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# CARACTERÍSTICAS DEL DESARROLLO DE LA CAPACIDAD DIGESTIVA DURANTE LA ONTOGENIA LARVARIA Y JUVENIL DEL ATERINÓPSIDO CHIROSTOMA ESTOR, SIN INTERFERIR LA MADURACIÓN DIGESTIVA

FEATURES OF DIGESTIVE ENZYME CAPACITY DEVELOPMENT DURING THE LARVAL AND JUVENILE ONTOGENY OF THE ATHERINOPSID CHIROSTOMA ESTOR, WITHOUT IMPAIRING DIGESTIVE MATURATION

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# Características del Desarrollo de la Capacidad Digestiva Durante la Ontogenia Larvaria y Juvenil del Aterinópsido Chirostoma Estor, sin Interferir la Maduración Digestiva

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**RESUMEN**

El pez blanco *Chirostoma estor* es una especie con alto potencial de cultivo y calidad nutricional. A pesar de los avances en su cultivo y alimentación, aún no se cuenta con una alimento balanceado que permita el buen crecimiento obtenido con el alimento vivo. En este trabajo se estudió el desarrollo de la actividad digestiva en la especie, desde la eclosión hasta los 150 días, usando solo alimento vivo para evitar afectar la maduración digestiva. Los análisis de actividad se realizaron con técnicas fluorómetricas (enzimas pancreáticas) y espectofotométricas (enzimas intestinales) validadas para evaluar maduración digestiva. El crecimiento observado fue comparable con el obtenido utilizando luz contínua y microdietas con proteína soluble y consorcio bacteriano. Se evidenció la necesidad de incluir rotíferos y nauplios de *Artemia* en los primeros estadíos. Los indicadores de maduración digestiva evidenciaron que *C. estor* sigue el modelo descrito para peces gástricos, con las particularidades de ser tardío -posterior a los 5 meses de vida-, presentar niveles muy elevados de actividad citosólica intestinal (leucin alanin peptidasa) y mantener esta actividad y la anclada a la membrana de los enterocitos durante el estadio juvenil. Por consiguiente, se recomienda un destete posterior al primer mes de vida, induciendo una maduración temprana.

***Keywords:*** agástrico, intestino corto, alimento vivo, maduración digestiva tardía

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# Features of Digestive Enzyme Capacity Development During the Larval and Juvenile Ontogeny of the Atherinopsid Chirostoma Estor, Without Impairing Digestive Maturation


## ABSTRACT

The silverside *Chirostoma estor* is a fish species with a high culture potential and nutritional quality. However, despite the advances in its culture, there is not yet a balanced diet that allows a similar growth obtained from feeding live feed. In this work, *C. estor* were fed only with live feed to avoid impairing the digestive maturation. The development of the digestive activity was studied using fluorometric (pancreatic enzymes) and spectrophotometric (intestinal enzymes) validated methods to evaluate digestive maturation, from the first until 150 days post-hatching (dph). The obtained growth was equivalent to that previously reported using continuous light and feeding fish with a microdiet containing soluble protein and a bacterial consortium. The results show the importance of feeding with rotifers and *Artemia* nauplii during early stages. Also, the digestive maturation indexes studied show that *C. estor* follows the maturation model already described for gastric fish, with some particularities: a late maturation, after 150 dph, very high levels of the intestinal cytosolic leucine alanine peptidase and the maintenance of high levels of this and the brush border enterocyte membrane digestive enzymes until the juvenile stage. Therefore, weaning after the first month of life is recommended, enhancing early digestive maturation.



***Keywords:*** agastric, short intestine, live feed, late digestive maturation

## INTRODUCTION

The silverside *Chirostoma estor*, endemic to Lake Patzcuaro, Mexico, is valued for its regional socioeconomic importance and nutritional content, particularly its high levels of long-chain omega-3 polyunsaturated fatty acids essential to human health (Martínez-Palacios et al., 2020). A member of the Atherinopsidae family, *C. estor* is agastric with a short intestine and lacks anatomical adaptations for its limited digestive system (Horn et al., 2006; Ross et al., 2006), aside from a well-developed branchial sieve and pharyngeal teeth suited to its zooplanktivorous habit (Ross et al., 2006; Martínez-Palacios et al., 2019).

As an ancient marine species, *C. estor* hatches from small eggs and requires live feed for the first three months of life (Martínez-Palacios et al., 2008). Early weaning was achieved using a high-protein microdiet supplemented with *Lactobacillus acidophilus* and *L. plantarum* (Martínez-Angeles et al., 2022), though final growth and specific growth rates did not surpass those from live feed in this or prior studies (Toledo-Cuevas et al., 2011). Frequent feeding of juveniles improved growth and body composition and reduced skeletal deformities (Melo et al., 2023). However, the knowledge of the development of key digestive enzymes during ontogeny is essential for understanding *C. estor*'s digestive physiology and designing species-specific diets.

In the first month post-hatching, fish larvae undergo significant anatomical and physiological changes, including pancreatic maturation and the onset of enzyme secretion. Enterocyte maturation follows, marked by increased activity of brush border enzymes and decreased cytosolic peptidase activity. The final stage involves stomach development, with pepsin activation and the start of acid digestion of proteins and lipids (Lazo et al., 2011; Zambonino-Infante et al., 2008). Understanding this maturation process is essential for selecting suitable ingredients and formulating digestible, balanced diets (Moyano et al., 2005).

A preliminary study on the digestive physiology of *C. estor* detected very high levels of the intestinal cytosolic leucine-alanine peptidase (leu-ala) activity (Toledo-Cuevas et al., 2011) that may suggest a functional compensation for an apparently restricted digestive anatomy, since this feature has also been found in other atherinopsids (Toledo-Cuevas et al., 2024).

Therefore, this study aimed to characterize the digestive ontogeny (pancreatic and intestinal) of *C. estor* during larval and juvenile development, and the use of these enzyme activities as markers to determine the model and timing of digestive maturation. To prevent the effects of poorly formulated diets or premature weaning (Hamlin et al., 2000; Zambonino-Infante & Cahu, 2001; Zambonino-Infante et al., 2008), fish were fed exclusively live feed for five months post-hatching.

## MATERIALS AND METHODS

**Source of fish and rearing conditions**. Fertilized eggs of *C. estor* were obtained from broodstock at the pilot farm of the Instituto de Investigaciones Agropecuarias y Forestales, Universidad Michoacana de San Nicolás de Hidalgo, México. Eggs were incubated at 25 °C in 1 L Zug jars with 7 g/L salinity and 100 ml/min water flow until hatching. Larvae were collected as they swam up and transferred to 2.5 L plastic tanks placed inside a 45 L tank with a closed recirculating system and a 50 L biological filter for the first 15 days post-hatching (dph). From day 15 to 45 dph, larvae were reared in the 45 L tank. Afterwards, they were moved to 90 L plastic raceways with a closed recirculation system and weekly water replacement. At 100 dph, juveniles were transferred to 1000 L tanks, remaining until the trial ended at 150 dph. All cultures were maintained in freshwater, under a natural photoperiod, at 23.31 ± 0.35 °C—close to the species' optimal rearing temperature (Martínez-Palacios et al., 2002a). Water temperature was recorded hourly using sensors (Thermotraker Pro), and physicochemical parameters were assessed every 10 days. Salinity was evaluated with a refractometer (ATAGO S/Mill-E), dissolved oxygen with an oximeter (YSI 55/25), pH with a potentiometer (Fisher, Accumet), and ammonia, nitrites, and nitrates with a photometer (YSI-9500). All parameters were kept within optimal ranges for the species (Martínez-Palacios et al., 2004).

Fish were fed live feed, *ad libitum*, three times a day (Martínez-Palacios et al., 2008). From hatching to 25 dph, they received *Brachionus plicatilis* rotifers, followed by co-feeding with *Artemia franciscana* nauplii from 15 to 60 dph. From 60 to 150 dph, feeding consisted exclusively of *A. franciscana* metanauplii.

