Lat. Am. J. Aquat. Res., 46(5): 909-923, 2018
DOI: 10.3856/vol46-issue5-fulltext-5

Research Article

Gene expression of digestive enzymes during the initial ontogeny of Mexican snook *Centropomus poeyi*

Gloria Gertrudys Asencio-Alcudia[1], Dariel Tovar-Ramírez[2], Hever Latisnere-Barragán[2] Rafael Martínez-García[1], Wilfrido Miguel Contreras-Sánchez[1] & Carlos Alfonso Alvarez-González[1]

[1]Laboratorio de Acuicultura Tropical, Universidad Juárez Autónoma de Tabasco
Villahermosa, Tabasco, México

[2]Instituto Politécnico Nacional, Centro de Investigaciones Biológicas del Noroeste
La Paz, B.C.S., México

Corresponding author: Carlos Alfonso Alvarez-González (alvarez_alfonso@hotmail.com)

ABSTRACT. Mexican snook (*Centropomus poeyi*) is one of the most important commercial species in Southeast Mexico, which has been overexploited by fisheries. For this reason, some recent studies related to its culture have been done. However, the basic biological knowledge of the digestive physiology is still unknown. In this study, the gene expression of trypsin, (TRY), bile-salt dependent lipase (BAL), amylase (AMY), and cytosolic non-specific dipeptidase (CNP) was investigated during the larval period from embryo (0 days after hatching, DAH), eleuteroembryo (1 DAH) until 25 DAH using RTq-PCR approach and was normalized with the elongation factor 1-α gene (EF1-α). TRY gene expression showed the maximum peak at 17 DAH; meanwhile, BAL gene expression showed variations during larviculture, however, three peaks were observed at 10, 17 and 25 DAH, which appears to correspond to changes in live food supply (rotifers and *Artemia* nauplii). AMY gene expression was low detected from hatching onwards and showed a slight peak at 10 DAH when larvae were fed with *Artemia* nauplii. Finally, CNP gene expression had presented three peaks at 2 DAH (yolk-sac absorption), 19 and 23 DAH when larvae were fed with *Artemia* nauplii. We conclude that *C. poeyi* larvae showed early and high digestive enzyme expressions, especially TRY, which indicate that could be possible to start the weaning process at 25 DAH, which could be shorter compared than other Centropomid species.

Keywords: *Centropomus poeyi*, snook, larviculture, gene expression, RT-qPCR, digestive enzyme.

INTRODUCTION

Snooks belong to the Centropomidae family, made up of the *Centropomus* gender, which contains 12 Amphi-American species, of diadromous behavior, typical of mixohaline environments and carnivore feeding (Castro-Aguirre *et al*., 1999). One of the highest commercial value species is the Mexican snook (*Centropomus poeyi*), a fish with great plasticity because it can be found during its first stages in high salinity waters and then, it can migrate to freshwater environments; although it concentrates in estuaries, its distribution covers the Gulf of Mexico from Tampico through Belize. This species has been studied in order to achieve its culture; however, it is of paramount importance to understand the physiological processes along its development stages allowing to encourage its culture in a commercial scale such as the cobia (*Trachycentrum canadum*), snappers (*Lutjanus* spp.), groupers (Serranidae, *Epinephelus* spp., *Paralabrax* spp.) (Avilés-Quevedo *et al.*, 1995).

In the present, the knowledge on the biology and handling of the Centropomidae is not enough, because we only count with the surveys performed by Stephen & Shafland (1982) and Jiménez-Martínez *et al*. (2012) with common snook (*Centropomus undecimalis*), meanwhile with fat snook (*C. parallelus*) authors like Cerqueira & Brügger (2001), Álvarez-Lajonchère *et al*. (2002) and Tarcisio *et al*. (2005), whom studied different topics such as reproduction, larviculture and the use of live feed among others, to encourage its development. The only article that has been published regarding the larval development of *C. poeyi* is that conducted by Kubicek *et al*. (2018), who studied ossi-

Corresponding editor: Crisantema Hernández

fication and pigmentation changes during early ontogeny. In this aspect, more detailed surveys on the fundamental aspects of its biology are required by using different techniques such as molecular tools, allowing the understanding of the physiology, especially in relationship with the morphophysiological changes showed during the larval stage and particularly to the expression of that specific gene's coding for digestive enzymes. These strategies allow the development of basic feeding schemes and to understand the capacity of the organism to use the nutrients coming from live food and to achieve a proper weaning process by using inert food. Considering the above mentioned, Kolkovski (2001) and Chakrabati *et al*. (2006), state that the nutrient assimilation depends directly on the digestive enzymes, and therefore to the nutrient digestibility and their absorbing capacity during the larval period.

