Latin American Journal of Aquatic Research, 52(3): 368-382, 2024
DOI: 10.3856/vol52-issue3-fulltext-3155

Research Article

Early anatomical development of the digestive system and high intestinal digestive enzyme activity in two silverside species, *Odontesthes bonariensis* and *O. hatcheri* (Atherinomorpha: Atherinopsidae)

Elva Mayra Toledo-Cuevas[1], María Cristina Chávez-Sánchez[2]
María Antonia Herrera-Vargas[1*], Selene María Abad Rosales[2]
Evangelina Cortés Ortiz[1], Carlos Augusto Strüssmann[3]**
María Gisela Ríos-Durán[1] & Luciana Raggi[4]
[1]Instituto de Investigaciones Agropecuarias y Forestales
Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México
*Instituto de Investigaciones sobre los Recursos Naturales, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México
**CRIAP Pátzcuaro, Pátzcuaro, Michoacán, México
[2]Unidad Mazatlán en Acuicultura y Manejo Ambiental, CIAD, A.C., Mazatlán, Sinaloa, México
[3]Division of Marine Sciences, Department of Aquatic Biosciences, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan
[4]Consejo Nacional de Ciencia y Tecnología-Instituto de Investigaciones Agropecuarias y Forestales
Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México
Corresponding author: María Cristina Chávez-Sánchez (marcris@ciad.mx)

ABSTRACT. Integrative studies are important for a better understanding of the functional development of fish digestive systems and the consequent design of suitable feeding regimes and *ad-hoc* diets. To characterize the ontogeny of digestive function in larvae and juveniles of two atherinopsids from South America, *Odontesthes bonariensis* and *O. hatcheri*, morphologic, histologic, and intestinal enzyme biochemical analyses were performed. Both species showed a functional digestive system from one week after hatching (wah) with a sustained high-level activity of the cytosolic enzyme leucine alanine peptidase (leu-ala) until 9 and 13 wah in *O. hatcheri* and *O. bonariensis*, respectively. Maximum specific leu-ala activities in both species were higher compared to other agastric and most gastric fish. These results and the abundant pinocytic vacuoles in the posterior intestine suggest that cytosolic digestion is crucial for these species. It seems that their apparent anatomically simple digestive system is compensated by an uncommonly early development and function of the digestive tract and accessory organs accompanied by high intestinal cytosolic activity. The above findings contribute to the understanding of the Atherinopsidae digestive model and, for the first time, suggest that under farming conditions, these species could be weaned at an early developmental stage with an appropriate balanced diet.

Keywords: *Odontesthes*; pejerrey; cytosolic and membranal, digestive system development, histology, intestinal activities

INTRODUCTION

Species of the genus *Odontesthes* are naturally distributed in fresh and brackish waters of Brazil, Chile, Uruguay, and Argentina. Some are important for recreational fishing and local fisheries in these countries. Silverside *Odontesthes bonariensis* (Valenciennes, 1835) and *O. hatcheri* (Eigenmann,

Associate Editor: Carlos Alvarez

1909), both known as pejerrey, are important economic resources for the region because of their good quality flesh (Somoza et al. 2008, Hualde et al. 2011) and high nutritional levels of DHA (Martínez-Palacios et al. 2020). Both species have been widely studied in biology and reproduction (Strüssmann & Takashima 1989a,b, Strüssmann et al. 1997), and research has been carried out to develop their culture (Somoza et al. 2008). Nevertheless, despite the high potential for aquaculture production of these species, studies on the development of suitable feeding regimes and efficient feeds for these species are limited, and specific diets for these species do not exist yet (Gómez-Requeni et al. 2013, Bertucci et al. 2022).

O. bonariensis and *O. hatcheri* are agastric fish, lacking pyloric caeca, with a short intestine like other members of the Atherinopsidae family (Toda et al. 1998, Ross et al. 2006). They have been described as zooplanktivorous fish, but benthic preferences have also been described for *O. hatcheri* (Hualde et al. 2011, Martínez-Palacios et al. 2019). These feeding habits agree with the morphology of their orobranchial structures (Martínez-Palacios et al. 2019).

Integrative studies of anatomical features and digestive enzymes along the ontogeny of a species provide the basis for understanding the functional development of its digestive system (Day et al. 2011, Solovyev et al. 2016) and contribute to the development of optimally balanced diets. Studies on developing the digestive tract, accessory organs, and digestive capacity in pejerrey species are scant. To our knowledge, there is no comparable information on the digestive system development of *O. hatcheri*. Regarding *O. bonariensis*, there are two studies on the histology of the digestive system of fed and starved larvae (Strüssmann & Takashima 1989a,b) and others describing the activity of pancreatic and intestinal enzymes during larval and juvenile stages (Toledo-Cuevas et al. 2011, Pérez-Sirkin et al. 2020). However, the latter studies were performed from the first week after hatching (wah). They did not include histological analysis, being the first time that a detailed histological analysis of the digestive system from day 1 till 13 wah is performed in larvae and juveniles of *O. bonariensis* and *O. hatcheri*, correlating from week 1 with the activity of intestinal digestive enzymes. Here, we provide detailed morphological/histological and biochemical observations in larvae and juveniles of *O. bonariensis* and *O. hatcheri* that support the assumption of an uncommon early development of the digestive system in both species and their ability to assimilate *ad-hoc* formulated diets from an early stage.

MATERIALS AND METHODS

Source of fish and rearing methods

Fertilized eggs from *O. bonariensis* and *O. hatcheri* were collected from broodstock maintained at the Yoshida Station, Field Research Center, and the Aquatic Animal Rearing Facilities at Shinagawa Campus, Tokyo University of Marine Science and Technology, Japan. Eggs were incubated at 19.5 ± 1.5°C until hatching.

During the first month, larvae of both species were reared at a density below 2 fish L^{-1} in two 200 L tanks under a 16:8 h light:dark photoperiod during the first month. After that, juveniles were reared at a density below 2 fish L^{-1} in two 1000 L semi-recirculated water tanks under a 14:10 h light:dark photoperiod until the end of the trial at 13 wah. In both periods, tanks were continuously supplied with a small flow of dechlorinated tap water and weak aeration to maintain adequate water quality and oxygen concentration. The salinity of the rearing water was maintained between 0.1 and 0.5 by the periodic addition of sodium chloride. Water temperature was kept constant during the experiment at 24 ± 0.5°C for *O. bonariensis* and 21 ± 0.5°C for *O. hatcheri* (Strüssmann et al.1997, Ito et al. 2005).

