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# Expression analyses of digestive enzymes during early development and in adults of the chame fish *Dormitator latifrons*

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## Abstract

This study describes the expression of genes that encode digestive enzymes (trypsin, pepsinogen, maltase-glucoamylase, sucrase-isomaltase, alkaline phosphatase and leucine aminopeptidase) using qRT-PCR in 0- to 7-day post-hatching (dph) chame (*Dormitator latifrons*). Additionally, adults liver and intestine were analysed for enzyme expression. Results showed that transcripts for all enzymes are present in both adult and larvae tissues. In adults, the expression of maltase-glucoamylase and sucrose-isomaltase showed the highest values in the middle intestine, while the liver and anterior and posterior intestine showed low expression levels. Other enzymes showed low expression in all tissues, with the exception of leucine aminopeptidase, which showed high expression in liver. In larvae whole-body samples of *D. latifrons*, maltase-glucoamylase and sucrose-isomaltase showed the highest expression from 3 to 5 dph, while other enzymes maintained low levels from hatching to 7 dph. The highest expression of disaccharidases such as maltase-glucoamylase and sucrose-isomaltase corresponded to the beginning of exogenous feeding, suggesting that this species exhibits an herbivorous profile. However, the presence of proteolytic enzymes may indicate that *D. latifrons* is programmed to be able to digest other substrates. This information will contribute to the development of larval feeding protocols to produce chame juveniles in laboratory conditions, as prior larval feeding essays have not been successful, using either live feeds or commercial fish larval diets.

KEYWORDS

chame, digestive enzyme, *Dormitator latifrons*, gene expression

## 1 | INTRODUCTION

The Pacific fat sleeper or chame (*Dormitator latifrons*) is a stomachless fish belonging to the Eleotridae family, whose distribution ranges from California to northern Perú. *D. latifrons* has very particular physiological attributes, because it tolerates drastic changes in salinity and temperature and long periods of hypoxia, as well as being resistant to manipulation (Larumbe, 2002; Yáñez-Arancibia & Díaz-González, 1976). These characteristics present an excellent alternative for the diversification of species for aquaculture in México, given that production statistics as farming already exist in countries such as Ecuador where wild juveniles are reared in extensive ponds, called chameras (Bonifáz, Campos, & Castelo, 1985), and previous research has been focused on the description of digestive tract morphological development in larvae from 1 to 6 dph, identifying that the larvae are able to initiate exogenous feeding from 4 dph (López-López et al., 2015). In addition, efforts have been made to find a suitable zooplanktonic prey once upon onset of exogenous

  

feeding, such as the minute rotifer *Proales similis*, given the small mouth gap that chame larvae exhibit (<100 microns) (Román- Reyes, López-Monteón, Castillo-Urreta, Monroy-Dosta, & Montes de Oca, 2017), but until now it has not been possible to establish a diet that allows for larvae to survive after 6–7 dph. The chame fish is known as a detritivore species, so it is necessary to obtain a profile of digestive enzymes on the early developmental stages of the species with the purpose of obtaining information about the digestive physiology, which can be used to develop adequate feeding strategies for increasing the larval survival of this fish.

Some studies have been carried out in stomachless species, where the main focus of research includes gene expression patterns and activity of digestive enzymes at early developmental stages, including research on ballan wrasse *Labrus bergylta* (Hansen, Folkvord, Grøtan, & Sæle, 2013), and bullseye puffer fish *Sphoeroides annulatus* (García-Gasca, Galaviz, Gutiérrez, & García-Ortega, 2006), as well as the description of structure and function of the digestive tract, including in topsmelt *Atherinops affinis* (Logothetis, Horn, & Dickson, 2001), jacksmelt *A. californiensis* and California grunion *Leuresthes tenuis* (Horn et al., 2006) and chame *D. latifrons* (López-López et al., 2005). However, aspects of both digestive enzyme expression and activity patterns and morpho-physiological digestive tract development for a species will depend on different factors such as habitat, food availability and feeding habits. In freshwater fish such as the carnivorous *Hoplias malabaricus* were reported higher acid protease and trypsin activities, while in herbivorous fish grass carp *Ctenopharyngodon idella* the higher activity enzymes were amylase and maltase. However, omnivorous species such as piava *Leporinus obtusidens* and silver catfish *Rhamdia quelen* showed intermediate activity values (Gioda et al., 2017). On the other hand, in the long-whiskered *Pimelodus maculatus* was reported a hepatic β-glucosidase activity higher in an oligotrophic reservoir, while the chymotrypsin-like activity was higher in eutrophic reservoir. These results could be related to the microbiota activity in the intestinal tract, as a result of the food habits and the environment, (Duarte, Bemquerer, & Araújo, 2015). In some species, it has been found that the morphology of the digestive tract and the digestive enzyme activity are related to the feeding habits during their ontogeny as in the characid fish *Brycon guatemalensis* (Drewe, Horn, Dickson, & Gawlicka, 2003) and that the structure of the digestive tract can be adapted to the diet as catfish *Pachypterus khavalchor* (Gosavi, Chandani, Sanjay, Manoj, & Kumkar, 2019).

For aquaculture species, it is necessary to know the digestive strategies in their natural habitats for implementing diets adequate (Wang et al., 2015). The major challenge has been to ensure proper quality feeds for first-feeding larvae and later juvenile production (Hamre et al., 2013). The onset of exogenous feeding is a crucial step in the development of any species of fish, given that it is fundamental for survival, somatic growth and health. Therefore, the digestive processes including the above-mentioned factors must be well known to establish a proper diet or prey selection and adequate feeding protocols to achieve juvenile production of chame fish.

**Highlights**

- An analysis of the relative expression of digestive enzymes was carried out in *Dormitator latifrons* larvae and adults.
- Digestive enzyme activity in adults corresponds to an omnivorous fish.
- New gene sequences for six digestive enzymes and two reference genes were identified for this fish.
- *mgam* and *si* disaccharidase enzymes showed a significant increase during the transition from endogenous to exogenous feeding in larvae.
- Larvae have an apparent preferential enzymatic activity for non-proteic substrates.
- Our results can be used to create adequate diets that allow larvae to survive after 6–7 dph.

