length (white circles) are expressed as mean ± SEM. Dotted lines indicate changes in the feeding protocol: 1) *Artemia* sp. nauplii supplied 5 times per day; 2) *Artemia* sp. nauplii supplied 3 times per day and artificial food, 2 times per day; 3) *Artemia* sp. nauplii supplied 2 times per day and artificial food, 3 times per day. Different letters indicate statistical differences.

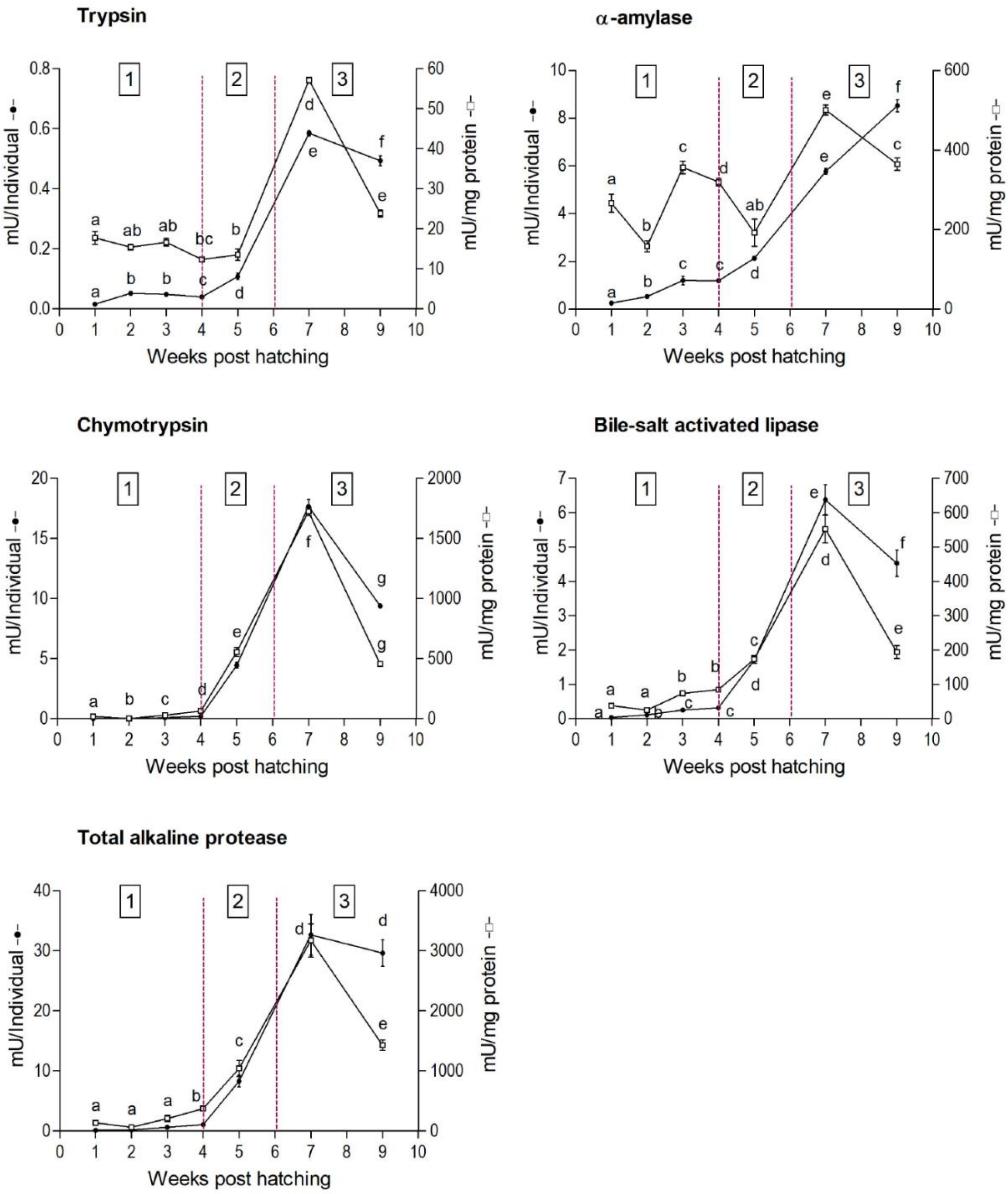

Figure 2. Total (black circles) and specific (white square) activity of pancreatic enzymes during pejerrey larval development. Results are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$). Dotted lines indicate changes in the feeding protocol: 1) *Artemia* sp. nauplii supplied 5 times per day; 2) *Artemia* sp. nauplii supplied 3 times per day and artificial food, 2 times per day; 3) *Artemia* sp. nauplii supplied 2 times per day and artificial food, 3 times per day.