Fish were fed according to the following regime. Larvae were fed twice daily *ad libitum* only with *Artemia franciscana* nauplii from hatching to 4 wah. Afterward, commercial diets were progressively incorporated into the fish feed, as indicated from now on. From 4 to 5 wah, fish were fed four times a day, alternating the administration of *Artemia* nauplii and a mixture of 50% trout (Maruha, Tokyo, Japan) and 50% carp (Nosan, Yokohama, Japan) commercial diets to apparent satiation. Both diets contained 3% lipids but differed in protein content (46% for the trout diet and 40% for the carp diet), and their mixture had particles with a ca. 100-800 μm diameter. Between 5 and 8 wah, fish were fed three times a day, the first two times with the mixture diet and then with *Artemia* nauplii by the end of the day. Complete weaning on the commercial diets was achieved at 8 wah, when the fish had about 2-3 cm standard length (SL), and they were fed twice a day only with the mixture diet.

Fifteen fish were collected every week between 1 and 9 wah and then at 11 and 13 wah to measure their growth in wet mass (mg) and SL (mm) following the methods reported in a previous study (Toledo-Cuevas et al. 2011). Fish were anesthetized and then sacrificed by prolonged exposure to ice-cold water.

Histological analysis

Thirty-two larvae were sampled daily between hatching and seven days after hatching (dah), and 10 individuals were sampled weekly between 1 and 9 wah and, subsequently, at 11 and 13 wah. In all cases, sampling was performed at the beginning of the week, before any change in feeding regime, and fish were fasted for at least 12 h before sampling. Animals were anesthetized and then sacrificed by prolonged exposure to ice-cold water for about 10 min. Samples were rinsed with distilled water, fixed in Bouin's solution for 24 to 72 h at 4°C, and then rinsed and stored in ethanol 70% at room temperature until processing. Samples were histologically processed following conventional procedures (Luna 1968, Drury & Wallington 1980). Serial longitudinal sections with a thickness of 5 µm were obtained from six to nine fish for each species between 1 and 8 dah and three fish between 2 and 13 wah. The slides were stained with hematoxylin-eosin (H&E). No specific stain was made to distinguish lipids or glycogen deposits in the liver. Slides were analyzed under an optical microscope (Carl Zeiss Axisocop 20) and photographed with a digital camera (Qimaging RoHS). All results are referred to in degree days after hatching (ddah) because each species was cultured at its optimal growing temperature (Strüssmann et al. 1997, Ito et al. 2005).

Digestive enzyme activity assays

Samples for analysis of leucine alanine peptidase (leu-ala), alkaline phosphatase (AP), and aminopeptidase N (APN) were collected between 1 and 13 wah, following the same protocols and care as for histological samples. Pancreatic digestive enzyme activities were not assessed here since these species were already measured in a similar age period (Toledo-Cuevas et al. 2011).

Samples consisted of whole fish to a minimum wet mass of 65 mg from 1 to 8 wah and dissected intestines from individual fish from 9 to 13 wah (n = 3 per week). Fish handling was performed over ice-cold metallic plates, and samples were immediately weighed and snap-frozen in liquid nitrogen. All materials were then stored at -80°C and subsequently lyophilized. Before digestive enzyme analysis, lyophilized whole larvae (1 to 2 wah), the trunk section (3 to 8 wah), or single intestines (9 to 13 wah) were homogenized in 600 µL of ice-cold deionized water by sonication (Sonicator, Model 150E, Fisher Scientific). The homogenate was centrifuged for 30 min at 15,700 *g* at 4°C (Microcentrifuge, Eppendorf 5415 R), and the clarified homogenate was stored at -80°C until analysis.

The activity of leu-ala was quantified according to Nicholson & Kim (1975), using Leu-Ala (SIGMA L9250) as substrate. Homogenates were incubated with Leu-Ala substrate and LAOR solution (Tris-HCl 50 mM, pH 8, O-dianisidina (Sigma D9143), L-amino oxidase (Sigma A5147), Horseradish peroxidase (Sigma P6782)), at 37°C. After 20 min, sulfuric acid at 50% was added, and absorbance at 530 nm was recorded. One unit of activity was defined as 1 nmol of hydrolyzed substrate released per minute AP activity was quantified based on Bergmeyer (1974), using 1 M diethanolamine, 1 mM $MgCl_2$ buffer, ρ-nitrophenyl phosphate (FLUKA 71770) as substrate at a final reaction concentration of 14.8 mM, at a pH of 9.8 and a temperature of 37°C. One unit of activity (U) was defined as 1 µmol of hydrolyzed substrate released per minute, recorded at 407 nm. APN activity was evaluated using 50 mM phosphate buffer, L-leucine p-nitroanilide (Sigma L9125) as substrate at a final reaction concentration of 1 mM, at a pH of 7.2 and a temperature of 37°C. U was defined as 1 µmol of hydrolyzed substrate released per minute, detected at 410 nm (Maroux et al. 1973).

Soluble protein was determined using Bradford's method (Bradford 1976). All assays were duplicated, and the values were averaged for further calculations. Enzyme activity was calculated as specific activity (U mg protein^{-1}) and relative activity (U g body wet mass^{-1}), and the results were plotted on a scale of thermal units (ddah).

Statistical analysis

The ontogenetic changes in growth rates of *O. bonariensis* and *O. hatcheri* were analyzed by applying least-squares linear regression of $\log_{10}$-transformed wet body mass (WBM) and SL. This analysis identified two distinct growth periods for each species corresponding to 0-4 and 5-13 weeks, and their slopes were then compared using the Student or Welch two-sample *t*-test (the latter when the homoscedasticity assumption was not met). The influence of the feeding regime on the $\log_{10}$-transformed WBM slopes was evaluated in three periods, where main changes in the feeding protocol occurred: 0-4, 5-8, and 9-13 wah, for each species. Shapiro-Wilk and Fligner-Killeen tests were used for normality and equal variance testing, respectively, followed by one-way ANOVA ($\alpha = 0.05$). Tukey's multiple range test was used to determine the significance of the differences between the three periods of growth.

Enzyme activity levels in both species for 1-8 wah were statistically analyzed separately from those of 9-13 wah because of the difference in sampling methods described above (whole body/trunk *vs*. only intestine).

One-way ANOVA or Kruskal-Wallis Analysis of Variance on Ranks ($\alpha = 0.05$) was used depending on normality and equal variance of the data. Tukey's Multiple Range Test, or Dunn's Pairwise Multiple Comparison Procedure, determined the significant differences between sampling times (wah), for each species, for all cases except for *O. hatcheri* between 9 and 13 wah. Since samples for 11-week-old *O. hatcheri* were accidentally lost during processing, the Welch two-sample t-test or Wilcox-Mann-Whitney was used. Longitudinal compa-risons on different enzyme activities between species from 1 to 8 wah were conducted using two-way ANOVA ($\alpha = 0.05$), followed by *post-hoc* comparison applying Tukey's Multiple Range Test. Log transfor-mations were conducted for specific and relative activity data of AP and APN to meet the normal distribution of residuals. The influence of the feeding regime on the specific digestive activities was also evaluated from 0 to 8 wah in both species. The analysis did not include the 9 to 13 wah period because of the different sampling protocols mentioned and because this period had no other changes in the feeding scheme. For this purpose, one-way ANOVA or Kruskal-Wallis Analysis of Variance on Ranks ($\alpha = 0.05$) was used, followed by Tukey's Multiple Range Test or Dunn's Pairwise Multiple Comparison Procedure. All statis-tical analyses were performed in R software 4.2.2 (R Core Team 2020).