One of the milestones of the larval period is the development of a juvenile-like digestive capacity during the first days of development to successfully acquire the proper nutrients for adequate growth and survival. Once the digestive enzymes appeared, the amount of enzyme activity is the next result of the interaction between the genes and other factors that might influence enzyme synthesis such as the availability and composition of the food, as well as the feeding patterns (Galaviz *et al*., 2015).

The physiological digestive development of the marine fishes larvae has been studied during the last years which has allowed for the design of diets to optimize its culture (Ueberschär, 1993; Gisbert *et al*., 2008; Zambonino-Infante *et al*., 2008; Lazo *et al*., 2011); in turn, multidisciplinary techniques have been used to increase the knowledge and understanding of the changes in the ontogeny of digestive physiology in marine fish larvae, including gene expression level in species such as *C. undecimalis* (Martínez-Burguete, 2013), white sea bass (*Atractoscion nobilis*) (Galaviz *et al*., 2011), barramundi (*Lates calcarifer*) (Srichanun *et al*., 2013), totoaba (*Totoaba macdonaldi*) (Galaviz *et al*. 2015), *C. parallelus* (Teles *et al*., 2015), Atlantic salmon (*Salmo salar*) (Sahlmann *et al*., 2015) and gilthead seabream (*Sparus aurata*) (Mata-Sotres *et al*., 2016). However, there are no studies on the digestive physiological changes in *C. poeyi*, and therefore the purpose of this study could contribute in the determination of the expression changes in trypsin (TRY), bile salt-dependent lipase (BAL), α-amylase (AMY) and nonspecific cytosolic dipeptidase (CNP) during *C. poeyi* initial ontogeny.

MATERIALS AND METHODS

Larval sampling

Centropomus poeyi eggs were obtained from an induced spawning (75,000 fertilized eggs) from broodstock (2 females and 4 males) at the Marine Aquaculture Station (Univer-sidad Juárez Autónoma de Tabasco). Larval culture lasted 25 days from hatching, for which embryos were placed in three cylindrical-conical fiberglass tubes of 500 L (1500 embryos per tube), coupled to an open system with daily changes of the 50% of the volume and keeping constant aeration. Temperature (25°C), dissolved oxygen (5.7 mg L^{-1}) were monitored daily with a YSI 85 oximeter and pH (8.2) with a Hanna potentiometer. The larvae feeding scheme was done from hatching. Once the eleuthero-embryos had consumed the yolk (24 h after hatching), they were fed with rotifer *Brachionus rotundiformis* (1-5 ind mL^{-1}) enriched with a lipidic emulsion (SuperHUFA®, Salt Creek) for 5 days after hatching (DAH) and mixed with microalgae *Isochrysis galvana* (100,000 cel mL^{-1}); from day 6 and until 18 DAH the larvae were fed with *Brachionus plicatilis* (1-5 ind mL^{-1}) enriched with the same lipidic emulsion. From 15 DAH, larvae were cofed with *Artemia* nauplii (0.5-2 ind mL^{-1}) enriched with SuperHUFA® (Salt Creek) until completing 25 DAH.

The sampling of larvae started from eleutheroembryo, first feeding, 3 DAH until 15 DAH. From here, the sampling was carried out every two days (days 17, 19, 21, 23 and 25 DAH), collecting 5 larvae per day in triplicate that were previously euthanized using an overdose of MS-222 (tricaine metasuphonate, 0.1 mg L^{-1}), until a total amount of 270 larvae which were stored in Eppendorf tubes with RNAlater® (Thermo-Fisher, Scientific) solution and were kept in ultra-freezing (Labconco VWR) at -80°C until processing at the Centro de Investigaciones Biológicas del Noroeste S.C.