**Sampling**. Sampling was conducted in the morning before the first feeding, following a 17-hour fast. Previous data showed trypsin activity remains stable up to 22 hours of fasting (not shown).

Four pools of fish of at least 65 mg wet body weight were collected every third day (1, 3, and 5 dph), then every five days (5 to 25 dph), and finally every 15 days (60 to 150 dph). From 45 dph onward, when fish exceeded 100 mg, four individual fish were sampled to reduce animal use. Additionally, 12–15 fish per time point were collected in individual tubes for fluorometric analyses. Fish were euthanized with ice-cold water, rinsed with distilled water, and excess water removed.

Samples consisted of whole fish aged 1 to 45 dph and dissected intestines and hepatopancreas from individual fish aged 60 to 150 dph. Handling was done on ice-cold metal plates; samples were weighed, snap-frozen in liquid nitrogen, and stored at -80°C. Wet body and organ weights were measured using a microbalance (Mettler Toledo MX5) and an analytical scale (Mettler Toledo AB204-S). To assess wet body weight (WBW; mg), larvae were pooled prior to each sampling: 60–160 individuals at 1–3 dph and 10 individuals from 5–150 dph. Total length (TL; millimeters) was measured for 10 larvae using a digital vernier.

**Growth data analysis**. The following formula was used to calculate the specific growth rate:

$SGR = 100 (Ln\ WBW_2 - Ln\ WBW_h)/ \Delta t$

Where $WBW_2$ is the mean weight body mass (mg) at the end of this study (150 dph) or either at 25 or 27 dph, as indicated in the discussion section; $WBW_h$ is the mean weight body mass (mg) at hatch; $\Delta t$ age at time 2 minus age at hatching (days).

The mean growth rate (mm/day) was calculated as the increment in TL from hatching to the periods 1 to 45 and 60 to 150 dph, based on previous reports (Alvarez et al., 2021):

$GR = (TL_2 - TL_1)/ \Delta t$

Where $TL_2$ = total length of fish at time 2 (either 45 or 150 dph); $TL_1$ = total length at time 1 (either 1 or 60 dph), and $\Delta t$ = age at time 2 minus age at time 1.

**Digestive enzyme activity assays**

**Fluorometric analysis**. Individual fish or digestive organs (hepatopancreas and intestines, from 60-150 dph; *n* = 12-15) were used to evaluate the activity of pancreatic enzymes: trypsin and lipase, as reported before (Rotllant et al., 2008). The homogenate was prepared as indicated by Toledo-Cuevas et al. (2011).

The measurements were carried out on a Fluoroskan Ascent fluorometer (Thermo Fisher Scientific) in duplicate. All activities were reported as arbitrary fluorescence units per minute per individual (U/individual) and as specific activity (U/mg of soluble protein). The percentage of secretion was calculated from 60 dph when the digestive organs (hepatopancreas and intestine) could be obtained (Zambonino-Infante & Cahu, 2001). In addition, the ratio of trypsin and lipase activities was calculated by dividing the total activity of each digestive enzyme. This was obtained from 60 days by summing the total activity levels obtained in the digestive organs.

**Spectrophotometric analysis**. Two samples from each pool (individuals or intestines) were used to measure the activity of cytosolic digestive enzymes (leucine alanine peptidase and acid phosphatase), while in the other two pool samples, the activity of intestinal brush border membrane (BBM) digestive enzymes (alkaline phosphatase and aminopeptidase N) was measured.

The homogenate for cytosolic analysis was prepared in cold distilled water using a tissue disruptor (Fisher Scientific Model 150E), with 10-sec pulses at an amplitude level of 10, until complete tissue homogenization. The tissues were always kept within ice-cold containers to prevent enzyme denaturation. The homogenates were centrifuged at 15,700 *g* for 30 min at 4°C, in a refrigerated microcentrifuge (Eppendorf 5415 R). The supernatants were stored in 0.1 ml aliquots at –20°C until analysis. Individual aliquots were used for each digestive enzyme determination to avoid the loss of enzyme activity by frosting/ defrosting cycles. BBM activities were analyzed in purified intestinal membranes prepared as reported before (Crane et al., 1979) and modified subsequently (Cahu & Zambonino, 1994), except for the centrifugation speed (see below). Whole individuals (1-45 dph) and intestines were homogenized in 30 volumes of cold buffer solution containing 50 mM mannitol, 2 mM Tris, pH 7. Subsequently, 0.1 M $CaCl_2$ was added to a final concentration of 10 mM, followed by a first centrifugation at 9000 *g* for 10 min at 4°C. The supernatant was recovered and centrifuged at 24,040 *g* for 20 min at 4°C. The sedimented BBM was homogenized by sonication for 10-15 seconds in 1 mL of 0.1 M KCl, 1 mM Dithiothreitol, 5 mM Tris-HEPES, pH 7.5, at 4°C.

The activity of leucine alanine-peptidase (leu-ala) was determined as reported by Nicholson & Kim (1975), while the acid phosphatase (AcP) activity was measured using the methodology of Bergmeyer et al. (1974).

The activity of these enzymes was measured at a final point in a spectrophotometer (Cary 50 Varian). Alkaline phosphatase (AP) activity was analyzed according to Bergmeyer et al. (1974), while Aminopeptidase N (APN) activity was assayed according to Maroux et al. (1973). The activity of these enzymes was kinetically recorded. For leu-ala, one unit of activity was defined as 1 nmol of substrate hydrolyzed per minute at 37°C. For all the other digestive enzymes, one unit of activity was defined as 1 µmol of hydrolyzed substrate per minute at 37°C. All assays were performed in triplicate, and the values were averaged for subsequent calculations.

The soluble protein concentration of all homogenates was determined using the Bradford method (Bradford, 1976), adapted to a microplate, measuring the absorbance in a photometer (ELIREAD, Kontrolab). Enzyme activity was calculated as total activity (U/individual or mU/individual) and specific activity (U/mg protein or mU/mg protein). The ratio of BBM and cytosolic activities was calculated by dividing the total AP and APN activities, respectively, by the total activity of leu-ala or AcP. For these calculations, the leu-ala activity was expressed in µmoles, as were the AP and APN activities.

**Statistics**

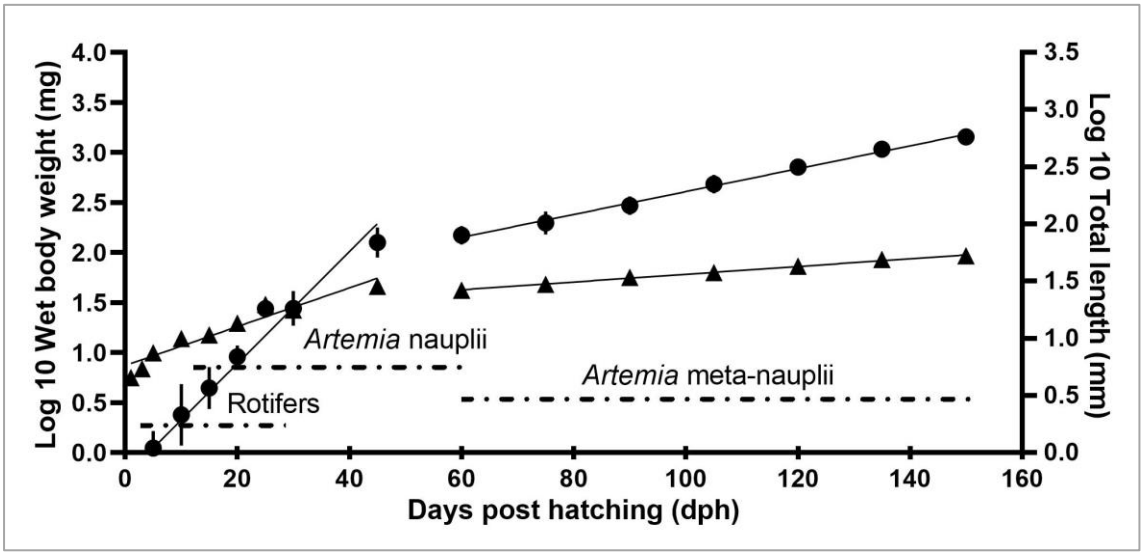
Welch's two-sample t-test was used to compare the slopes of the decimal-log transformed body weight and total length, and growth rate from 1-45 and 60-150 dph. One-way analysis of variance or Kruskal-Wallis's analysis of variance on ranks ($\alpha = 0.05$) (depending on the normality and equal variance of the data) was used to evaluate: a) the influence of the different feed types on the slopes of decimal log wet body weigh at 1-15, 20-25, 30-45, and 60-150 dph, and on the specific activity of each digestive enzyme from 1-45 dph; b) the differences on total activities of digestive enzymes throughout the ontogeny; c) on specific activities, from 1 to 45 dph and 60 to 150 dph, for the sample differences explained above (whole body versus hepatopancreas or intestine); d) the differences on the trypsin/lipase and intestinal brush border/cytosolic digestive activities ratios along the fish ontogeny; and e) the differences on trypsin and lipase secretion values from 60-150 dph. The Shapiro-Wilk and Fligner-Killeen tests were used to test normality and equality of variance, respectively. A post hoc comparison, Tukey's Multiple Range Test or Dunn's Pairwise Multiple Comparison Procedure, was used to determine the significance of differences.