RESULTS

Growth measurements

Newly hatched larvae of *O. hatcheri* were larger and heavier (7.8 ± 0.6 mm and 2.4 ± 0.0 mg; mean ± standard deviation) than those of *O. bonariensis* (6.3 ± 0.6 mm and 1.3 ± 0.6 mg). Final body mass and SL at 13 wah were 1108.3 ± 241.3 mg and 50.1 ± 3.8 mm for *O. bonariensis* and 972.6 ± 282.6 mg and 52.3 ± 4.9 mm for *O. hatcheri*. Two distinct phases of growth were visualized when WBM and SL data were decimal log-transformed (Fig. 1). Growth in WBM and SL was significantly faster in the period between hatching and 4 wah than it was between 5 and 13 wah ($P < 0.001$) in both species (df = 28 and 23.5, for WBM and SL, respectively, for *O. bonariensis* and df = 28 for both WBM and SL, for *O. hatcheri*). *O. hatcheri* showed a significant stepper regression line slope (df = 28; $P = 0.001$) for WBM and SL (df = 24.9, $P < 0.001$) in the first period than *O. bonariensis*. In the second period, the slope for the WBM regression was similar for *O. bonariensis* and *O. hatcheri* (df = 28, $P = 0.054$), whereas that for the SL regression was higher in *O. hatcheri* than in *O. bonariensis* (df = 28, $P = 0.001$).

Regarding the influence of the feed on the WBM growth slope, it was observed that the lower regression line slope (between 5 and 13 wah) was significantly related ($P < 0.001$) to the partial (4-8 wah) and complete weaning (9-13 wah), for both species (Table 1).

Histological findings

The main anatomical features during the development of the digestive system of *O. bonariensis* and *O. hatcheri* in the first 3 wah are summarized in Table 2. With the few exceptions noted below, the development of the digestive tract on a daily age scale was similar in both species and differed only slightly on a degree-day scale. The digestive tract, which is open from mouth to anus already at hatching in both species, consists of the oral cavity (Fig. 2a-b), pharynx, esophagus (Fig. 2c-d), and intestine (Fig. 2e-f). The two species are typically agastric. Taste buds were found in the buccal region of both species right from hatching (Fig. 2a-b). Goblet cells in this region and mandibular teeth appeared first in *O. hatcheri* (0 and 294 ddah, respectively; Fig. 2d), than in *O. bonariensis* (48 and 336 ddah, respectively). Both species had pharyngeal teeth (not yet emerged) from 0 ddah, whereas goblet cells in this area were noted first in *O. hatcheri* (0 ddah) than in *O. bonariensis* (48 ddah). Goblet cells in the esophagus were noted from hatching in both species (Fig. 2c-d).

The intestine was short and consisted of two regions, anteromedian and posterior, without a clear structural separation (e.g. no readily identifiable sphincter-like structure) but histologically distin-guishable by the patterns of vacuolation. A single loop was present at about half the length of the anteromedian intestine (Fig. 2e-f). The intestinal mucosa was already well developed in both species at 0 ddah, and supranuclear vacuoles in the posterior intestine were also observed at this age (Fig. 3a-b). Intestinal goblet cells appeared at 24 and 21 ddah in *O. bonariensis* and *O. hatcheri*, respectively. Undulations in the apical area of the villi of the anteromedian intestine, which significantly increased the microvillar area, were observed from 72 and 63 ddah for *O. bonariensis* and *O. hatcheri*, respectively (Fig. 3c-d). Absorptive lipidic vacuoles were noted for the first time in enterocytes of the anteromedian intestine from 72 and 63 ddah in *O. bonariensis* and *O. hatcheri*, respectively (Fig. 3c-d).

Lipidic and glycogenic inclusions in the liver were present at 0 ddah (Fig. 4a-b), and their abundance increased with fish development. The endocrine pancreas and a functional exocrine pancreas loosely interspersed among hepatic lobes (Fig. 4a-b) and along

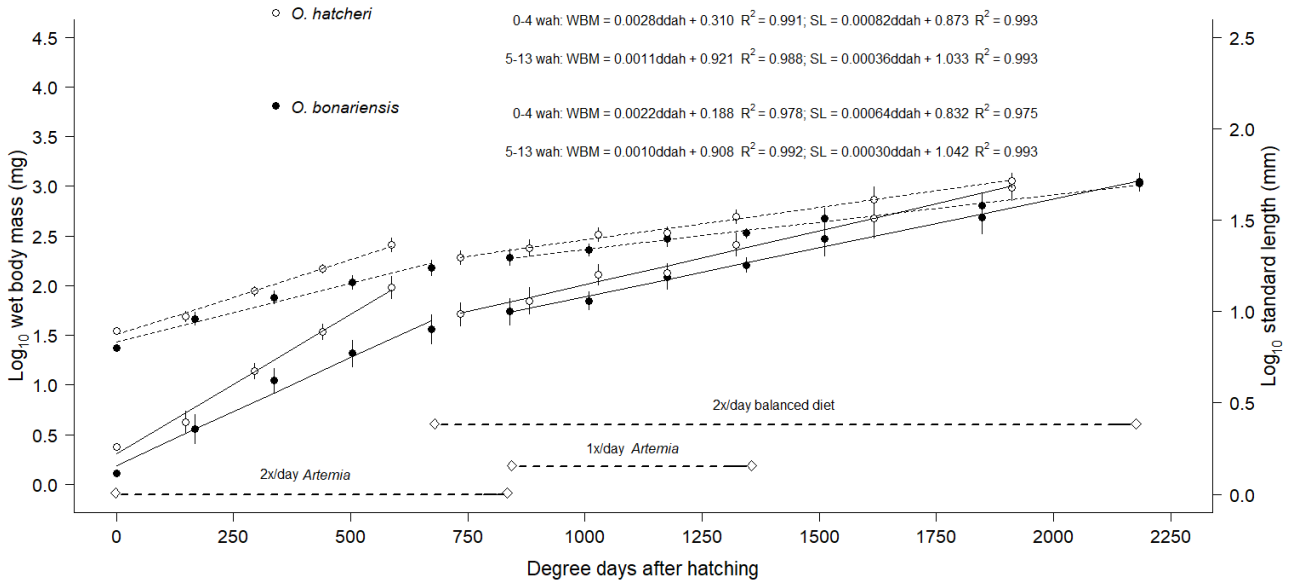

Figure 1. Decimal-log wet body mass (WBM; mg ± standard deviation, SD; n = 15; solid line) and decimal-log standard length (SL; mm ± SD, n = 15; broken line) of *Odontesthes bonariensis* (black circle) and *O. hatcheri* (white circle) during the experiment. Results are shown on a scale of degrees days after hatching (ddah). Growth is shown separately with the respective regression lines for two periods (0-4 and 5-13 weeks after hatching (wah)) for each species. The linear equations are also shown for both species. Horizontal broken lines indicate the duration of different feeding regimes. Only one tank was used for the culture of each species during the study's development period.