Primers design

The oligonucleotide design was performed by using the free access software Primer 3 Web [http://primer3 plus.com/web_3.0.0/primer3web_input.htm] from a consensus region derived from an alignment of gene sequences coding for the different fish digestive enzymes obtained from the GeneBank database. Oligos were designed with the following features: 20 to 22 nucleotide long, an alignment temperature of 58 to 60°C, CG (guanine-cytosine) content of 40 and 60% and finally the secondary structure formation was assessed using the free access software Oligo Calculator version 3.27 [http://biotools.nubic.north-western.edu/OligoCalc.html] (Table 1).

RNA Extraction and cDNA synthesis by RT-PCR in *Centropomus poeyi* larvae

Total ARN extraction was done by using a larvae homogenate (0 to 25 days after hatch). The process was done with the commercial kit SV Total RNA Isolation

Table 1. Oligonucleotide primer sequences, melting temperatures and amplicon size of the TRY, BAL, AMY, CNP and EF1-α from *Centropomus poeyi*.

Target genes	Nucleotide sequence (5'-3')		Melting Temperature (ºC)	Amplicon size (pb)	GeneBank reference
	Forward	Reverse			
qPCR					
Trypsin	CCCAACTACAGCTCCTACAAC	CAGCTTGTTCCTGTCAGCAGT	60	183	KR349663
Cytosolic non-specific dipeptidase	TGGAGGTACCCATCTCTTTCTC	AGTCGATAACCTGCTTCTCCAC	60	154	KR349662
Bile-salt dependent lipase	GCAGGTGGAGGTAGTGTTAGCT	ACCTTCAGAGCAATCTCCTCAG	60	188	
Amylase	CAGATCTCCCCTCCAAATGAG	GATGACAGCGTCCACATAGATG	60	177	
Elongation factor 1-α	GAGGAAATCACCAAGGAAGTGA	CTTGAACCAGCTCATCTTGGAG	60	154	

System (Promega, Madison, WI, USA), and finally, the RNA samples were resuspended in RNase-free water. All RNA samples were treated with DNAse according to the RQ1 RNAase-Free DNase kit (Promega, Madison, WI, USA) instructions previous to cDNA synthesis. Finally, RNA concentration and integrity were checked with a spectrophotometer NanoDrop 2000 (Thermo Scientific) and agarose electrophoresis.

The cDNA synthesis was done with 1µg of total RNA according to the Improm II kit (Promega, Madison, WI, USA) instructions in a thermal cycler iCycler (Bio-Rad, Berkeley, CA, USA).

qPCR analysis

cDNA pooled from the common snook larvae were used to construct standard curves for each analyzed gene using the primers described in Table 1. Real-time PCR efficiencies (E) were automatically determined for each primer pair using standard curves obtained from serial dilution from de cDNA pool. E was calculated according to the equation: E = 10e(-1/slope). This analysis ensures that E ranged from 97% to 100% for each primer pair.

Once the dilution was established, we quantify the expression level of the digestive enzymes along the ontogeny using the SsoAdvanced™ SYBR Green Supermix (Bio-Rad, Berkeley, CA, USA). The elongation factor gene (EF1-α) was used to normalize the Cq values obtained from the different genes analyzed according to the algorithms in the CFX-Manager (BIORAD) software. All the RT-qPCR assays were performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Berkley, CA, USA).

RNA extractions and homogenates for this effect were performed by pooling 5 ind per replicates (3X), and the expression mean ± standard error. All gene expression analyzes were carried out using the Ct method (Livak & Schmittgen, 2001) with the Bio-Rad CFX Manager 3.1 Gene Study software (Bio-Rad, Berkeley, CA, USA) using the efficiency-corrected values of Ct. The qPCR data were normalized to the geometric mean of the expression efficiency-corrected data for EF1. The expression difference between samples (days after hatching) was tested by significant differences in Bio-Rad CFX Manager 3.1 Gene Study, using a two-tailed, unpaired t-test (Augimeri & Strap, 2015). Differences were considered statistically significant if $P < 0.05$.

Sequencing and analysis of TRY, BAL, CNP, AMY, and EF1- α genes

PCR fragments obtained for each gene were sent to Genewiz Inc. (MD, USA) for direct sequencing.

Nucleotide sequences were analyzed with Chromas Pro software [http://www.technelysium.com.au/], and its ORFs deduced from the codon translation. Nucleotide sequences, as well as those of the amino acids, were compared with the BLASTn and BLASTp tools to evaluate the identity of each one.