The influence of diet (type of live feed) and age on the specific activity of digestive enzymes was analyzed using ANCOVA, considering age as a continuous covariate and diet as a factor. All statistical analyses were performed using R 4.2.2 software (R Core Team, 2020).

**RESULTS**

This is the first study describing the species' growth from hatching to 150 days after hatching. A potential growth profile of *C. estor* was observed during the larval and juvenile stages for WBW and a linear profile for TL, with the following equations: $WBW = 0.005dph^{4.24}$, $R^2 = 0.98$; $TL = 3.21dph - 6.18$, $R^2 = 0.97$ (Fig. S1). When both growth parameters were transformed to decimal base logarithms, two phases were observed (Fig. 1). The growth slope in WBW and TL was significantly greater in the period between one to 45 dph (log10 WBW = 0.06dph - 0.26, $R^2 = 0.97$; log10 TL = 0.02 dph + 0.75, $R^2 = 0.92$; mg or mm ± standard deviation) than between 60 to 150 (log10 WBW = 0.01dph + 1.46, $R^2 = 1.00$; log10 TL = 0.003dph + 1.22, $R^2 = 1.00$) (d.f. = 16 and d.f. = 18, respectively, $P < 0.00001$). In addition, the mean growth rate (GR) for the period from 1 to 45 dph was higher (0.54 ± 0.02) than for 60 to 150 dph (0.29 ± 0.01) (d.f. = 17.39, $P < 0.00001$). On the other hand, the specific GR for the larval period (30 dph) was 13.69 g/100 g, while for the entire period it was 5.39 g/100 g.

**Figure 1.**




Decimal-log wet body weight (mg ± standard deviation, SD, *n* = 10 (except for 1 to 10 dph, where pools of 60-160 individuals were needed), black circles) and decimal-log total length (mm ± SD, *n* = 10, black triangles) from *Chirostoma estor* during the larval and juvenile development. Growth is

shown separately with the respective regression lines, with the following equations: 1 to 45 dph: log10 WBW = 0.06dph - 0.26, $R^2$ = 0.97; log10 TL = 0.02 dph + 0.75, $R^2$ = 0.92; 60 to 150 dph: log10 WBW = 0.01dph + 1.46, $R^2$ = 1.00; log10 TL = 0.003dph + 1.22, $R^2$ = 1.00. Horizontal broken lines indicate the period of the different feeding regimes. WBW: Wet body weight; TL: Total length.

The influence of the different live feeds on the log 10 WBW slope during the development of *C. estor* is shown in Table 1. No significant differences were observed in the growth slope when feeding rotifers alone or when co-feeding rotifers and *Artemia* nauplii. Nevertheless, the growth slope decreased when fish were fed on *Artemia* nauplii and *Artemia* metanauplii.

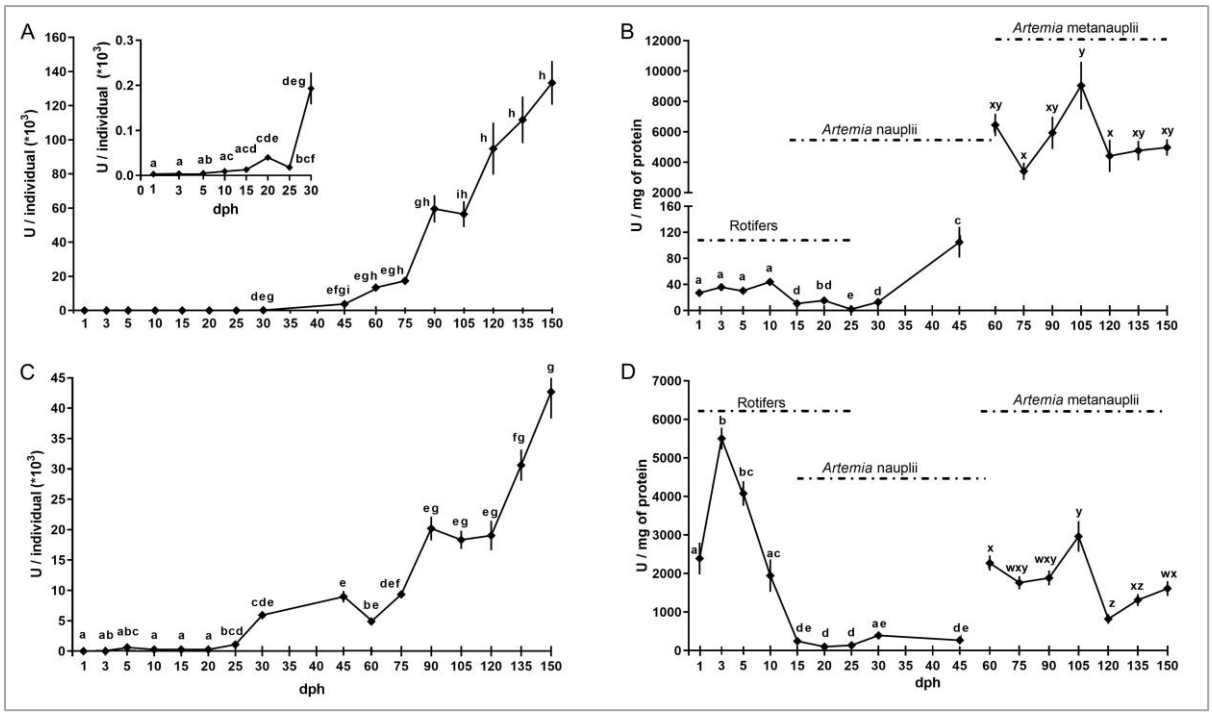
**Table 1.** Effect of the feeding regime on the slope of decimal-log wet body weight at different age periods of Chirostoma estor.

| | **Age (dph)** | | | | | |
|---|---|---|---|---|---|---|
| | **1 to 15** | **20 to 25** | **30 to 45** | **60 to 150** | **Statistics** | |
| Daily feeding regime | Rotifers | Rotifers + Artemia nauplii | Artemia nauplii | Artemia metanauplii | d.f. | F |
| Slope | 0.07 ±0.01[a] | 0.10 ±0.01[a] | 0.03 ±0.00[b] | 0.01 ±0.00[c] | 3 | 118.97 |

Different letters indicate significant differences between age periods (slope mean ± standard error, S.E.; $n$ = 10, $P$ <0.00001). dph: days post-hatching.