Table 1. Effect of the feeding regime on the slope of decimal-log wet body mass growth at different age periods for *Odontesthes bonariensis* and *O. hatcheri*. *****From 4 to 5 weeks after hatching (wah), fish (both species) were fed on 2x/day *Artemia* + 2x/day balanced diet. Only one tank was used for the culture of each species during the study's development period. Different letters indicate significant differences between age periods for each species (mean ± standard error, SE; n = 15, $P < 0.00001$). df: degrees of freedom, F: F-test from One-Way ANOVA.

		Slope			Statistics	
Period of age (wah)		0 to 4	4 to 8*	9 to 13	df	F
Daily feeding regime		2x *Artemia*	1x or 2x *Artemia* + 2x balanced diet	2x balanced diet		
Species	*O. bonariensis*	0.00215 ± 0.00007^{a}	0.00103 ± 0.00009^{b}	0.00089 ± 0.00007^{b}	41	89.53
	O. hatcheri	0.00243 ± 0.00004^{a}	0.00104 ± 0.00009^{b}	0.00098 ± 0.00008^{b}	42	131.75

the peritoneum, and already containing zymogen granules, were also observed along with a pancreatic duct at hatching in both species. A clear hepatopancreas was observed by 336 and 294 ddah for *O. bonariensis* and *O. hatcheri*, respectively (Fig. 4c-d). Changes in the number and size of zymogen granules or intensity of eosinophilia seemed to occur along fish ontogeny, although these could not be quantified. The gallbladder was evident from hatching (0 ddah) in both species (results not shown). Yolk reserves were completely exhausted by 168 and 147 ddah (1 wah) for *O. bonariensis* and *O. hatcheri*, respectively. Fat deposition in the mesentery as adipocytes were noted in 3 wah in *O. bonariensis* (504 ddah) and *O. hatcheri* (441 ddah). There were no obvious qualitative differences in the digestive structures of both species after 336 ddah (*O. bonariensis*) and 294 ddah (*O. hatcheri*) (2 wah, for both species), except for the growth of the organs and the deposition of mesentery fat just mentioned.

Digestive enzyme activity

The specific activity of leu-ala in *O. bonariensis* increased gradually from 168 to 840 ddah (1 to 5 wah), and the levels after that remained constant (Fig. 5a). Values were similar in isolated intestines between 1512 and 2184 ddah (9-13 wah). In *O. hatcheri*, the specific activity of leu-ala was significantly higher at 147 ddah (1 wah) than on any other degree day until 1176 ddah

Table 2. Ontogenetic changes in the anatomy of the digestive system of *Odontesthes bonariensis* and *O. hatcheri* during the first three weeks after hatching, referred in daily or weekly age and degree days after hatching to account for the different rearing temperatures (24 and 21ºC, respectively). Only one tank was used for the culture of each species during the study's development period. Letters indicate the first time a feature appeared in *O. bonariensis* (B) *and O. hatcheri* (H). For clarity, only observation points (days or weeks) where changes were noted for the first time are shown.

	Time (age) of first appearance					
Days or weeks after hatching (dah or wah)	0 dah	1 dah	2 dah	3 dah	2 wah	3 wah
Degree days for *O. bonariensis* (24ºC)	0	24	48	72	336	504
Degree days for *O. hatcheri* (21ºC)	0	21	42	63	294	441
Anatomical features						
Open digestive tract, from mouth to anus	B, H					
Buccal structures						
Taste buds	B, H					
Goblet cells	H		B			
Mandibular teeth					B, H	
Pharynx						
Teeth not yet emerged	B, H					
Goblet cells	H		B			
Oesophagus						
Goblet cells	B, H					
Intestine						
Differentiated intestine with a folded mucosa	B, H					
Pinocytic supranuclear vacuoles in the posterior intestine	B, H					
Goblet cells		B, H				
Undulations on the apical area of villi of the anteromedian intestine				B, H		
Lipidic vacuoles in anteromedian intestine enterocytes				B, H		
Liver and pancreas						
Liver with lipids or glycogen inclusions	B, H					
Exocrine pancreas	B, H					
Pancreatic zymogens	B, H					
Endocrine pancreas	B, H					
Pancreatic duct	B, H					
Hepatopancreas					B, H	
Associated organs and tissues						
Gallbladder	B, H					
Mesenteric fat deposits						B, H

(8 wah). A transient increase was observed at 588 ddah (4 wah). Values in isolated intestines of this species were significantly higher at 1323 ddah (9 wah) than in 1911 ddah (13 wah). The specific activity of leu-ala was significantly lower in *O. bonariensis* than in *O. hatcheri* at 1 wah but higher at 7 wah (1176 and 1029 ddah, respectively). The relative activity of leu-ala had a general decreasing profile for both species (Fig. 5b). Differences in relative activity levels were observed between both species. On the other hand, including the mixture-balanced diet in fish feeding increased the specific leu-ala activity for *O. bonariensis* since 5 wah (840 ddah) (Table 3).

The specific activity of AP in *O. bonariensis* showed a significant rise at 840 ddah (5 wah), followed by a brief decrease, and then a tendency to increase again at 1344 ddah (8 wah) (Fig. 5c). Activity in isolated intestines was significantly higher in 1848 ddah (11 wah) than at 1512 ddah (9 wah) and 2184 ddah (13 wah). In *O. hatcheri*, AP-specific activity gradually increased from 147 to 1176 ddah (1-8 wah). Specific activity in isolated intestines was higher at 1323 ddah (9 wah) than at 1911 ddah (13 wah). The relative activity levels of AP in *O. bonariensis* showed significant fluctuations (Fig. 5d). *O. hatcheri* showed relatively high values in the first two weeks (147 to 294 ddah) and stable levels after that. As with specific activity, the relative activity of AP was higher in *O. bonariensis* than in *O. hatcheri* at 5 wah (840 and 735 ddah, respectively). Additionally, as observed for the specific activity of leu-ala in *O. bonariensis*, including the mixture diet in the feeding resulted in higher levels of the specific activity of AP (Table 3).