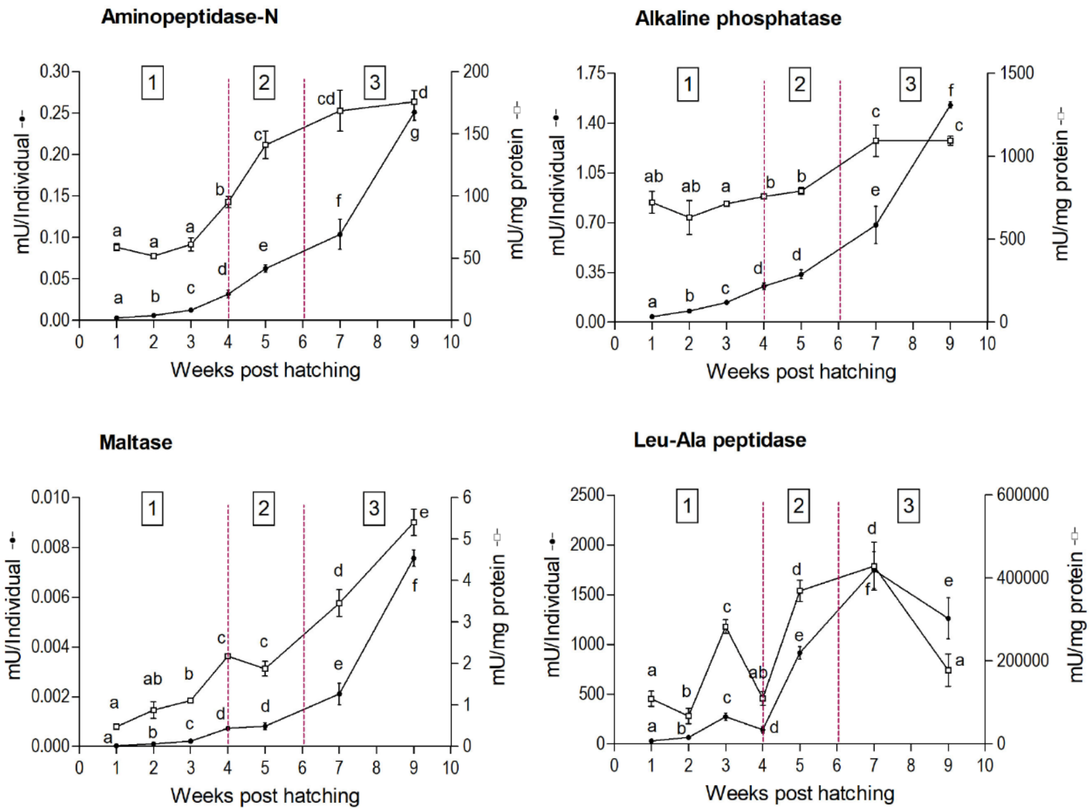

Figure 3. Total (black circles) and specific (white square) activity of brush border (alkaline phosphatase, maltase and aminopeptidase N) and cytosolic (leucine-alanine peptidase) intestinal enzymes during pejerrey larval development. Results are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$). Dotted lines indicate changes in the feeding protocol: 1) *Artemia* sp. nauplii supplied 5 times per day; 2) *Artemia* sp. nauplii supplied 3 times per day and artificial food, 2 times per day; 3) *Artemia* sp. nauplii supplied 2 times per day and artificial food, 3 times per day.

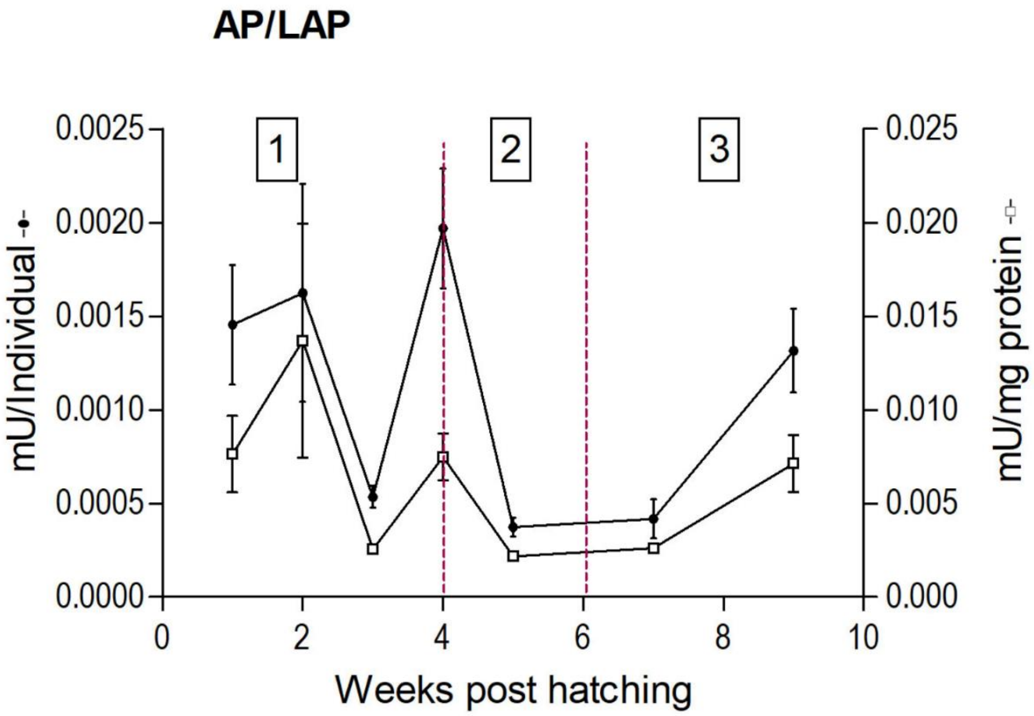

Figure 4. Calculated ratio of total (black circles) and specific (white square) activity of alkaline phosphatase to leucine-alanine peptidase during pejerrey larval development. Results are expressed as mean ± SEM. Dotted lines indicate changes in the feeding protocol: 1) *Artemia* sp. nauplii supplied 5 times per day; 2) *Artemia* sp. nauplii supplied 3 times per day and artificial food, 2 times per day; 3) *Artemia* sp. nauplii supplied 2 times per day and artificial food, 3 times per day.