Regarding the effect of age on digestive activities, total trypsin and pancreatic lipase activity increased throughout the development of *C. estor* (Fig. 2A, C). In contrast, the specific trypsin activity showed a fluctuating profile during the first 45 dph, with a significant increase at the end of this period (Fig. 2B). When specific activity was measured in the digestive organs (hepatopancreas and intestines) from 60 to 150 dph, the profile was high and stable. The specific lipase activity showed a significant increase on the third day, after which it decreased and remained at low levels until 45 dph. However, specific activity during the 60 to 150 dph period was high and relatively stable (Fig. 2D).

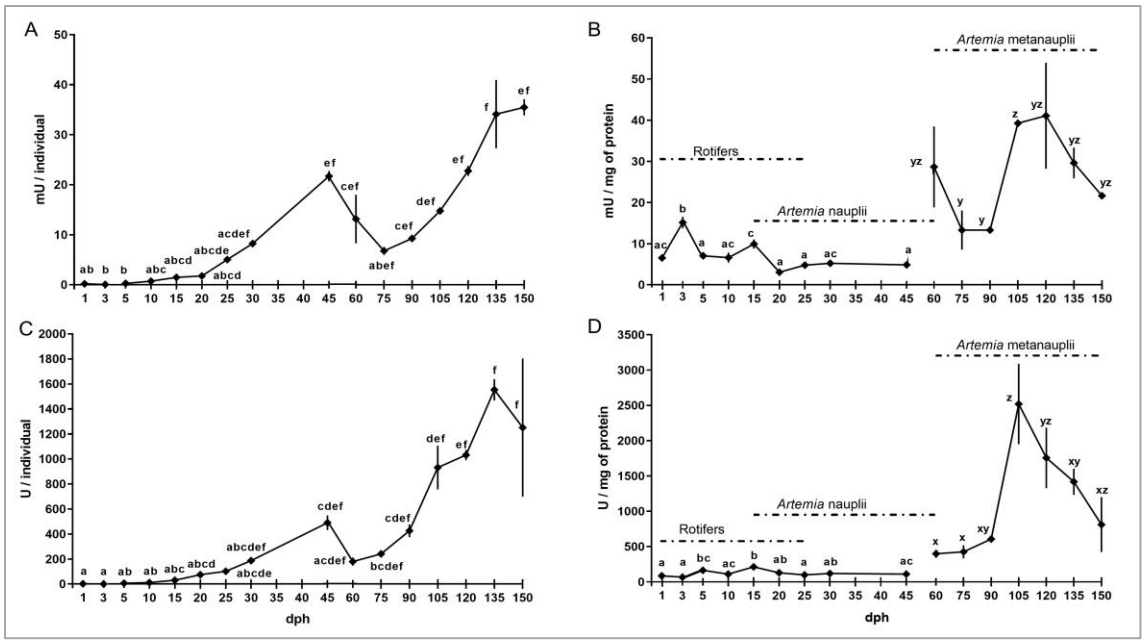
**Figure 2**




Total and specific activity of pancreatic digestive enzymes during the larval and juvenile development of *Chirostoma estor*. A and B: trypsin; C and D: lipase. Statistical analysis of the specific activity was performed in two periods: 1 to 45 and 60 to 150 dph due to tissue differences (digestive organs from 60 to 150 dph). Each circle represents the mean of twelve to fifteen individuals. Different letters indicate significant differences ($P < 0.05$) between days post-hatching. 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. The internal graph in A shows the levels of trypsin-specific activity during the first 30 dph. Horizontal broken lines indicate the period of the different feeding regimes. dph: days post-hatching.

The total activity of the cytosolic digestive enzymes, AcP and leu-ala, increased throughout larval and juvenile development of *C. estor* (Fig. 3A, C). The AcP specific activity levels were very low and stable during the first 45 dph. Similarly, the activity recorded between 60 to 150 dph was stable, but with higher levels since dissected intestines were analyzed in this period (Fig. 3B). In contrast, the specific activity of leu-ala remained constant during the first 45 days. Subsequently, from 60 to 150 dph, the activity showed a significant increase at 105 dph, with similar activity levels thereafter (Fig. 3D).

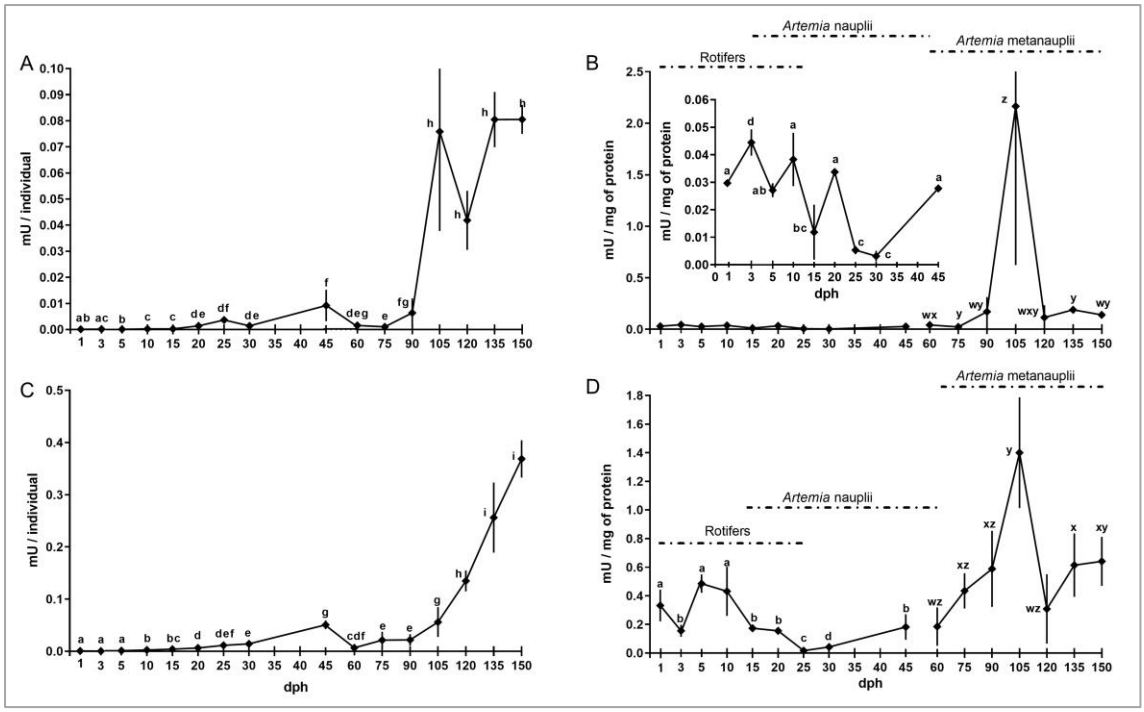
**Figure 3.**




Total and specific activity of the intestinal cytosolic digestive activities during larval and juvenile development of *Chirostoma estor*. A and B: acid phosphatase; C and D: leucine alanine peptidase. The statistical analysis of the specific activity was performed in two periods: 1 to 45 and 60 to 150 dph, due to tissue differences. Each circle represents the mean of two pools of individuals or two intestines. Different letters indicate significant differences ($P < 0.05$) between days post-hatching. 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. Horizontal broken lines indicate the period of the different feeding regimes. dph: days post-hatching.

The total activity of the intestinal brush border membrane digestive enzymes, AP and APN, increased during larval and juvenile development of *C. estor*, albeit at very low levels (Fig. 4A, C). On the other hand, the specific AP activity was also low but fluctuated during the first 45 days. In the second developmental period, the activity significantly rose until 105 dph, falling afterwards (Fig. 4B). APN-specific activity was low and fluctuating during the first 45 dph, while for the period from 60 to 150 dph, APN activity was analogous to that observed for AP. However, a second increase was observed after the significant increase at 105 dph (Fig. 4D). Interestingly, the specific activity of all the intestinal digestive enzymes cytosolic and membrane-bound) showed an abrupt increment at 105 dph.