For this reason, in this study we selected some genes that code for proteases and carbohydrases that have been studied in other stomachless fish (Day et al., 2011). The transcripts of proteolytic enzymes such as trypsin and pepsinogen and intestinal enzymes such as alkaline phosphatase, leucine aminopeptidase, maltase-glucoamylase and sucrase-isomaltase were analysed. The selection of disaccharidases maltase-glucoamylase and sucrose-isomaltase was analysed because their activity is highest in the intestinal mucosa of herbivorous rather than carnivorous species, and it has been observed that the intestinal mucosa shows a high capacity for degrading maltose over other disaccharides (Krogdahl, Hemre, & Mommsen, 2005). This will allow us to know more about the food habits of chame larvae, under the assumption that enzyme expression patterns are genetically encode at the unset of exogenous feeding, even under starvation conditions (Zambonino-Infante & Cahu, 2001).

In this study, the relative expression levels of genes that encode for digestive enzymes were determined in 0- to 7-dph whole-body samples of chame larvae and >150 g adult fish. The analysis was also carried out in adult organisms to establish the site of expression of digestive enzymes in the digestive system.

## 2 | MATERIALS AND METHODS

### 2.1 | Larval fish rearing and sample collection

Adult chame (*Dormitator latifrons*) >150 g and 14–25 cm mean individual weight and length, respectively, were collected from an extensive pond in shrimp farm 40 km south of Mazatlán, Sinaloa, México in October 2016. Animals were used as broodstock to produce the experimental pool larvae at the Fish Reproduction and Culture Laboratory at the Facultad de Ciencias del Mar (FACIMAR-UAS). These fish were kept in a recirculation system (for nitrogen waste control) with six 250-L tanks with constant aeration and

natural photoperiod, fed daily with a 32% crude protein 6% lipid commercial diet for tilapia at satiation, one ratio before noon. Fish were induced to spawn and spermiate following the protocol reported by Rodríguez-Montes de Oca et al. (2012) using LRHRa injections within 3–5 days after arrival. Once gametes were obtained, artificial fertilization was performed, and the eggs were placed in a transparent 1-L plastic container, filled with filtered water (5 µm) and irradiated with UV light, using constant aeration at room temperature (26 ± 2°C) for 14–16 hr until hatching. Thereafter, the obtained larvae (individual length around 1.2 mm per larvae) were transferred to three holding tanks (150 L) with constant aeration with airstone diffusers at 15 g/L salinity, at room temperature (27.3 ± 1.7°C) with daily partial water exchange (at least 25% total volume) at same salinity during sample collection; subsequently, chame larvae were reared until seven dph with no feeding (starvation). A pool of larvae (~100–120 larvae) were sampled daily from zero hours post hatching every 24 hr until 7 dph; each sample was preserved in RNA stabilization solution (RNAlater, Ambion®) and stored at −20°C until processing.

## 2.2 | Adult fish samples

Fish from the breeder group were selected for sampling 2 weeks upon arrival at our facilities (specimens were kept in same recirculation system and conditions as mentioned above). Samples from a posterior portion of the liver and intestine (anterior, middle and posterior portions) were taken from three adult chame (mean weight 150 g). All samples were preserved in RNA stabilization solution (RNAlater, Ambion®) and stored at −20°C until further analysis.

## 2.3 | Selection of target and reference genes and oligonucleotide design

Specific oligonucleotides were designed for beta-actin (*act-β*) using the software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) from a sequence of *Dormitator latifrons* deposited in GenBank (accession no. KU958389.1). The oligonucleotides for alkaline phosphatase (*alp*), maltase-glucoamylase (*mgam*), sucrase-isomaltase (*si*) and elongation factor alpha (*ef-α*) were designed from well-conserved regions of an alignment of marine teleost homolog sequences. Oligonucleotides for digestive enzymes and reference genes were selected from those reported in other species. Trypsin (*tryp*) and ribosomal 18S-rRNA (*18S*) were reported for spotted rose snapper (Moguel-Hernández et al., 2016); leucine aminopeptidase (*lap*) and alpha-tubulin (*tub-α*) were reported for Asian seabass (Srichanun, Tantikitti, Utarabhand, & Kortner, 2013); pepsin (*pep*) was reported for totoaba (Galaviz et al., 2015). The oligonucleotides of digestive enzymes and reference genes used for qPCR analyses are shown in Table 1. The most stable reference gene found for larvae and adult tissues was *act-β*. Additionally, the second most stable reference gene was selected for each analysis. For the analysis of larvae, *ef-α* was selected, and for the analysis of tissues, *18S* was selected. The geometric mean of the two

**TABLE 1** Real-time PCR primer sequences for analysis

| Gene name | Gene abbreviation | Oligonucleotides 5′–3′ | Annealing temperature (°C) | Fragment size (bp) | Access number |
|---|---|---|---|---|---|
| Trypsin | *tryp* | F:ATGAGCTCCACTGCTGACAGGAAC<br>R:AGAGTCACCCTGGCAAGAGTCCTT | 60.0 | 156 | MG373167.1 |
| Leucine aminopeptidase | *lap* | F:TGGATGCAATGAGAGCTGAT<br>R:TGGTTTAGTGGCTTTTCCAC | 62.0 | 143 | MG373157.1 |
| Pepsinogen | *pep* | F:CTCTGACAACGTCGTGCCTGTCTT<br>R:CAGAGGTCAGAGGGATCCAGGTGA | 60.0 | 172 | MG373164.1 |
| Alkaline phosphatase | *alp* | F:GAGATGGACAAGTTCCCTTTTGTG<br>R:GCCTGCATCCTTAGCCCATCTGA | 60.0 | 213 | MG373158.1 |
| Maltase-glucoamylase | *mgam* | F:ATGGGGGAACACACAACCTC<br>R:AGGACTCCTCCGATGGTTC | 60.0 | 160 | MG373163.1 |
| Sucrose-isomaltase | *si* | F:TGAACAGCAATGCCATGGAGGTG<br>R:AGCTGGAAGCCCAGGGACCAGTA | 60.0 | 179 | MG373165.1 |
| Elongation factor 1 alpha | *ef-1α* | F:TCAAGTACGCCTGGGTGTTG<br>R:CGCAGTCAGCCTGAGAGGTA | 60.0 | 174 | MG373161.1 |
| Actin-beta | *act-β* | F:GGGTATGGGCCAGAAAGACA<br>R:CTTCTCCCTGTTGGCTTTGG | 60.0 | 177 | – |
| 18S ribosomal | *18S* | F:CTGAACTGGGGCCATGATTAAGAG<br>R:GGTATCTGATCGTCTTCGAACCTC | 60.0 | 165 | – |
| Tubulin-alpha | *tub-α* | F:GGCACTACACAATCGGCAAAGAGA<br>R:TCAGCAGGGAGGTAAAGCCAGAGC | 60.0 | 144 | MG373159.1 |