Phylogenetic inference

A phylogenetic approximation was built with the CNP and Try amino acid sequences by Neighbor-Joining method. 1000 bootstrapping replicates evaluated the nodes support in MEGA 6.0 (Tamura *et al*., 2013).

RESULTS

Sequencing and analysis of TRY, BAL, CNP, AMY, and EF1- α genes

The sequences showed fragments of 154 bp, 183bp, 188bp, 177bp and 154bp for CNP, TRY, BAL, AMY and EF1-α respectively. The nucleotide analysis of Trypsin (Fig. 1) showed the highest identity percentage (98%) against the *C. undecimalis* (JX128261) sequence while the next highest percentile value (95%) was against spotted sand bass (*Paralabrax maculatofasciatus*, AJ344566). In the same way, the amino acid analysis (Fig. 2) showed the highest identity percentage (98%) against *C. undecimalis* (AKP92840). However, the next highest percentage value was against European sea bass (*Dicentrarchus labrax*, CAA07315) and orange-spotted grouper (*Epinephelus coioides*) both with 96% identity indicating that the amino acid sequence belongs to a type 1a trypsinogen. Figure 2 shows an alignment where the specific residues of the protease's series are highlighted; it also shows the conserved amino acids that shape the catalytic triad of the active site of the trypsin.

In the case of BAL (Fig. 3), AMY (Fig. 4) and EF1-α (Fig. 5) sequences, the identity percentages of the nucleotidic identity reached the 92% against large yellow croaker (*Larimichthys crocea*), 95% yellowtail amberjack (*Seriola lalandi*) and 96% Atlantic bluefin tuna (*Thunnus thynnus*,) respectively. Due to the size of the fragments, it was not possible to characterize in greater detail the deducted proteins.

CNP gene showed the highest nucleotide identity percentage (98%) against the sequence reported for *C. undecimalis* (JX128263) whereas the next highest percentage value (91%) was against *T. thynnus* (FM995225) (Fig. 6). On its side, when comparing the amino acid sequences, the highest percentage observed (97%) was again against the *C. undecimalis* (AFP86442) sequence while the next highest percentage (94%) value was against the Leopard grouper (*Mycteroperca rosacea*).

Phylogenetic inference

Phylogenetic inference of CNP showed that the amino acid sequence obtained was grouped with the only additional specimen of the *Centropomus* gender (Fig. 7). The rest of the phylogenetic arrangement showed specimens of different classes. Likewise, the trypsin dendrogram (Fig. 2) showed a Group with *undecimalis* and with tongue sole (*Cynoglossus semilaevis*) both members of the *Acanthopterygii* suborder.

Gene expression of digestive enzymes in *Centropomus poeyi*

After the RT-qPCR we were able to confirm the specificity of primers, because of the absence of secondary structures, primer-dimer or non-specific amplification fragments using the melting peaks analysis, using for this the CFX Manager (BioRad). After that analysis, we determined the relative quantification of the trypsin, bile salt depending lipase, amylase and cytosolic non-specific dipeptidase using the EF1 as reference gene and we observed the following. A slight increase of early expression of trypsin was detected by 6 DAH and having a steady increase by 7 DAH and showing a significant peak at 9 DAH, it kept on fluctuating until reaching the highest expression peak reflected on 17 DAH, to then continue with a fluctuant irregular pattern until the end of the experiment (Fig. 8a). In the case of BAL, it shows a fluctuating behavior during the 25 days of the survey, having the 10, 17 and 25 DAH as those of the highest expression (Fig. 8b). Regarding the amylase enzyme, the highest peak is shown by 10 DAH, keeping its fluctuations throughout the larval culture where it reflects a low expression different from other enzymes (Fig. 8c). Finally, the expression of the CNP is detected from the first days of larval culture, having its maximum expression on 2 DAH, keeping fluctuations from 4 to 17 DAH, and increasing the expression on 19 and 23 DAH (Fig. 8d).