**Figure 4.**




Total and specific activity of intestinal border membrane-digestive enzymes during larval and juvenile development of *Chirostoma estor*. A, B: alkaline phosphatase; C, D: aminopeptidase N. Statistical analysis of specific activity was performed in two periods: 1 to 45 and 60 to 150 dph due to tissue differences. Each circle represents the mean of two pools of individuals or two intestines. Different letters indicate significant differences ($P < 0.05$) between days post-hatching. Statistical significance was based on One-way ANOVA followed by Tukey's Multiple Range Test. The internal graph in B shows the levels of alkaline phosphatase specific activity during the first 45 dph. Horizontal broken lines indicate the period of the different feeding regimes. dph: days post-hatching.

The analysis of the influence of feed regime (feed type) on the specific digestive activity is shown in Table 2. As is observed, the activity of trypsin increased when fish were fed exclusively with *Artemia* nauplii. Although fish were fed on *Artemia* nauplii from day 30, the sample at this age was taken before feeding. Therefore, the influence of this feed was noted up to 45 dph. The lipase activity showed a fluctuating profile, with a tendency to decrease. However, feeding fish with *Artemia* nauplii also positively influenced lipase activity. No significant differences were detected in the activity of intestinal cytosolic digestive enzymes in relation to the feeding regime. Regarding the brush border membrane enzymes, co-feeding with rotifers and *Artemia* nauplii had a negative effect on AP and

APN activities, while their activity increased when fish were fed exclusively on *Artemia* nauplii (Table 2).

**Table 2.** Effect of feeding regime on the specific digestive enzyme activity during the ontogeny of Chirostoma estor.

| **Feeding regime** | **Rotifers** | | | | | **Rotifers + *Artemia* nauplii** | | ***Artemia* nauplii** | |
|---|---|---|---|---|---|---|---|---|---|
| **Age (dph)** | **1** | **3** | **5** | **10** | **15** | **20** | **25** | **30** | **45** |
| Trypsin | 27.18 ± 4.91$^{a}$ | 36.04 ± 5.36$^{a}$ | 30.45 ± 3.96$^{a}$ | 44.08 ± 5.12$^{a}$ | 10.83 ± 1.31$^{a}$ | 15.56 ± 2.54$^{a}$ | 2.02 ±0.19$^{a}$ | 13.02 ± 2.52$^{a}$ | 104.93 ±23.46$^{b}$ |
| Lipase | 2391.88 ±412.47$^{a}$ | 5502.70 ±284.52$^{a}$ | 4078.86 ±321.33$^{a}$ | 1945.61 ±420.28$^{a}$ | 246.30 ±26.38$^{a}$ | 100.60 ±14.64$^{a}$ | 140.19 ±18.74$^{b}$ | 397.45 ±29.55$^{b}$ | 268.82 ±31.79$^{b}$ |
| Alkaline phosphatase | 29.69 ± 0.38$^{a}$ | 44.49 ±1.96$^{a}$ | 27.13 ±1.28$^{a}$ | 38.32 ±4.85$^{a}$ | 11.84 ±6.89$^{a}$ | 33.75 ±0.02$^{a}$ | 5.37 ±0.77$^{b}$ | 3.18 ±0.98$^{b}$ | 27.77 ±0.39$^{ab}$ |
| Aminopeptidase N | 332.47 ± 49.52$^{a}$ | 155.64 ±17.12$^{a}$ | 484.77 ±28.61$^{a}$ | 432.03 ±65.12$^{a}$ | 173.25 ±0.12$^{a}$ | 155.50 ±8.74$^{a}$ | 16.97 ±0.50$^{b}$ | 42.54 ±6.19$^{b}$ | 182.15 ±36.36$^{ac}$ |

For the one-way ANOVA analysis, feeding regime was considered from 1 to 20 with rotifers, 25 to 30 with co-feeding on rotifers and *Artemia* nauplii, and from 45 dph with exclusively *Artemia* nauplii. This was because, on days 20 and 30, fish were sampled before feeding and, therefore, before the change in feed type. Alkaline phosphatase and aminopeptidase N are expressed as mU/mg of protein $\times 10^{-3}$. dph, days post-hatching.

When analyzing the influence of the feeding regime and age of the fish, it was found that the activities of digestive pancreatic enzymes were significantly swayed by the interaction of both variables (Table 3).

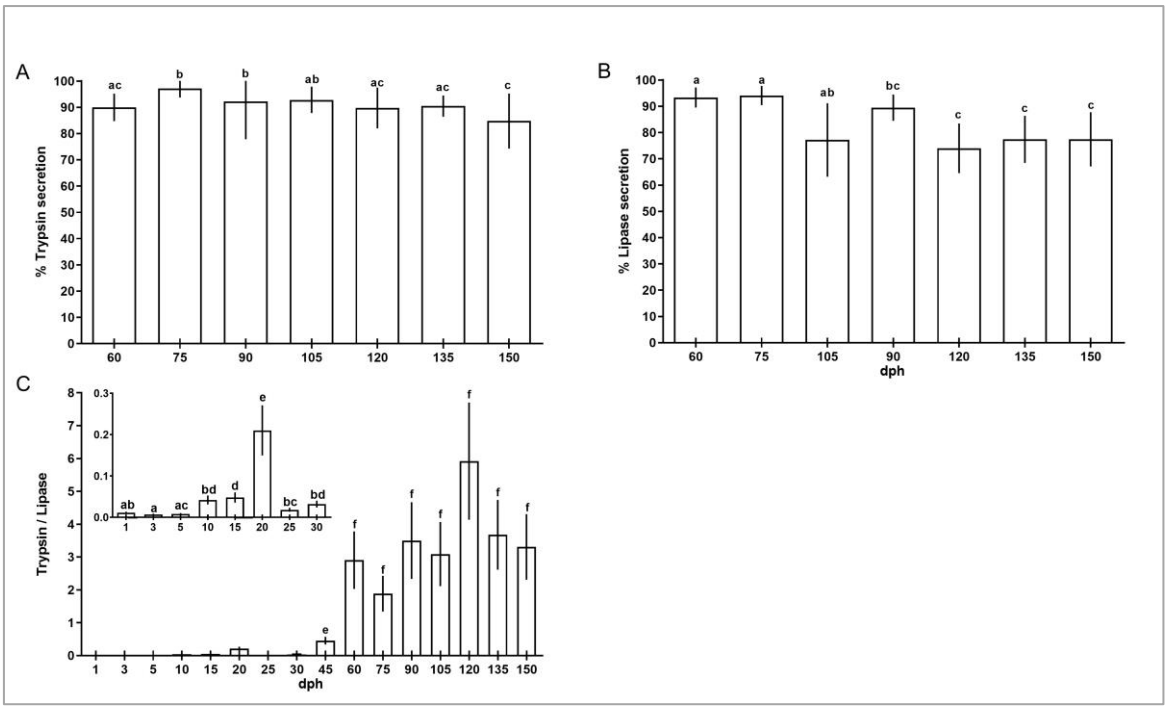
Also, diet and its interaction with age affected the specific activity of leu-ala. Meanwhile, acid phosphatase activity was influenced by both diet and fish age. Likewise, age and its interaction with diet influenced AP activity, while specific APN activity was controlled by age, feeding regime, and their interaction (Table 3).