most stable reference genes was used to obtain the normalization factor (*nf*) for each analysis.

## 2.4 | RNA extraction, reverse transcription and PCR assays

Total RNA was extracted from each pool of whole larvae divided in three replicates (~20–30 larvae per replicate) and posterior liver and intestine (anterior, middle and posterior portions) obtained from chame adults, which were removed from the RNAlater® (Ambion) and homogenized in 800 µl of TriPure Isolation Reagent (Roche Diagnostics) following the manufacturer's guidelines. RNA integrity was confirmed on a TBE 1% agarose gel and was quantified spectrophotometrically (NanoDrop Lite, Thermo Fisher Scientific®). The RNA obtained was treated with RQ1 RNase-Free DNase (Promega) according to the manufacturer's instructions. The absence of genomic DNA contamination was confirmed by performing a PCR non-amplification using *beta-actin* (*act-β*, Table 1) oligonucleotides (0.24 µM each), 0.25 U GoTaq Flexi DNA Polymerase (Promega), 2.5 mM $MgCl_2$, 1× Go Taq Flexi Buffer, 0.25 mM dNTP Mix (Promega) and 1 µl de RNA treatment, in a final volume of 12 µl. The PCR was performed in a thermocycler T-1000 (Bio-Rad Laboratories) using the following conditions: initial denaturation at 95°C for 3 min, 35 cycles at 95°C for 30 s, 60°C for 30 s and a final extension at 72°C for 10 min. PCR non-amplifications were checked on a TBE 1% agarose gel with 1× GelRed (Biotium Inc.) and visualized in a Gel Doc™ EZ (Bio-Rad). Two microgram of RNA was reverse-transcribed using Improm-II Reverse Transcriptase (Promega) according to the manufacturer's instructions, with RNAsin Plus RNase inhibitor (Promega) and a mix of oligo $(dT)_{18}$ synthesized using IDT (Integrated DNA Technologies Coralville), with random primers (Promega) in a volume of 40 µl. The resulting complementary DNA (cDNA) was kept at −20°C. The oligonucleotides of digestive enzymes and reference genes were first tested using end-point PCR amplification with a cDNA from pool larvae and chame tissues. The PCR conditions were as mentioned above, using 1 µl of each cDNA and 0.48 µM each primer, in a final volume of 12 µl (the concentration of oligonucleotides was modified depending on the presence of primer dimers). The PCR programs and the visualization of PCR products were as mentioned above. Positive amplifications were rerun in a TBE 2% agarose gel with 1× GelRed. The fragments of the predicted size for each oligonucleotides pair were cut and eluted in ~50 µl of nuclease-free water at room temperature overnight. A total of 1 µl of the eluted fragments was reamplified with the same PCR conditions and oligonucleotides used to produce them, using a PCR program with 20 cycles. The amplifications were verified using electrophoresis as described above and were sent to Macrogen Inc. for both forward sequencing and reverse sequencing. The sequences obtained were aligned using BIOEDIT software version 7.2.5 (Hall, 1999), and the consensus sequences were subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) and were submitted to GenBank (accession numbers are shown in Table 1).

## 2.5 | Molecular phylogenetic analysis of pepsinogen of *Dormitator latifrons*

The alignment of the amino acid sequences of fish pepsinogens (Figure 1) and construction of the phylogenetic tree (Figure 2) were carried out to validate that the sequence was pepsinogen, because *D. latifrons* is an agastric fish. The alignment was performed based on the amino acid sequences of pepsinogens using the program of Clustal Omega (Sievers et al., 2011). The phylogenetic analyses were performed including fish pepsinogens A and pepsinogens C amino acid sequences. The amino acid sequences were obtained from the GenBank sequences databases. Model testing and phylogenetic analysis were carried out in MEGA 7.01.21 (Kumar, Stecher, & Tamura, 2016), where the evolutionary history was inferred using the maximum likelihood (ML) method based on Whelan and Goldman (2001) model, based on the Tamura 3-parameter model (Tamura, 1992) using a discrete Gamma distribution (TN93 + G). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining method and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model.

## 2.6 | Real-time quantitative PCR analysis of digestive enzymes from *Dormitator latifrons*

The expression of digestion-related genes of *D. latifrons* was determined using real-time PCR. To analyse the transcriptional profile of each digestive enzyme, gene expression levels were determined in three larval whole-body pooled samples from 0 to 7 dph, as well as tissues from adult chame (posterior liver and portions of intestine). qPCR reactions were performed in a total volume of 15 µl with 0.45 U of GoTaq Flexi DNA (Promega), 2.5 mM $MgCl_2$, 1× Go Taq Flexi Buffer, 0.2 mM dNTP Mix (Promega), 1× EvaGreen fluorescent dye (Biotium), 0.15–0.40 µM each primer and 5 µl cDNA (50 ng/µl) as reported in Llera-Herrera, García-Gasca, Huvet, and Ibarra (2012). Each individual sample was measured in triplicate, and a no-template control was added as a negative control. The real-time PCR conditions were as follows: denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s and the annealing temperature from Table 1 for each oligonucleotides pair for 1 min. At the end of each PCR reaction, a melting curve analysis was performed to verify the specificity of the PCR products, confirming that only one PCR product was present. PCR amplification was conducted using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) with 96-well clear low-profile PCR microplates and ultraclear sealing film (Axygen Scientific). The appropriate reference genes as endogenous controls were determined through four computational algorithms ($\Delta C_t$, NormFinder, BestKeeper and GeNorm) that are integrated into the RefFinder software package (http://leonxie.esy.es/RefFinder), using the Cqs values to evaluate the relative stability. The stability of reference genes was analysed for larvae and tissues independently. Standard curves were made for each gene with six different dilutions (factor 1:5) in triplicate of the cDNA pool samples, amplification efficiencies (E) were obtained from the slopes of