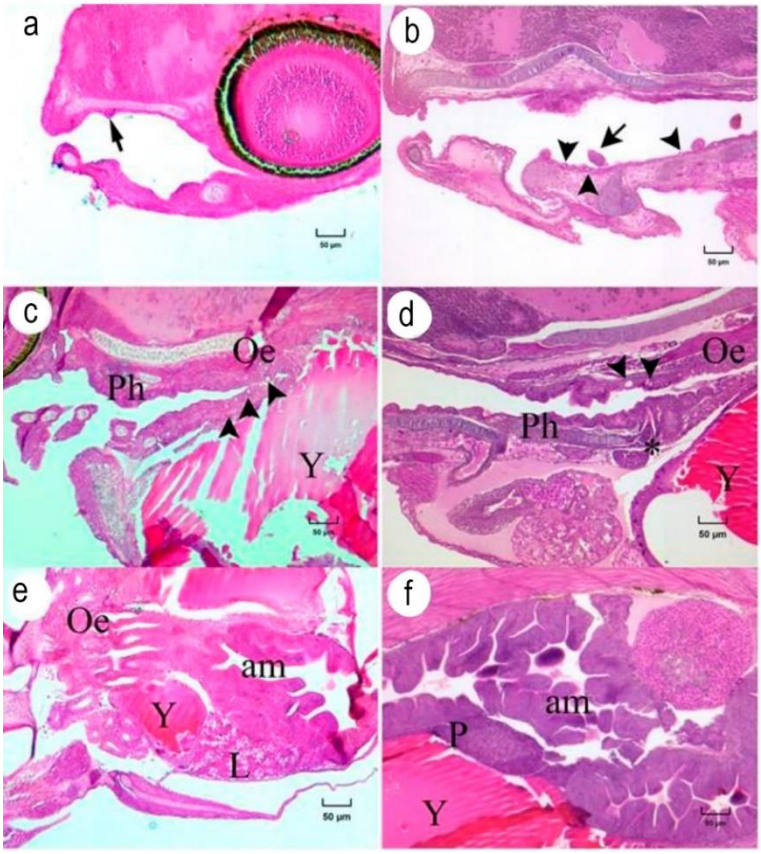

Figure 2. Longitudinal histological sections (HE staining) of the digestive system of a,c,e) *Odontesthes bonariensis*, and b,d,f) *O. hatcheri* at 0-degree days after hatching. Panels show a-b) the oral cavity with open mouths and taste buds (arrow), present in both species, and goblet cells (arrowheads) in *O. hatcheri*; c-d) pharynx (Ph) and esophagus (Oe) regions in both species, the goblet cells (arrowheads) and pharyngeal teeth (*) are present; e-f) abdominal cavity showing anteromedial intestine (am), liver (L) with lipids and glycogen inclusions, and pancreas (P). Yolk reserves are present (Y).

The specific activity of APN in *O. bonariensis* was relatively stable except for a pronounced peak at 672 ddah (4 wah) and another small but significant rise at 1344 ddah (8 wah) (Fig. 5e). Values were also stable in the isolated intestines between 1512 and 2184 ddah (9 to 13 wah). There were no significant changes in the specific activity of APN in *O. hatcheri* between 147 and 1176 ddah (1-8 wah), but the values seemed to increase slightly after 1029 ddah (7 wah). Levels were stable in isolated intestines of *O. hatcheri* between 1323 and 1911 ddah (9-13 wah). The specific activity of APN in *O. bonariensis* was significantly lower at 1 wah and higher at 4 wah than in *O. hatcheri* (168 and 672 ddah for *O. bonariensis*, 147 and 588 ddah for *O. hatcheri*). The relative activity of APN in *O. bonariensis* showed a conspicuous peak at 672 ddah (4 wah) amidst an otherwise gradual decrease between 168 and 1344 ddah (1-8 wah) (Fig. 5f). The decreasing trend continued in isolated intestines from this species between 1512 and 2184 ddah (9-13 wah). In *O. hatcheri*, relative APN activity was nearly constant between 147 and 1176 ddah (1-8 wah) but decreased in isolated intestines between 1323 and 1911 ddah (9-13 wah). The relative APN activity was higher in *O. bonariensis* than in *O. hatcheri* at 4 wah (672 and 588, respectively), whereas the opposite was observed at 7 wah (1176 and 1029, respectively). Regarding the effect of the feeding schedule on the specific activity, including the balanced diet in the feeding of *O. hatcheri* resulted in significantly higher levels of APN-specific activity, but only when the frequency of *Artemia*/day diminished (Table 3).

DISCUSSION

There needs to be more information on the morphological development of the digestive system in agastric fish larvae (Zambonino-Infante et al. 2008, Gisbert et al. 2013, Le et al. 2019). Previous studies have reported

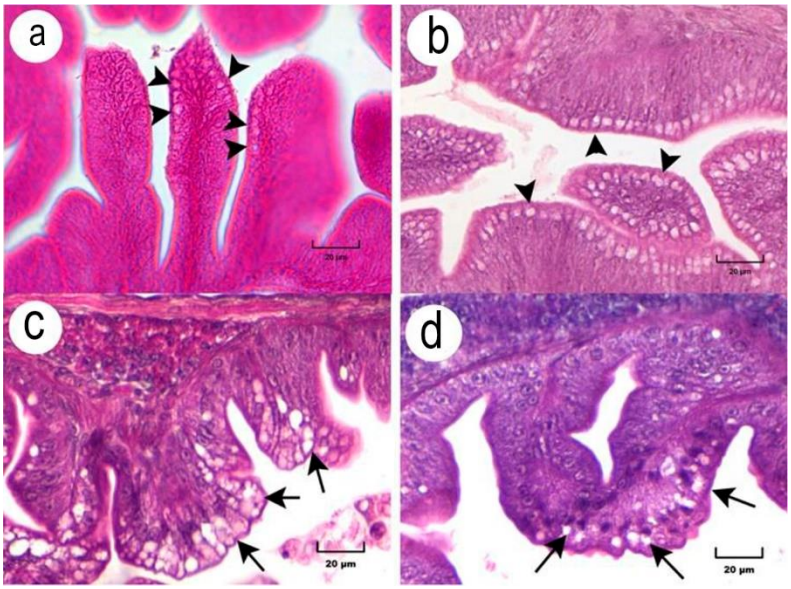

Figure 3. Longitudinal histological sections of the intestine of a,c) *Odontesthes bonariensis* and b,d) *O. hatcheri* (HE staining). Panels show a-b) supranuclear vacuoles (arrowheads) in enterocytes of the posterior intestine at 0 degree days after hatching (ddah); c-d) the microvillar area with undulations and absorptive lipidic vacuoles (arrows) in enterocytes of the anteromedian intestine at 72 and 63 ddah for *O. bonariensis* and *O. hatcheri*, respectively.