**Table 3.** Results of the ANCOVA model for the effect of diet (feeding regime) and fish age on the digestive enzyme activities of *Chirostoma estor* during its larval and juvenile ontogeny. The model considered age as a covariate and diet as a factor.

| | **Covariate and factor** | **Specific activity** | | | |
|---|---|---|---|---|---|
| | | **d.f.** | **SS** | **F** | ***P*** |
| Trypsin | Age | 1 | 22992 | 21.53 | <0.00001 |
| | Diet | 2 | 38337 | 17.95 | <0.00001 |
| | Age:Diet | 2 | 53629 | 25.11 | <0.00001 |
| Lipase | Age | 1 | 244657268 | 136.80 | <0.00001 |
| | Diet | 2 | 46535987 | 13.01 | <0.00001 |
| | Age:Diet | 2 | 59980386 | 16.77 | <0.00001 |
| leu-ala | Age | 1 | 56 | 0.05 | 0.83 |
| | Diet | 2 | 13545 | 5.79 | 0.01 |
| | Age:Diet | 2 | 22922 | 9.79 | 0.01 |
| Acid phosphatase | Age | 1 | 104.96 | 9.72 | 0.00 |
| | Diet | 2 | 80.17 | 3.71 | 0.03 |
| | Age:Diet | 2 | 5.74 | 0.27 | 0.77 |
| Alkaline phosphatase | Age | 1 | 0.00 | 17.45 | 0.00 |
| | Diet | 2 | 0.00 | 0.68 | 0.51 |
| | Age:Diet | 2 | 0.00 | 22.23 | <0.00001 |
| APN | Age | 1 | 0.42 | 25.93 | <0.00001 |
| | Diet | 2 | 0.21 | 6.52 | 0.00 |
| | Age:Diet | 2 | 0.12 | 3.67 | 0.03 |

On the other hand, a significant increase in the percentage of trypsin secretion was observed on day 75, unlike lipase secretion (Fig. 5A, B).

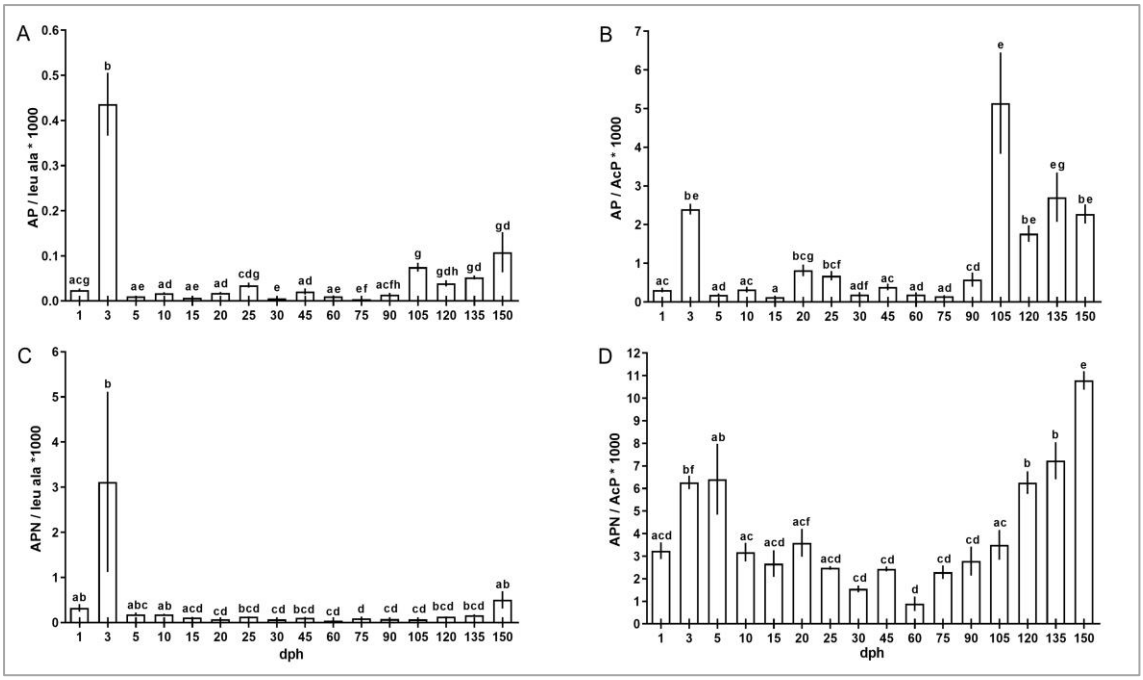
**Figure 5.**




Percentage of secretion. A: Trypsin and B: lipase. C: Trypsin/lipase activity ratios. Each bar represents the mean of twelve to fifteen individuals or organs. Different letters indicate significant differences ($P < 0.05$) between days post-hatching. Statistical significance was based on Kruskal-Wallis Analysis of Variance on Ranks followed by Dunn's Pairwise Multiple Comparison Procedures. The internal graph in C shows the levels of the trypsin/lipase activity ratio during the first 30 days. dph: days post-hatching.

Regarding the trypsin/lipase ratio, three significant increases were observed at 20, 45, and 60 dph (Fig 5C). With respect to the ratios of BBM/cytosolic activities, two common and statistically significant increases were observed for AP/leu-ala and AP/AcP, at 3 and 105 dph. The increase at 3 dph was also observed for APN/leu-ala and APN/AcP, but another significant increase was observed at 120 dph for the latter (Fig. 6).

**Figure 6.**




Intestinal brush border and cytosolic activity ratios. A and B: AP/leu-ala and AP/AcP activities ratios, respectively. C and D: APN/leu-ala and APN/AcP activities ratios, respectively. Each bar represents the mean of two pools of individuals or digestive organs. Different letters indicate significant differences ($P < 0.05$) between days post-hatching. 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. dph: days post-hatching.

## DISCUSSION

### Growth performance and the impact of nutrients and weaning time

The growth of *C. estor*, in terms of body weight and total length, in this study resembles that found in other studies for the species, under similar conditions (Martínez-Palacios et al., 2002b; Navarrete-Ramírez et al., 2011), although this is the first time that the growth profile has been analyzed from the larval and juvenile stages up to 150 dph. The specific growth rate found for the larval period is also analogous to previous studies (Toledo-Cuevas et al., 2011; Martínez-Chávez et al., 2014; Juárez-

Gutiérrez et al., 2021), suggesting a common and normal development for the species in the present study.

On the other hand, a higher decimal-log wet body weight and mean growth rate were found for the first period (1-45 dph), compared to the second period (60-150 dph). A similar performance has been reported in the anchovy *Engraulis encrasicolus* and the sardine *Sardine pilchardus*. These species showed higher growth rates than mackerel *Scomber scombrus*, horse mackerel *Trachurus trachurus* and hake *Merluccius merluccius*, suggesting that, for the cupleoids, swimming ability is more important than the development of a large mouth (Alvarez et al., 2021). Also, for the thick lip grey mullet *Chelon labrosus*, improving locomotor function is critical for food capture and predator avoidance (Gilannejad et al., 2020). Constant movement and a functional mouth have been described in *C. estor* one-day post-hatch larvae (Martínez-Palacios et al., 2006), and food has been found in their digestive tract at two days of age (Martínez-Angeles et al., 2022), suggesting a fast-growing larval stage in *C. estor*, which supports our findings. Similar fast larval growth has been described in two other atherinopsids, *Odontesthes bonariensis* and *O. hatcheri* (Toledo-Cuevas et al., 2024).

Although different growth phases are related to the morphophysiological changes occurring during fish development, these can also be influenced by the composition of the live feed. The SGR obtained in this study (at 25 days) is higher than that obtained by Juárez-Gutiérrez et al. (2021) at 27 days (16.47 vs 14.43) and by Martínez-Angeles et al. (2022) (13.69 vs 10.87, at 30 days), suggesting a better response of *C. estor* larvae to co-feeding on rotifers and *Artemia* rather than feeding exclusively on rotifers. Similar results were obtained for the pikeperch *Sander lucioperca* fed on a combination of rotifers and *Artemia* nauplii (Imentai et al., 2020; Yanes-Roca et al., 2018). A higher average protein and free amino acids content in *Artemia* nauplii than in rotifers can result in higher larval growth (Hamre et al., 2002; Carvalho et al., 2003; Conceição et al., 2010; Hamre, 2016).