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pepsinogen C
Trematomus_bernacchii_gastricsin_CAD80098.1                  SYPSISAGGETPVVDNMISQNLLSANIFSFYLSRGGQQGSVLSFGGVDTSLYRGQIYWTP  237
Epinephelus_coioides_pepsinogen_C_ABX44849.1                 SYPTISAGGETPVMDNMINQNLLSSNIFAFYLSRGGQQGSVLSFGDVDNSLYEGQIYWTP  238
Mastacembelus_armatus_gastricsin_XP_026166324.1              SYPAISAGGETPVMDNMISQNLLNADIFAFYLSRGGQQGSVLSFGGVDNMYQGQVYWTP  238
Siniperca_chuatsi_pepsinogen_C_ACF18591.1                    SYPTISAGGATPVMDNMISQNLLNADIFAFYLSSGEQQGSELSFGGVDSSMYQGQIYWTP  238
Siniperca_scherzeri_pepsinogen_C_ACF18590.1                  SYPTISAGGATPVMANMISQNLLNADIFAFYLSSGEQQGSELSFGGVDSSMYQGQIYWTP  238
pepsinogen A
Dormitator_latifrons_pepsinogen_AVI57488.1                   ------SDNVVPVFNTMVKQGLLPQNYFSVYLSRNDDQGSEVIFGGIDSSHFTGSITWIP   54
Boleophthalmus_pectinirostris_pepsin_A-like_XP_020785298.1   AFQSIAADNVVPVFKNMVAQGLLGQPLFSVYLSSNDQQGSEVIFGGIDVSYYTGSIYWIP  239
Anabas_testudineus_pepsin_A-like_XP_026224825.1              AFQSIASDDVVPVFVNMVSQGLVSQPMFSVYLSSNSEQGSEVVFGGVDSSHYTGEITWIP  237
Mastacembelus_armatus_pepsin_A-like_XP_026188356.1           AFQSIASDNVVPVFDNMINQGLVSQSMFSVYLSSNSDQGSEVVFGGVDSNHYTGQIAWIP  239
Monopterus_albus_pepsin_A-like_XP_020470274.1                AFQSIASDNVVPVFYNMIKQGLVSQSMFSVYLSSNSEQGSEVVFGGVDSNHYTGQITWIP  238
Epinephelus_coioides_pepsinogen_A1_ABY87034.1                AFQTIAADNVVPVFDNMVKQGLVSQPLFSVYLSSHGEQGSEVVFGGIDSSHYTGQVTWVP  237
Micropterus_salmoides_pepsinogen_2_BAU37038.1                AFQSIASDNVVPVFDNMIQQGLVSQPMFSVYLSGNSDQGSEVVFGGVDSSHYTGQITWIP  237
Siniperca_chuatsi_pepsinogen_A2_ACT35560.1                   AFQTIASDNVVPVFDNMVKQGLVSQPLFSVYLSSNSEQGSEVVFGGIDSSHYTGQITWIP  237
Siniperca_scherzeri_pepsinogen_A2_ACT35559.1                 AFQTIASDNVVPVFDNMVKQGLVSQPLFSVYLSSNSEQGSEVVFGGIDSSHYTGQITWIP  237
Pagrus_pagrus_pepsinogen_AAZ29603.1                          AFQSIASDNVVPVFDNMIKQGLVSQPMFSVYLSGNSEQGSEVVFGGTDSNHYTGQITWIP  118
Pagrus_major_pepsinogen_2_BAL14143.1                         AFQSIASDNVVPVFDNMIKQGLVSQPMFSVYLSGNSEQGSEVVFGGTDSNHYTGQITWIP  238
                                                                   :.. .**. .*: *.*:    *:.***   :*** : **. * . : *.: * *