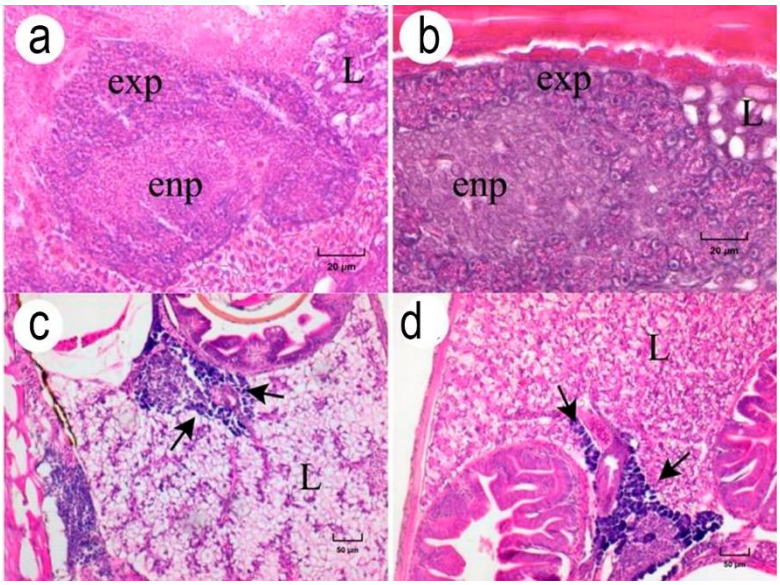

Figure 4. Longitudinal histological sections of the pancreatic tissue and liver of a,c) *Odontesthes bonariensis* and b,d) *O. hatcheri* (HE staining). a-b) The exocrine pancreas (exp), endocrine pancreas (enp), and a section of the liver (L) are shown, with lipidic and glycogenic inclusions in hepatocytes at 0 degree days after hatching (ddah); c-d) L with a pancreatic gland (arrows) at 336 (*O. bonariensis*) and 294 (*O. hatcheri*) ddah.

digestive enzyme activity from 1 wah up to 9 wah in *O. bonariensis* (Toledo-Cuevas et al. 2011, Pérez-Sirkin et al. 2020). Still, this information did not match the morphological development of the digestive system, particularly in the first wah. For *O. hatcheri* the lack of information is even more striking as, to our knowledge, no study has ever examined its digestive system histologically.

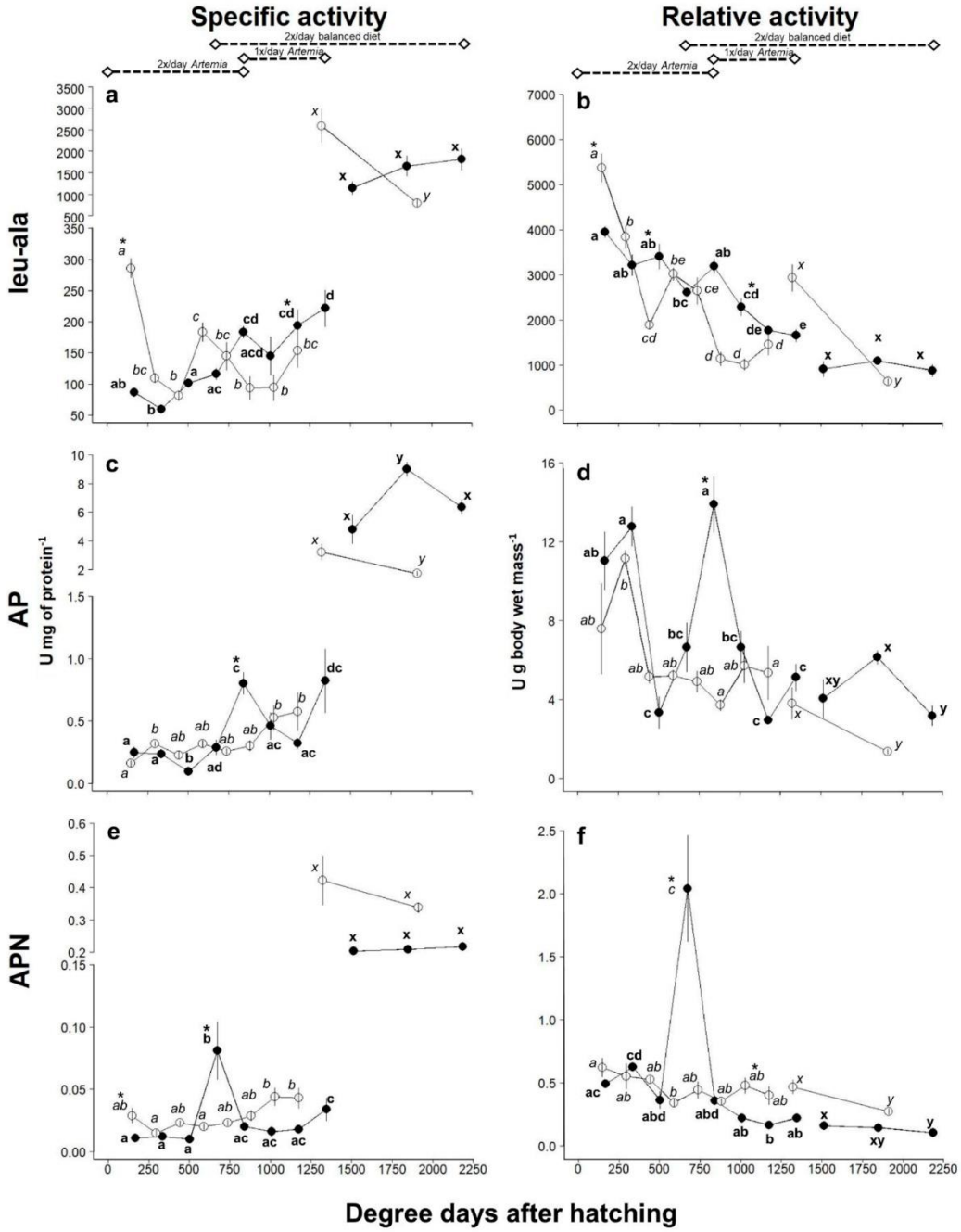

Figure 5. Changes in leucine alanine peptidase (leu-ala), alkaline phosphatase (AP), and aminopeptidase N (APN) activity during larval and juvenile development of *Odontesthes bonariensis* (black circle) and *O. hatcheri* (white circle). Only one tank was used for the culture of each species during the study's development period. Enzyme activity is shown as specific (U mg protein^{-1}) and relative (U g of body wet mass^{-1}) activity on a scale of degree days after hatching. Each circle represents the mean of three replicates (pools of larvae for 1-8 weeks after hatching (wah) and only intestines for 9-13 wah) ± standard error (SE). Different letters indicate significant differences ($P < 0.05$) between weeks for the same species (bold and italicized letters represent *O. bonariensis* and *O. hatcheri*, respectively). Note that 1-8 and 9-3 wah were analyzed separately (see Materials and Methods). Statistical significance was based on one-way ANOVA or Kruskal-Wallis Analysis of Variance on Ranks followed by Tukey's Multiple Range Test or Dunn's Pairwise Multiple Comparison Procedure, depending on normality and variance, except for *O. hatcheri* activity data between 9 and 13 wah, were the Welch two-sample t-test or Wilcox-Mann-Whitney was used. Asterisks indicate significant differences between species in the same week of age (two-way ANOVA followed by Tukey's Multiple Range Test; $P < 0.05$). Horizontal lines at the top of the figure indicate the duration of different feeding regimes, as in Figure 1.