DISCUSSION

As part of the basic knowledge regarding gene studies for *Centropomus poeyi*, it was possible to obtain and registered two sequences for digestive enzymes, trypsin (TRY) and nonspecific cytosolic dipeptidase (CNP). In this sense, TRY gen is highly conservative when comparing its sequence, with those of the other carnivore marine fishes such as *C. undecimalis, Epinephelus coioides* (Liu *et al*., 2012) and *Dicentrarchus labrax* (Péres *et al*., 1998) (GeneBank accession number: JX128261.1, JN848593.1, AJ006882.1), according to the dendrogram. In this way, trypsin is a digestive protease present in the gut of fishes under different

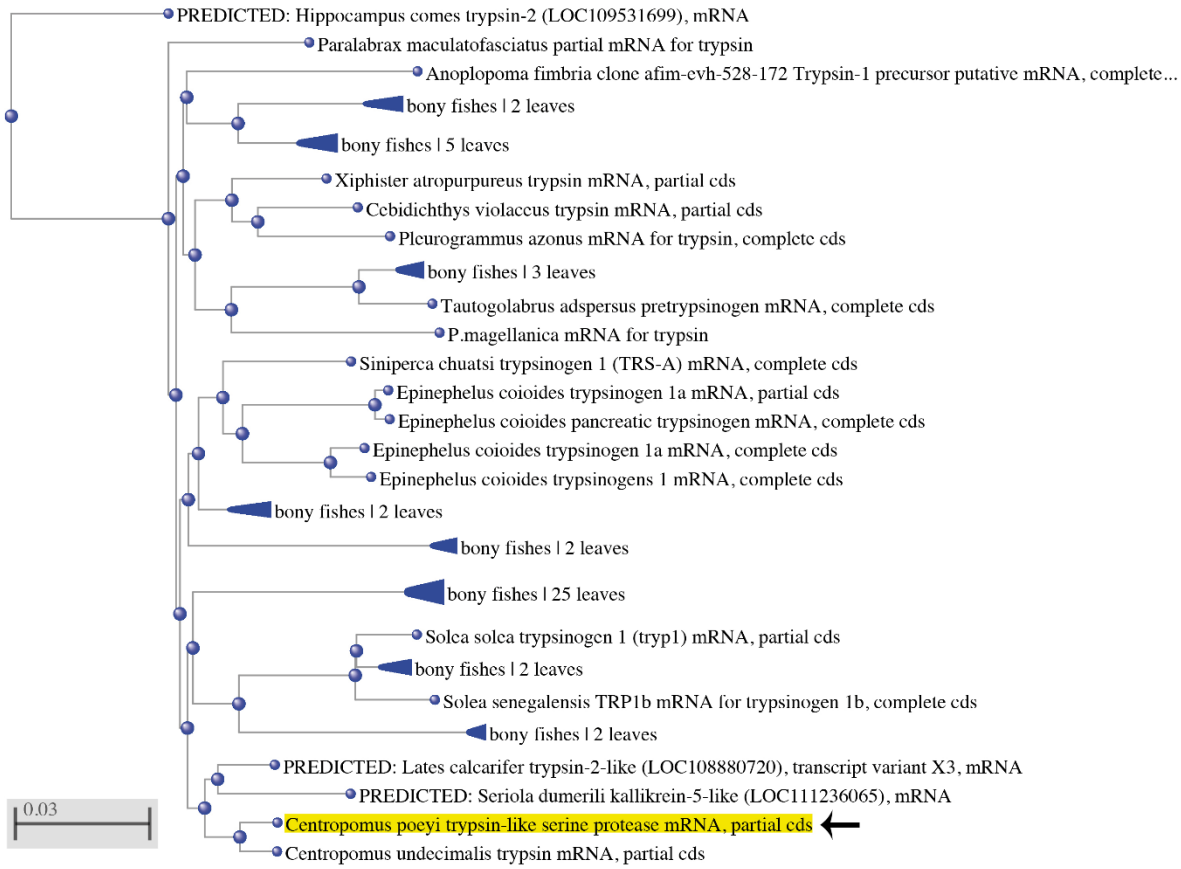

Figure 1. Similarity analysis of TRY gen in *Centropomus poeyi*.

isoforms and is of principal importance to achieve the digestion of proteins in the intestine (Cara *et al*., 2007). Also, it has been proved that the trypsinogen is secreted in the light of the digestive tract, is activated by an enterokinase, and this is so that the activation of the trypsin activates the other digestive enzymes such as chymotrypsin, elastase, collagenase, and lipase (Rust, 2002). Trypsin activity began firstly as a zymogen. Then, to the light of the digestive tract as an active endopeptidase, which cleaves peptide chains mainly in the carboxyl side of lysine and arginine, except when either is followed by proline, releasing smaller peptides and allowing other proteases to perform the hydrolysis process by other parental digestive proteases such as carboxypeptidases and aminopeptidases (Moyano *et al*., 1996).