pepsinogen C
Trematomus_bernacchii_gastricsin_CAD80098.1                  VTSETYWQIGVQGFEINGRETGWCSQGCQSIVDTGTSTLTAPSHFIGDIMQAIGAQRSQN  297
Epinephelus_coioides_pepsinogen_C_ABX44849.1                 VTSETYWQIGIEGFEINGRQTGWCSQGCQAIVDTGTSTLTAPGQLLGYIMQAIGAQQSQY  298
Mastacembelus_armatus_gastricsin_XP_026166324.1              VTSQTYWQIGVNGFAINGQETGWCSQGCQSIVDTGTSMLTAPSQYLGSLMQAIGAQQNQY  298
Siniperca_chuatsi_pepsinogen_C_ACF18591.1                    VTSETYWQIGVQGFQINGQESGWCSQGCQSIVDTGTSMLTAPSQLLGYIMQAIGAQQNQY  298
Siniperca_scherzeri_pepsinogen_C_ACF18590.1                  VTSETYWQIGVQGFQINGQESGWCSQGCQSIVDTGTSMLTAPSQLLGYIMQAIGAQQSQY  298
pepsinogen A
Dormitator_latifrons_pepsinogen_AVI57488.1                   LTS-------------------------------------------------------- 57
Boleophthalmus_pectinirostris_pepsin_A-like_XP_020785298.1   LSSATYWQIKMDSVTINGQI-VACADGCQAIIDTGTSLIVGPNTGINKINSRLGASLNQY  298
Anabas_testudineus_pepsin_A-like_XP_026224825.1              LTSATYWQIKMDSVTINGQT-VACSGGCQAIIDTGTSQIVGPTTDINNMNSKVGASTDQY  296
Mastacembelus_armatus_pepsin_A-like_XP_026188356.1           LTSATYWQIQMDSVTINGQT-VACSGGCQAIVDTGTSLIVGPTSDINNMNAKVGATTDQY  298
Monopterus_albus_pepsin_A-like_XP_020470274.1                LTSATYWQIKMDSVTINGQT-VACSGGCQAIIDTGTSLIVGPTSDINNMNSKVGATTDQY  297
Epinephelus_coioides_pepsinogen_A1_ABY87034.1                LTSATYWQIKMDGVKINGQT-VACAGGCQAIIDTGTSLIVGPTNDINNMNSKVGASTNQY  296
Micropterus_salmoides_pepsinogen_2_BAU37038.1                LTSATYWQIQMDSVTINGQT-VACSGGCQAIIDTGTSLIVGPTSDISNMNSKVGATTDQY  296
Siniperca_chuatsi_pepsinogen_A2_ACT35560.1                   LSSATYWQIKMDSVTINGQT-VACSGGCQAIIDTGTSLIVGPTSDINNMNAKVGASTNQY  296
Siniperca_scherzeri_pepsinogen_A2_ACT35559.1                 LSSATYWQIKMDSVTINGQT-VACSGGCQAIIDTGTSLIVGPTSDINNMNAKVGASTNQY  296
Pagrus_pagrus_pepsinogen_AAZ29603.1                          LSSATYWQISMDSVTINGQT-VACSGGCQAIIDTGTSLIVGPTNDINNMNSKVGASTNQY  177
Pagrus_major_pepsinogen_2_BAL14143.1                         LSSATYWQISMDSVTINGQT-VACSGGCQAIIDTGTSLIVGPTNDINNMNSKVGASTNQY  297
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**FIGURE 1** Amino acid sequence alignments of fish pepsinogens and their phylogenetic tree. The prepro-forms of pepsinogen C are included for comparison. Amino acid sequences aligned using the program Clustal Omega (Sievers et al., 2011) with default adjustments. Asterisks, two dots and single dots indicate identical residues, chemically similar residues and residues with weak chemical similarity, respectively

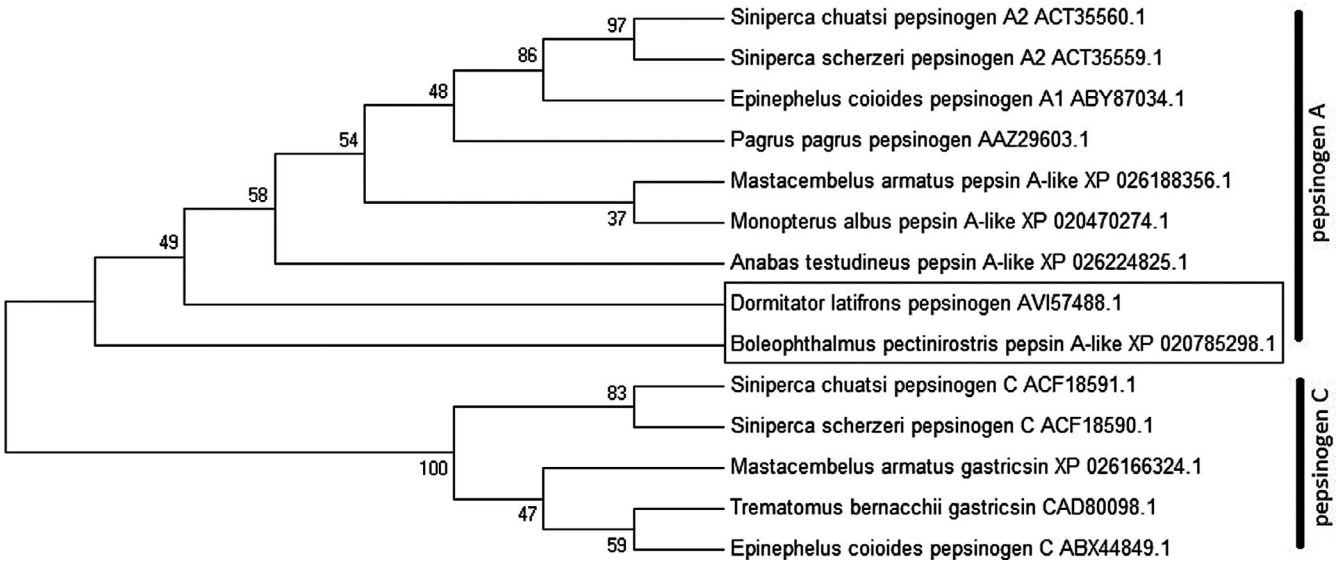


**FIGURE 2** Phylogenetic tree constructed with pepsinogen A and C amino acid sequences from different species of teleost, using the program MEGA. The percentage identity is shown in each branch. The species within a box belong to the family Eleotridae

the log-linear function of the dilution factor versus fluoresce, and these were analysed according to the following equation $E = 10^{(-1/\text{Slope})} - 1$ (Bustin et al., 2009). Relative expression (RE) of each target gene was calculated from the ratio of relative quantities (RQ) of each sample with the equation $RE = RQ_t/RQ_{nf}$, where *t* is the target gene and *nf* is the normalization factor obtained from the geometric mean calculated from the RQs of the most stable reference genes. The relative quantities of each sample of target and reference genes were calculated with the equation $RQ = (1 + E^{(Cq\ mean - Cq)})$ (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007).

### 2.7 | Data analyses

Digestive enzyme expression differences in the early development and in the digestive system tissues were analysed using one-way analysis of variance after transforming the data to natural logarithms, followed by Tukey's post hoc test, which was used to make pairwise comparisons between dph and tissues. Data are presented as the means ± *SE* (standard error). Statistical analyses were performed using STATISTICA 8.0 (Statsoft, Tulsa). A *p*-value < .05 was considered significant. The results are presented back-transformed.