On the other hand, the reduction in the slope of the decimal-log body weight between 60 to 150 dph may be attributed to the different nutrient and energy content of *Artemia* nauplii compared to *Artemia* metanauplii (Sorgeloos et al., 2001; Guevara & Lodeiros, 2003), which could cause an imbalance in the nutritional requirement for the juvenile stage. Alternatively, the growth slowdown could also be caused by the difficulty of *Artemia* in meeting the energy and nutrient requirements of fish after 60

days, related to the small size of *Artemia* metanauplii and the large amount of live feed required by larger fish, as suggested in other studies (Hamre et al., 2002).

Fish are known to maximize their energy gain by ingesting larger, higher-calorie prey (Sorgeloos et al., 2001; Hernández-Rubio et al., 2006; Bittar et al., 2012).

It is important to note that feeding only live feed for this study was performed to seek the maturation of the digestive system of the species, under cultured conditions, avoiding the introduction of a balanced diet, which could impair the maturation process (Zambonino-Infante & Cahu, 2001; Zambonino-Infante et al., 2008). Previous studies have determined that the digestive maturation in *C. estor* did not occur during the larval stage, that is, the 30 days post-hatching (Toledo-Cuevas et al., 2011). This may be the reason of why the best SGR obtained before (Martínez-Angeles et al., 2022) at 30 dph (13.79 %/day) is barely comparable with the one obtained in this study (13.69 at 30 dph) when it should have been much higher in the former study due to various factors that should have improved growth: 1) the fish were fed on a microdiet containing 278 g of soluble protein/kg and a mixture of *Lactobacillus* (0.5% *L. acidophilus* and 0.5% *L. plantarum*). The very high levels of the cytosolic (lysosomal) leu-ala activity found in *C. estor* (Toledo-Cuevas et al., 2011) suggests its great capacity to digest soluble proteins that would enter the intestine by pinocytosis, already demonstrated in juveniles of the species (Toledo-Cuevas et al., unpublished); 2) the inclusion of probiotics in feeds accelerates digestive maturation (Tovar et al., 2002; Ringø et al., 2020), and 3) the use of continuous illumination (24L) in the culture of *C. estor* also increases growth (Martínez-Chávez et al., 2014; Corona-Herrera et al., 2022 ). However, in the study of Martínez-Angeles et al. (2022), larvae were fed only on rotifers, and weaning occurred at 10 dph. Better results would undoubtedly have been obtained if the fish had been fed on rotifers and *Artemia*, and especially if weaning were performed at a later stage of development, since, as discussed later, the maturation of the *C. estor* digestive system seemed to occur after 105 dph. The nutrigenomics study conducted on *C. estor* weaned at 10 dph clearly shows that, at this early stage, larvae are not ready to be fed on microdiets (Juárez-Gutiérrez et al., 2021).

A similar negative impact on larval growth of weaning at an early stage has previously been reported in other fish species (Chen et al., 2022).

**Impact of age and nutrients on digestive enzyme activities**

The feeding regime influenced the digestive activity of most of the analyzed enzymes. Although the nutritional content of the live feed used in this study was not assessed, differences have been reported between rotifers and different life stages of *Artemia* (Hamre et al., 2002; Carvalho et al., 2003; Guevara & Lodeiros, 2003; Guermazi et al., 2008; Conceição et al., 2010; Hamre, 2016; Ringø et al., 2020). The protein content of *B. plicatilis* and *Artemia* nauplii varies depending on several factors (Guevara & Lodeiros, 2003; Guermazi et al., 2008; Øie et al., 2011; Peykaran Mana et al., 2014), although higher protein levels have occasionally been reported in the latter (Hamre et al., 2002; Conceição et al., 2010; Kotani et al., 2017; Peykaran Mana et al., 2014). This could explain the increase in trypsin activity from 45 dph. Higher trypsin activity was also reported in *S. lucioperca* when feeding on rotifers, followed by *Artemia* (Imentai et al., 2022). Furthermore, *C. estor* larvae could acquire more protein when feeding on *Artemia* nauplii since their larger size (compared with rotifers) makes them easier to capture (Guevara & Lodeiros, 2003; Conceição et al., 2010), which would increase trypsin activity since it is stimulated by its substrate (Cahu et al., 2004). On the other hand, although a higher lipid content is sometimes reported in *Artemia* nauplii than in rotifers (Hamre et al., 2002; Conceição et al., 2010; Peykaran Mana et al., 2014; Hamre, 2016), which may explain the gradual increase in lipase activity at 30 and 45 dph, the opposite trend of trypsin and lipase activities during the first 45 days is also related to the progressive shift from the use of lipids as the main nutrient to proteins, as reported for this and other species (Cara et al., 2003; Toledo-Cuevas et al., 2011). The higher phospholipid levels reported in rotifers than in *Artemia* nauplii (Øie et al., 2011; Dhont et al., 2013) could explain the decrease in alkaline phosphatase during the larval stage of *C. estor*, since AP activity is stimulated by nutrients containing organic phosphate, such as phospholipids (Lallès, 2020). However, AP activity may also be positively influenced by a possible higher consumption of *Artemia* nauplii by *C. estor* larvae, whose larger size than rotifers would facilitate it. AP activity is also significantly stimulated by food intake (Lallès, 2020). Higher AP levels were observed in *S. lucioperca* fed on *Artemia* or in combination with rotifers (Imentai et al., 2022). Finally, the different protein content between rotifers and *Artemia* nauplii could explain the differences in APN activity.

An increase in APN activity was found to be related to the shift of rotifers to *Artemia* in *G. morhua* larvae (Kvåle et al., 2007) and *S. lucioperca* (Imentai et al., 2022). APN activity is influenced by the level and source of dietary protein (Nicholson et al., 1974; Zambonino Infante & Cahu, 1994; Kvåle et al., 2007). However, APN is present in other organs besides the BBM of the intestine (Tang et al., 2016), which may influence the profile found for APN in *C. estor* larvae since their whole body was used until 45 dph. Future studies should include the evaluation of the nutritional content of the live feed to clarify all these diet-related results of digestive enzyme activity.

On the other hand, age (throughout larval and juvenile stages) and the type of live feed were also observed to affect all the digestive enzymes studied in *C. estor*. Changes in the digestive enzyme activity during fish development have been widely described in other species, as has the influence of the nutrient content and their form (Zambonino-Infante et al., 2008; Zambonino-Infante & Cahu, 2010). In addition to the above-mentioned differences in the nutritional content between rotifers and *Artemia*, nutrients also differ across the different *Artemia* stages. The nutritional content of *Artemia* is influenced by abiotic and biotic factors, feeding strategy, food bioavailability, and development stage, especially since *Artemia* feeding begins at the metanauplii stage (Guevara & Lodeiros, 2003; Guermazi et al., 2008; Peykaran Mana et al., 2014).

**Digestive enzyme activity features and maturation markers in *C. estor***

The maturation process of the digestive system can be monitored through the profile and activity levels of the different digestive enzymes synthesized in their respective organs, which has led to the discovery of biochemical markers of maturation in fish. Nevertheless, all these studies have been conducted mainly in commercial species that develop functional stomachs (Zambonino-Infante et al., 2008). Most studies on agastric fish have not been designed to investigate the profile of digestive enzyme activities during fish ontogeny, or they did not assess intestinal enzyme activities (Zambonino-Infante et al., 2008; Gisbert et al., 2013; Le et al., 2019). However, some studies of this kind have been carried out on South American silversides (known as pejerreyes) and on *C. estor*, the silverside of the Lake of Patzcuaro, to elucidate the model and timing of their digestive maturation (Pérez-Sirkin et al., 2020; Toledo-Cuevas et al., 2011, 2024). All these atherinopsid species are not only agastric but also have a short intestine (Toda et al., 1998; Ross et al., 2006; Toledo-Cuevas et al.,

2024). The study of some digestive maturation markers for *C. estor* describes that this process does not occur before the first 3 months of life (Toledo-Cuevas et al., 2011), and digestive maturation markers are not yet detected in pejerreyes up to 10 weeks post-hatching (Pérez-Sirkin et al., 2020).