Table 3. Effect of the feeding regime on the activity of intestinal digestive enzymes of *Odontesthes bonariensis* and *O. hatcheri*. Only the combinations of species and enzyme-specific activity, which showed statistically significant differences with the changes in feeding regime, are shown. Only one tank was used for the culture of each species during the study's development period. Different letters indicate significant differences between age (wah: week after hatching; mean ± standard error; n = 6, $P < 0.001$).

Species and specific digestive enzyme activity (U mg of protein^{-1})	Age (wah)							
	1	2	3	4	5	6	7	8
	Daily feeding regime							
	2x *Artemia*			2x *Artemia* + 2x diet	1x *Artemia* + 2x diet			
O. bonariensis leu-ala	0.248 ± 0.043^{a}	0.236 ± 0.016^{a}	0.236 ± 0.019^{a}	0.290 ± 0.057^{a}	0.802 ± 0.086^{b}	0.462 ± 0.107^{b}	0.322 ± 0.042^{b}	0.823 ± 0.255^{b}
O. bonariensis AP	86.973 ± 7.002^{a}	59.669 ± 4.435^{a}	101.474 ± 6.955^{a}	116.093 ± 8.901^{a}	183.467 ± 9.966^{b}	145.053 ± 30.543^{b}	194.022 ± 25.502^{b}	221.584 ± 29.400^{b}
O. hatcheri APN	0.029 ± 0.006^{a}	0.015 ± 0.003^{a}	0.023 ± 0.002^{a}	0.020 ± 0.001^{a}	0.023 ± 0.001^{ab}	0.029 ± 0.004^{b}	0.044 ± 0.007^{b}	0.043 ± 0.008^{b}

Growth performance

Both species showed negligible mortality and exponential growth during the first 13 weeks (2184 ddah for *O. bonariensis* and 1911 ddah for *O. hatcheri*), which are indications of optimal rearing conditions during the experiment (Tsuzuki et al. 2000, Ito et al. 2005). Both species showed an initial phase of fast growth from 0 to 4 wah, followed by a decrease in growth rates. The early phase of fast growth in fish is characterized by a substantial improvement in locomotive function, which is likely crucial for food capture and predator avoidance (Gilannejad et al. 2020). Active movements and swimming near the surface were detected at 2 dah for *O. bonariensis* (Chalde et al. 2011), and feeding activity from this age in the two species examined in this study has been observed before (Strüssmann & Takashima 1989a, Chalde et al. 2011, Hualde 2020, *pers. comm.*). Growth rates in the two *Odontesthes* species were similar to that of the anchovy *Engraulis encrasicolus*, which has been considered a species with rapid growth and good swimming capabilities at larval stages (Alvarez et al. 2021). On the other hand, the observed slow growth period (from 5 to 13 wah) of both *Odontesthes* species may correspond to the common isometric profile reported in juvenile stages (Gilannejad et al. 2020). In fact, according to Chalde et al. (2011), *O. bonariensis* specimens are considered juveniles around 20.1 to 23 mm in total length, corresponding to the 5th to 6th wah (19.9 to 21.7 mm SL) in this study. Alternatively, this lower growth could be caused by non-optimal balanced diets for the species. Bertucci et al. (2022) reported that 12% of lipids in the diet is optimal for this species, with similar concentrations of dietary protein.

Morphological and histological features

Both species seemed ready to initiate digestion, absorption, and utilization of nutrients as soon as the exogenous feeding started, probably immediately after hatching, as suggested by Strüssmann & Takashima (1989a) for *O. bonariensis*. Important structures necessary for these processes were observed in both species from the first dah. These include taste buds, pharynx teeth, and buccal, pharyngeal, esophageal, and intestinal goblet cells, which, along with the opening of the digestive tract, are known to match the start of exogenous feeding in other fish species (Yúfera & Darias 2007, Zambonino-Infante et al. 2008). However, the appearance of buccopharyngeal goblet cells differed somewhat between species, in concordance with the different ontogeny of these cells among fish species (Gisbert et al. 2013). Mandibular teeth appeared until 336 and 294 ddah in *O. bonariensis* and *O. hatcheri*, respectively, suggesting that it is until that moment that both species can feed on bigger and harder food as do juveniles (Martínez-Palacios et al. 2019). An early differentiated intestine with a folded mucosa and supranuclear vacuoles (in the posterior intestine) was already observed at hatching in both species, as reported for *O. bonariensis* by Strüssmann & Takashima (1989a). The observation of lipid absorption vacuoles in the anteromedian section of the intestine of *O. bonariensis* and *O. hatcheri* agree with reports about this being the main site for lipid absorption in many fish species (Strüssmann & Takashima 1995, Le et al. 2019). The appearance of undulations in the microvilli area of the anteromedian intestine of *O. bonariensis* and *O. hatcheri* as early as at 72 and 63 ddah, respectively,

could be related to enhanced activity of membrane-linked digestive enzymes, as has been reported for other fish species (Zambonino-Infante & Cahu 1994). Unfortunately, we could not verify these results because the assessment of the activity of intestinal digestive enzyme activities was performed later, at the beginning of the first wah (168 and 147 ddah for *O. bonariensis* and *O. hatcheri*, respectively).

A well-differentiated liver was observed in both species at hatching. The hepatocytes showed lipids and glycogen accumulation, indicating that this organ was already active in the assimilation of either yolk or exogenous nutrients (Strüssmann & Takashima 1989a, Zambonino-Infante et al. 2008). The exocrine pancreas showed zymogen granules, a pancreatic duct, and a functional gallbladder already by day 0. These indicate a potentially functional digestive system (Zambonino-Infante et al. 2008). By 2 wah, the two species developed a pancreatic gland spreading into the hepatic tissue, known as a hepatopancreas by 2 wah, an organ not present in almost any other fish species (Wilson & Castro 2011).

All the above findings place these two species among the fish with the earliest development of digestive capability. The early development of the digestive system in both species could explain their fast initial growth phase. It may represent digestive compensation for their lack of stomach and the possession of a short and thin intestine.