## 3 | RESULTS

### 3.1 | Gene expression of digestive enzymes in *Dormitator latifrons*

In adult *D. latifrons*, the expression of genes encoding digestive enzymes such as trypsin (*tryp*), pepsinogen (*pep*), alkaline phosphatase (*alp*), leucine aminopeptidase (*lap*), maltase-glucoamylase (*mgam*) and sucrase-isomaltase (*si*) was detected, and these were analysed in four tissues (Table 2). The highest expression was detected in the middle intestine, where *mgam* shows significant differences between tissues ($p = .0491$) with levels of mRNA expression of 28.191-fold, followed by *si* with mRNA expression of 20.637-fold (which did not show significant differences), while the other enzymes showed low mRNA expression levels such as *alp* with 0.467-fold, followed by *tryp* with mRNA levels of 0.353-fold. The enzymes *mgam* and *si* also showed higher mRNA expression levels with 3.861-fold and 3.091-fold, respectively, in the anterior intestine with respect to the other enzymes and the lowest expression in the posterior intestine such as *tryp* with mRNA levels of 0.258-fold followed by *lap* with 0.362-fold. Inversely, the enzyme *lap* exhibited higher expression in the liver with mRNA levels of 5.644-fold, showing significant differences between tissues ($p = .005$), while the lowest expression was detected in the anterior intestine. The enzymes *tryp*, *pep* and *alp* did not show significant differences, but *tryp* is the enzyme with the lowest expression in all four tissues, while *pep* and *alp* showed a significant increase in the posterior intestine with mRNA levels of 1.971- and 1.894-fold, with the lowest mRNA levels in the anterior and middle intestine.

The expression levels of proteolytic and intestinal genes were analysed in larvae from *D. latifrons* during the first 7 days post hatching (Figure 3). The lowest expression during 0–2 dph was shown by the enzymes *mgam* and *si* with mRNA levels of 0.0113 ± 0.0733-fold, subsequently significantly increasing their expression from 3 to 5 dph ($p = .000$), where the highest expression was observed on day 5 with mRNA levels of 16.85- and 16.497-fold, respectively, and a decrease in both enzymes was observed from 5 to 7 dph with mRNA levels of −7.422 ± 7.913-fold. The changes in the expression of *pep* were not significant, but showed the trend of increase between 3 and 4 dph with mRNA levels of 1.377- and 1.527-fold, respectively. The expression of *tryp* was present at low levels from hatching (0 dph) to 7 dph with mRNA levels of 0.351 ± 0.667-fold. The transcripts of *alp* were detected at relatively low levels but showed significant differences ($p = .000$) between hatching (0 dph) with mRNA levels of 2.29-fold and 6–7 dph where present at lowest mRNA levels of 0.492 ± 0.415-fold, while maintaining constant expression from 2 to 4 dph. For *lap*, expression was observed at low mRNA levels, fluctuating between 0.591 ± 1.597-fold without significant changes from 0 to 7 dph.

### 3.2 | Phylogenetic tree of pepsinogen sequence of *Dormitator latifrons*

The sequence of nucleotides obtained from pepsinogen of *D. latifrons* encode for a sequence of 57 amino acids. Pepsinogen sequence was aligned with other fish sequences. The identities were 77% with *Siniperca chuatsi* and *S. scherzeri*, 75% with *Boleophthalmus pectinirostris*, 74% with *Monopterus albus* and *Anabas testudineus* and 72% with *Pagrus pagrus*, *Epinephelus coioides* and *Mastacembelus armatus*, all sequences with the 100% of similarity with pepsinogen A. The sequences of pepsinogen C selected for the analyses were taken at random.

We have constructed the respective phylogenetic trees to compare the sequence obtained with other sequences of pepsinogens reported and demonstrate their homology. The amino acid sequence of pepsinogen from *D. latifrons* is grouped with the sequences of pepsinogen A, closely related to *B. pectinirostris*, a member of the Eleotridae family, such as *D. latifrons*.

## 4 | DISCUSSION

The present study is the first report of the digestive enzyme expression of those that are activated during yolk sack absorption,

**TABLE 2** One-way ANOVA results for the relative expression of six digestive enzymes measured in *Dormitator latifrons* adults

| Tissue | *mgam* | *si* | *pep* | *tryp* | *alp* | *lap* |
|---|---|---|---|---|---|---|
| Liver | $0.335^{AB}$ | $0.377^{A}$ | $1.872^{A}$ | $0.573^{A}$ | $1.690^{A}$ | $5.644^{B}$ |
| Anterior intestine | $3.861^{AB}$ | $3.091^{A}$ | $0.449^{A}$ | $0.258^{A}$ | $0.670^{A}$ | $0.362^{A}$ |
| Middle intestine | $28.191^{B}$ | $20.637^{A}$ | $0.604^{A}$ | $0.353^{A}$ | $0.467^{A}$ | $0.620^{A}$ |
| Posterior intestine | $0.027^{A}$ | $0.042^{A}$ | $1.971^{A}$ | $0.199^{A}$ | $1.894^{A}$ | $0.788^{A}$ |
| *p*-value | .049 | .080 | .354 | .868 | .425 | .005 |
| Standard error | 4.416 | 4.412 | 1.980 | 2.549 | 1.965 | 1.477 |

*Note:* Different letters indicate statistically significant differences of expression between tissues of chame adults (Tukey's HSD test, $p < .05$). Data expressed as means, and *SE* (standard error) ($n = 3$)