Among the known markers of digestive maturation, cytosolic (lysosomal) intestinal digestive activity is known to be relevant during early stages of life when the digestive system has not fully matured. During the process of maturation, this lysosomal activity decreases, coinciding with a pronounced increase in the activity of digestive enzymes linked to the brush border (Zambonino-Infante & Cahu, 2001; Zambonino-Infante et al., 2008). In *C. estor*, the present study shows that the cytosolic activity levels of leu-ala and AcP do not decrease during the first 45 dph but significantly increase at 105 dph. Since the role of these enzymes has been described in the digestion of nutrients acquired by pinocytosis (Henning, 1987; Zambonino-Infante & Cahu, 2001; Lazo et al., 2011), and because pinocytosis has been demonstrated in juveniles of *C. estor* (Toledo-Cuevas et al., unpublished), it seems that the species maintains the activity of these enzymes as a functional compensation for its lack of a stomach and its possession of a thin and short intestine. This digestive strategy appears to be shared by other aquatic organisms that possess a "primitive" digestive system, such as the pejerreyes *Odontesthes bonariensis* and *O. hatcheri* (Toledo-Cuevas et al., 2024) and the sea cucumber *Isostichopus badionotus*, which does not possess a functional stomach (Martínez-Milián et al., 2021). Although there are reports of leu-ala activity that persists beyond the first month of life in some fish species, such as the cod *Gadus morhua* and the Atlantic halibut *Hippoglossus hippoglossus*, up to 75 and 117 dph, respectively (Kvåle et al., 2007), the activity levels are much lower than those found in *C. estor*. Thus, not only is the prolonged presence of leu-ala and AcP a characteristic of this type of digestive system, but also the very high levels of leu-ala, several times higher than in other agastric fish with a long intestine and in gastric fish (Toledo-Cuevas et al., 2024). Specialized enterocytes in zebrafish internalize dietary protein for intracellular digestion throughout their life and are essential for the growth and survival of fish larvae and mice (Park et al., 2019).

The activity of BBM enzymes, APN and AP, was detected on the first day post-hatching of *C. estor* (just like the activity of all the cytosolic and pancreatic digestive enzymes herein studied), and like the cytosolic digestive activities, their activity increases with age.

The BBM activity is, however, much lower than that previously reported for *C. estor* (Toledo-Cuevas et al., 2011). This, even though in the present study, BBM were purified, suggesting difficulties in the analytical methods. Notwithstanding this, a significant increase in the activity of both enzymes was detected at 105 dph and constant levels thereafter. BBM enzymes have been reported as markers of digestive maturation (Kvåle et al., 2007; Zambonino-Infante & Cahu, 2011), but their specific activities decline a few weeks after hatching in several fish species (Zambonino-Infante et al., 2008; Solovyev et al., 2016; Koven et al., 2019; Mozanzadeh et al., 2021). The high and constant levels of BBM activities found for *C. estor* suggest that this species maintains all the digestive activity it possesses (cytosolic and BBM-linked) as a digestive strategy due to its digestive anatomical limitations: absence of a stomach and a thin and short intestine.

Findings of digestive maturation were observed in *C. estor*. The ratios of trypsin/lipase activities suggest the early acquisition of pancreatic function, between 20-45 dph, since at those ages, lipase activity decreases in relation to protein digestion by trypsin, as also described for the white seam bream *Diplodus sargus* (Cara et al., 2003), a profile that resembles previous studies in *C. estor* (Toledo-Cuevas et al., 2011). On the other hand, the secretion function of pancreatic enzymes appears to be acquired between 60-75 dph. It has been suggested that the pancreatic function is completed shortly after hatching, while secretion begins later in the development, followed by intestinal maturation (Zambonino-Infante et al., 2008; Lazo et al., 2011). In *C. estor*, the BBM/cytosolic ratios showed significant increases at 105, 120 and 150 dph, suggesting a late maturation of intestinal function (Zambonino Infante & Cahu, 1994), as previously reported (Toledo-Cuevas et al., 2011). However, it is important to highlight that these ratios were not found in *O. bonariensis* (Pérez-Sirkin et al., 2020), suggesting that either the high and prolonged levels of cytosolic enzymes impaired the finding of this maturation index or that intestinal maturation occurs quite late in development, as observed for *C. estor* (Toledo-Cuevas et al., unpublished). Interestingly, at 105 dph, almost all the analyzed digestive enzyme activities peaked, suggesting that some changes in the microanatomy of the digestive tract might occur at this age. This should be clarified with future histological studies.

## CONCLUSIONS AND IMPLICATIONS

This study suggests that the combined feeding on rotifers and *Artemia* nauplii is relevant for optimal growth of *C. estor* larvae. It was also found that *C. estor* digestive system maturation occurs around 105 dph, indicating that weaning should occur after the third month post-hatching. Furthermore, the maturation indexes described for gastric fish (pancreatic secretion, trypsin/lipase and the BBM/cytosolic intestinal digestive ratios) are present in *C. estor*, despite its absence of a stomach. Nevertheless, some of the compensations for its "limited" digestive system are the prolonged presence of both cytosolic and brush-border digestive enzymes, at least until 150 dph, in addition to the unusually high levels of leu-ala activity.

**Acknowledgements and conflict of interest**

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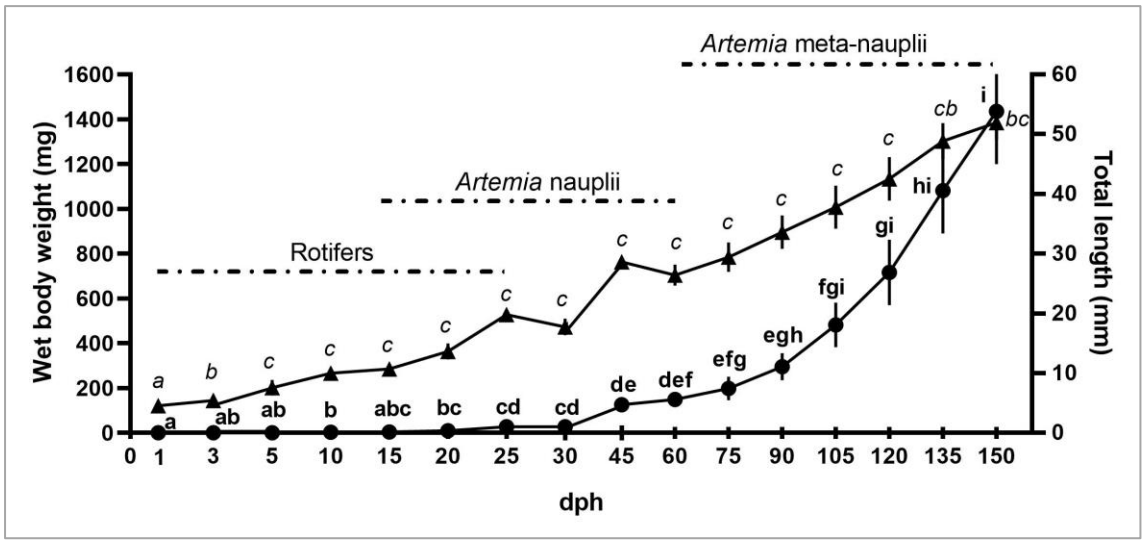
**ANEXO**

**Supplementary material**

**Fig. S1.**




Growth profile of *Chirostoma estor* during its larval and juvenile development, until 150 dph. Wet body weight (WBW, mg ± standard deviation, SD, $n$ = 10 (except for 1 and 5 dph, where pools of 60-130 individuals were used), black circles) showed a potential profile with the equation: $WBW = 0.005dph^{4.237}$, $R^2 = 0.983$. On the other side, Total length (TL; mm ± SD, $n$ = 10, black triangles) show a linear profile described by the equation: $TL = 3.206dph - 6.176$, $R^2 = 0.968$. Horizontal broken lines indicate the period of the different feeding regimes. dph. Days post-hatching.