Intestinal digestive enzymatic activity

The general profile of AP and APN-specific activity during the development of these two silversides matched what has been reported before for gastric marine species and other agastric fish (Zambonino-Infante et al. 2008, Toledo-Cuevas et al. 2011, Pérez-Sirkin et al. 2020). A general increasing profile is observed, with decreases in activity at certain ages, reaching stable levels afterward. As it is known, the reduction in specific activities during fish development is attributed to an increase in tissue proteins. Such rise in soluble protein (measured as WBM) was translated as a general decreasing profile in the relative activity of the assayed digestive enzymes. Concerning leu-ala, a decrease in its specific activity did not occur during the 13 wah of culture, a fall described for many fish species concerning digestive system maturation (Zambonino-Infante et al. 2008). Nevertheless, this constant high cytosolic activity seems to be a peculiar feature of these agastric species with a short intestine (see below), even though a digestive maturation seems to occur (Pérez-Sirkin et al. 2020).

The most notorious biochemical feature observed for the two *Odontesthes* studied species was their higher leu-ala specific activity in the first months compared to other agastric (about 20 times higher) and gastric fish (about 2.5 times higher) (Ma et al. 2005, Hamza et al. 2007, Kvåle et al. 2007, Suzer et al. 2007, Zouiten et al. 2008, Tong et al. 2012, Martínez-Lagos et al. 2014, Solovyev et al. 2016, Castro-Ruiz et al. 2019, Koven et al. 2019, Mozanzadeh et al. 2021), and the fact that total leu-ala activities increased during development, opposite to what is observed in most of fish during their development (Ma et al. 2005, Hamza et al. 2007, Kvåle et al. 2007, Suzer et al. 2007, Zambonino-Infante et al. 2008, Zouiten et al. 2008, Tong et al. 2012, Martínez-Lagos et al. 2014, Solovyev et al. 2016, Castro-Ruiz et al. 2019, Koven et al. 2019, Mozanzadeh et al. 2021). The high levels of leu-ala may be a key feature of the short intestine agastric digestive systems of atherinopsid fish (Toledo-Cuevas et al. 2011, unpubl. data for *Chirostoma estor*).

These very high specific activities of leu-ala and the abundance of pinocytic vacuoles (supranuclear vacuoles) observed in the posterior intestine strongly suggest that cytosolic digestion may be an important mechanism for protein assimilation in atherinopsids as in other aquatic agastric species with a short intestine, like sea cucumbers, whose digestive apparatus need to compensate for the functional limitations of a "simple digestive system" (Toledo-Cuevas et al. 2011, Martínez-Milián et al. 2021). This type of cellular digestion has been previously described in the larval stage of several fish species (Walford & Lam 1993). Still, it seems essential for vertebrate growth and survival (Park et al. 2019).

There was no clear relationship between digestive enzyme activity and structural changes in the digestive system of the two studied species, probably because digestive activities were not assessed before 1 wah when the digestive system seemed already complete and functional. However, a lack of these relationships has been reported for species that show early digestive development (He et al. 2012, Gilannejad et al. 2020). Nevertheless, the increase in exocrine pancreatic tissue, zymogen granules, and eosinophilic intensity detected throughout development in this study might be related to the changes in pancreatic enzyme activities previously reported in these *Odontesthes* fish species (Toledo-Cuevas et al. 2011). On the other hand, the profiles of some intestinal enzyme activity were affected by the feeding protocol. The administration of the balanced diet from 4 wah was significantly related to an increase in the specific activity of leu-ala in *O. bonariensis*. Variations in this enzyme's activity levels

have been observed when modifications to the dietary protein content are implemented (Nicholson et al. 1974). Feeding with balanced diets impacted the AP and APN activity of the two species. In *O. bonariensis*, an increase in the AP-specific activity was observed from the 5 wah (the balanced diet was offered after the sampling at 4 wah). For *O. hatcheri*, feeding on a balanced diet induced a tendency to increase the specific APN activity at 5 wah, which turned significantly higher afterward when a reduction in the frequency of *Artemia* occurred. These enzyme activity responses are explained by the changes in the diet composition, specifically modifications in the levels of proteins and lipids, which affects the activity of AP (Lallès 2019) and APN (Nicholson et al. 1974, Kvåle et al. 2007, Tang et al. 2016). The distinct responses of the digestive activities to the feeding protocol may be explained by species differences which, for example, were observed in the distinctive slope of the WBM and SL in similar periods of growth, the dissimilar timing of development of some of the structures of the digestive system, the different levels of digestive activities at similar ages (in wah) and the reported distinct orobranchial structures of these species (Martínez-Palacios et al. 2019). On the other hand, the increase in the specific activity of AP in *O. bonariensis* after feeding with balanced diets might indicate that this species is efficiently digesting the balanced diets offered (see Tonheim et al. 2005).

The patterns of change in the activity of APN and AP were highly correlated in the two species, although sometimes there was a one-week time lag between the two enzymes of ca. 200 ddah. There is a correlated expression of these enzymes during normal development with similar posttranslational modifications regulating their activity (Tang et al. 2016). Also, coordinated increases in activity levels of these two enzymes are likely related to a heightening in the microvilli areas, where both enzymes are located (Hamza et al. 2007, Kvåle et al. 2007, Solovyev et al. 2016). However, we could not quantify changes in the microvilli area in this study.

Our results on *O. bonariensis* are generally consistent with those reported by Pérez-Sirkin et al. (2020) on the same species. However, there were substantial differences regarding growth rate and enzymatic activity levels, which are higher in this study. These differences may be due to the plasticity of the digestive system concerning environmental and dietary factors (Pittman et al. 2013) as well as differences in broodstock origin, domestication status, and husbandry practices between studies (Chalde et al. 2011, Gisbert et al. 2013, Pérez-Sirkin et al. 2020).

CONCLUSIONS

This study provides for the first time evidence of the exceptional early anatomical and functional development of the digestive system in *O. bonariensis* and *O. hatcheri*, whereby the digestive tract and accessory organs are completely developed by the end of the first wah, and the main intestinal digestive enzymes are also active. We propose that this early development and the very high and sustained intestinal cytosolic digestive activity (leu-ala) may be a compensation mechanism in response to the agastric condition and the short and thin intestines these species possess. These findings contribute to understanding the digestive mechanisms in atherinopsid fish and suggest a potential for earlier and complete weaning in farming *O. bonariensis* and *O. hatcheri* by feeding with suitable *ad-hoc* species-specific diets.

ACKNOWLEDGMENTS

We thank Zhang Yan and Estefany L. García Cruz for their help rearing fish for this study. Also, to Dr. Carlos A. Álvarez González for his digestive activity determination expertise counseling and Dr. Carlos A. Martínez-Palacios for contributing ideas for the manuscript preparation and edition.

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Received: October 3, 2023; Accepted: January 18, 2024