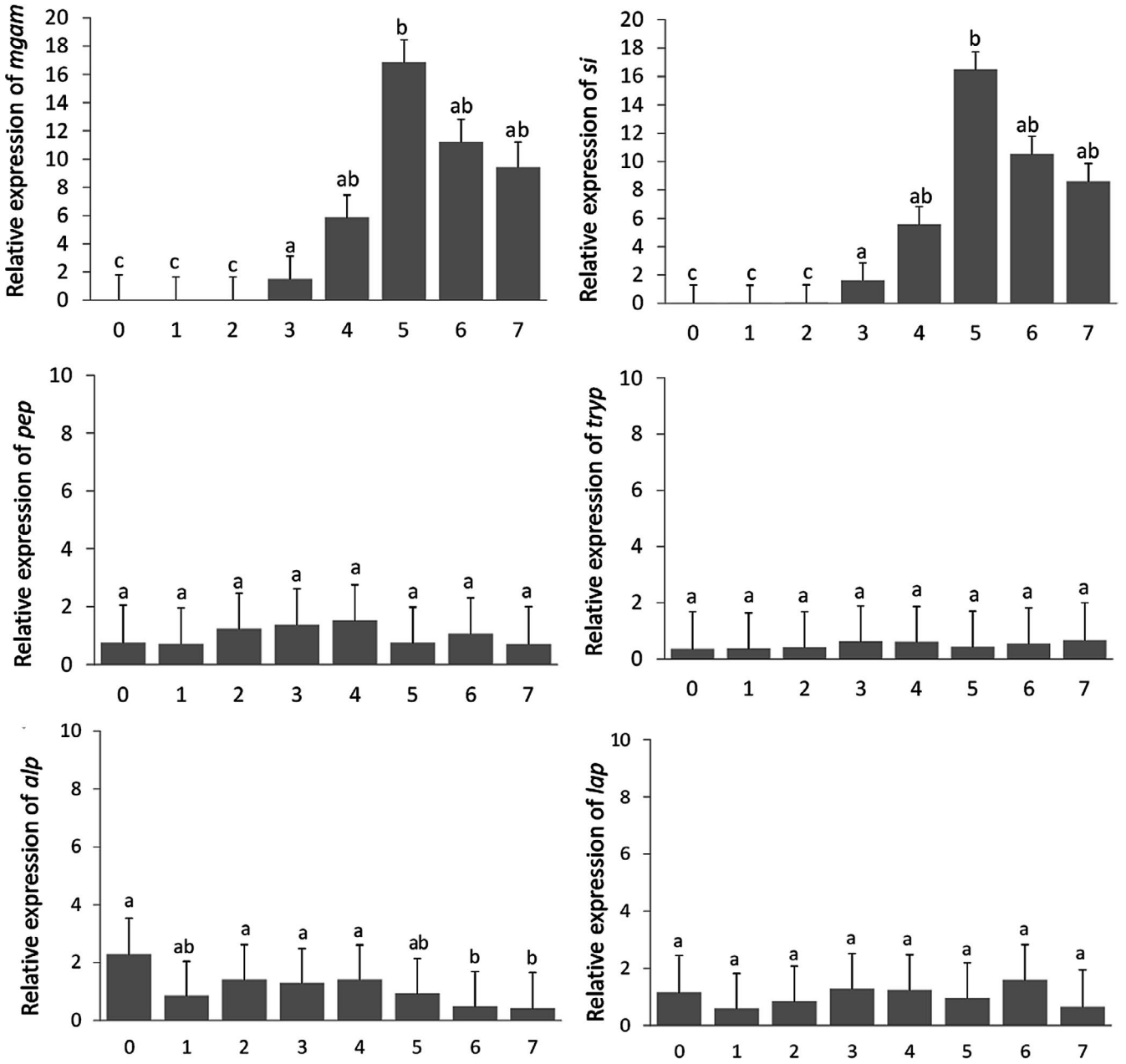


**FIGURE 3** Relative gene expression of six digestive enzymes normalized with the geometric mean of *act-β* and *tub-α*, measured during the first days of development of *Dormitator latifrons*. The mRNA levels measured at each developmental stage are shown as the means of 3 pooled larvae with standard error ($n = 3$). Different letters indicate statistically significant differences in expression between days of development (Tukey's HSD test, $p < .05$)

the immediate time in preparation to food ingestion once mouth opens and the following days until 7 dph, in larvae of the chame *Dormitator latifrons*. Likewise, the expression of digestive enzymes in the digestive tract of adult organisms has been determined to expand the information of this species with aquaculture potential. As additional information, many larval feeding trails have been carried out in our laboratory for the last 8 years, comprising many zooplanktonic prays including our Mexican strain of the minute rotifer *Proales similis* (Román- Reyes et al., 2017), only observing survival beyond 10 dph when larvae were fed with microbial flocs produced with fermented corn meal, rice bran and molasses; preliminarily suggesting an apparent preference for this kind of organic particulated compounds as initial food over zooplankton.

In this work, we wanted to describe the expression of digestive enzymes in chame *D. latifrons* during the transition from endogenous to exogenous feeding without the influence of the diet, to establish its digestive strategy or preferred metabolic substrate. It has been suggested that feeding does not affect the expression of the genes that code for digestive enzymes during the first days of development because they are genetically programmed (Zambonino-Infante & Cahu, 2001; Zambonino-Infante et al., 2008), but other species such as *Solea senegalensis* shows an effect of the diet during

the first days of life in this species (Gamboa-Delgado et al., 2011; Hachero-Cruzado et al., 2014) changing enzymatic activity patterns. Nevertheless, given the poor knowledge of the actual feeding habits of the larvae, in both their natural habit of this species or laboratory rearing conditions, is not known, we consider that carrying out the analyses in starvation conditions provides a baseline for future feeding trials.

It was observed in adult organisms that the disaccharidases *mgam* and *si* showed the highest expression in the middle intestine compared with the other digestive enzymes analysed. This is in agreement with dietary habits reported in *D. latifrons*, as a detritivorous fish, although occasionally presenting omnivorous habits by consuming annelids and copepods (Bonifáz et al., 1985). Similar results have been shown in species with different feeding habits such as carnivorous hybrid striped bass, omnivorous tilapia and herbivorous silver carp, where the highest activity of maltase and sucrose was found in the midgut (Harpaz & Uni, 1999). On other stomachless species such as ballan wrasse was determined that the major digestion of carbohydrates is carried out in the anterior intestine and that most of these digested foods are transported to the middle intestine (Le et al., 2019). This could be related to the highest expression of the *mgam* and *si* enzymes in the middle intestine in chame adults, because these are two brush border-anchored disaccharidases (glycosidases) involved in the breakdown of maltose, which is a product of starch hydrolysis and is the last step in carbohydrate cell transport and absorption (Naumoff, 2007; Nichols et al., 2003; Quezada-Calvillo et al., 2007). After disaccharidase hydrolysis, the carbohydrate units (monosaccharides) are transported by enterocytes across the villi (Krogdahl et al., 2005).

On the other hand, we analysed the expression of digestive enzymes during the first seven dph from chame larvae with the purpose of providing information about their nutritional requirements at the start of exogenous feeding under starving conditions. Our results show that the carbohydrases such as the disaccharidases *mgam* and *si* increased in expression significantly between 3 and 5 dph. The other enzymes analysed, including *tryp*, pepsin *pep*, *lap*, and *alp*, were detected much earlier (dph 0) than the *mgam* and *si*, presenting low levels of expression from hatching to 7 dph. The increase in the expression of the disaccharidases in chame larvae corresponds to the beginning of exogenous feeding as depicted by López-López et al. (2015). The authors describe that absorption of the yolk sac occurs at 3–4 dph and that the intestinal lumen is visible presenting brush border projections and ciliated epithelial cells. Likewise, at 4 dph the authors observed a diffuse exocrine pancreas, a functional mouth, and found that mandibular activity is also evident in this period (Rodríguez-Montes de Oca et al., 2012). At 5 and 6 dph, a bulge was observed in the anterior intestine as an extension of a functional oesophagus. Similar results were found in common dentex, where maltase was undetected before 2 dph and was detected after the onset of exogenous feeding, presenting higher expression levels from 6 to 50 dph (Gisbert, Giménez, Fernández, Kotzamanis, & Estévez, 2009; Santamaría et al., 2004). Kuźmina (1996) found that maltase activity increased in the transition to exogenic feeding in rainbow trout and common carp, while in several species of carp the maltase activity increased when the larvae were fed with phytoplankton and zooplankton. Other disaccharidases such as amylase have shown high expression levels in European seabass *Dicentrarchus labrax* larvae even before mouth opening (Zambonino-Infante & Cahu, 2001).

Alternatively, the expression of proteolytic enzymes observed in chame larvae does not show an increment when the yolk sack is absorbed but is present after hatching until 7 dph. Alvarez-González et al. (2006) suggest that the expression of preprogrammed digestive enzymes such as *lap* is to allow the correct absorption of the nutrients contained in the yolk sac. The expression of *tryp* has been detected during larval development of different stomach less fish, such as the bullseye puffer fish and ballan wrasse (García-Gasca et al., 2006; Hansen et al., 2013) and zebra fish (Guerrera, Pasquale, Muglia, & Caruso, 2015). Some enzymes related to the metabolism of the egg and the yolk sac will be present from fertilization until the larva opens the mouth and exogenous feeding (Perez-Casanova et al., 2006; Sveinsdóttir, Thorarensen, & Gudmundsdóttir, 2006). Nevertheless, the expression of pepsin is not present in stomachless species such as ballan wrasse *L. bergylta* (Hansen et al., 2013) and is negligible in *Atherinops affinis* (Logothetis et al., 2001), due to their lack of a functional stomach. Some studies have shown that stomachless fish such as ballan wrasse (Lie et al., 2018), zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) have lost the pepsinogen gene, and this loss is correlated with the lack of a stomach function (acid secretion and pepsin activity) (Castro et al., 2014). It has been suggested that the source of food and changes in the diet to which these organisms were subjected, acted as a driving force for the loss of the stomach (Castro et al., 2014). However, in stomachless fish such as pufferfish *Takifugu rubripes* and green-spotted pufferfish *Tetraodon nigroviridis* the expression of pepsinogen mRNA has been detected, and only one copy of this gene has been identified (Castro et al., 2014). In pufferfish *T. rubripes*, this gene is expressed in the skin and the gill but not in digestive organs (Kurokawa, Uji, & Suzuki, 2005) which may indicate the loss of the function of this gene. On other stomachless fish such as Jullien's golden carp *Probarbus jullieni*, the activity of acid proteases was demonstrated in larvae, suggesting that these participate in the digestion of the yolk sac components (Limtipsuntorn, Rungsin, Thongprajukaew, Boonyung, & Rangsin, 2018). The comparison of the amino acid sequence obtained from the pepsinogen in *D. latifrons* and the phylogenetic analyses show high homology with the pepsinogen A of other teleost fish species, so the possibility that it is another acid protease is null. Our results suggest that the regulation of the expression of the pepsinogen gene in chame *D. latifrons* or its inhibition could be through a post-transcriptional process. Therefore, the presence of these enzymes, including *tryp*, *pep* and *alp* in chame larvae, a species without a gastric gland or a functional stomach, can indicate that this species is programmed to be able to digest other substrates besides carbohydrates, in a similar fashion as an omnivorous species.

These results are in accordance with the hypothesis that during the early stages of fish development the expression of the genes that code for digestive enzymes is genetically preprogrammed

(Zambonino-Infante & Cahu, 1994; Zambonino-Infante et al., 2008) to allow larvae to take advantage of the nutrients in their initial feed intake. The genetic predetermination of the expression pattern of disaccharidases in chame larvae indicated the importance of highly metabolized carbohydrates in the diet during early life stages (Lazo, Darias, & Gisbert, 2011; Mata-Sotres, Martos-Sitcha, Astola, Yúfera, & Martínez-Rodríguez, 2015). The expression of genes encoding enzymes of the brush border membrane is fundamental for nutrient absorption, which are utilized to fulfil metabolic needs (Hakim, Harpaz, & Uni, 2009). In a stomachless fish, the foregut leads directly into the intestine and apparently secretes mucus but no other digestive components (Horn et al., 2006).

In conclusion, with the results of this study, we can infer that chame is genetically programmed to digest glycogenic disaccharides such as maltose and sucrose, because the changes in the digestive enzyme expression were age-related and not dietary-induced (Pinoni, Iribarne, & Mañanes, 2011), and appeared to correspond with the feeding habits in the adult age. These findings allow us to establish an initial highly digestible carbohydrate-based diet as a predominant substrate at the beginning of exogenous feeding, along with the observed presence of protease enzymes that allow for determination of a successful dietary protocol in the transition from endogenous to exogenous feeding, aiding in the selection of most suitable ingredients or prey items for its nutrition at early life stages.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interests.

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