Considering the above mentioned, alignments obtained from the protein sequences showed on one side, the close phylogenetic distance for TRY and CNP of *C. poeyi* to *C. undecimalis*, which is the only species of the same family registered at GenBank. On the other hand, it is important to highlight that for the CNP is an enzyme that has been previously reported under different denominations (glycine-glycine dipeptidase, carnosine dipeptidase) agreeing to the MEROPS peptidase database (http://merops.sanger.ac.uk/index. shtml) ID: 20.005, it is considered a carnosine dipeptidase II (Boldyrev *et al*., 2004). These authors have hypothesized that CNP is a non-secreted enzyme and differs from those secreted such as serum carnosinase and anserinase, with a limited expression, and that arise by the effect of the second duplication of the gene once the urochordates separated from the vertebrate to evolve as secreted enzymes. So, it seems this secretion quality is an important classification and group differentiation factor when vertebrates and invertebrates are phylogenetically compared (Oku *et al*., 2011). CNP is a Mn^{+2} dependent protease, which hydrolyzes dipeptides such as L-carnosine, preferentially hydrophobic dipeptides including prolyl amino but has a strong preference for Cys-Gly (Teufel *et al*., 2003). This enzyme has several functions such as peptide reuptake in the kidney, intracellular protein turnover, pH buffering, anti-oxidation, neuronal regulation and protein digestion in the gut releasing β-alanine and histidine from carnosine (Bauer, 2005; Okomura &

```
C. poeyi AKP92840                1   ----------------------------------------------ENWVVSAAHCYKSRVEVRLGEHNIRVTEGT  30
C. undecimalis AFP86440          1   ----------------------------QVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIRVTEGT  47
E. coioides ACM41844             1  [11]LIGAAFAT---EDDKIVGGYECTPHSQAHQVSLNSGYHFCGGSLVSANWVVSAAHCYKSRVEVRLGEHNIKVTEGN  84
D. labrax CAA07315               1   ----------------------------QVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIRVTENT  47
C. semilaevis NP 001281136       1  [ 8]LIGAAFSL---EDDKIVGGYECQPYSQPHQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIRVTEGT  81
P. perryi BAJ12017               1  [ 8]LIGAAFAT---EDDKIVGGYECKAYSQPHQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIKVTEGS  81
S. salar CAA49677                1   ----------------------KAYSQAHQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIKVTEGS  54
C. violaseus AAX83265            1   ----------------------------------GYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIRVTEGN  41
X. atropurpureus AAX85688        1   ------------------------------L--GYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIRVTEGN  42
O. masou BAG84558                1  [ 8]LIGAAFAT---EDDKIVGGYECKAYSQPHQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRLGEHNIKVTEGS  81
D. mawsoni AEA08589              1  [ 7]LIGAAFAT---EEDKIVGGKECAPYSMPYQVSLNSGYHFCGGSLVNENWVVSAAHCYKSRVEVRMGEHNIRVTEGN  80

C. poeyi AKP92840               31  EQFISSSRVIRHPNYSSYNINNDIMLIKLSKPATLNQYVQPVALPTSCAPAGTMCNVSGWGNTMSSTADRNKLQCLNIPI  110
C. undecimalis AFP86440         48  EQFISSSRVIRHPNYSSYNINNDIMLIKLSKPATLNQYVQPVALPTSCAPAGTMCNVSGWGNTMSSTADRNKLQCLDIPI  127
E. coioides ACM41844           85  EQFISSSRVIRHPNYTSYNINNDIMLIKLSEPATLNQYVQPVALPTSCAPAGTMCTVSGWGNTMSSTADRNKLQCLNIPI  164
D. labrax CAA07315              48  EQFISSSRVIRHPRYSSYNIDNDIMLIKLSKPATLNQYVQPVALPTSCAPAGTMCTVSGWGNTMSSTADRNKLQCLNIPI  127
C. semilaevis NP 001281136      82  EQFISSSRVIRHPNYSSYNINNDVMLIKLSKPATLNQYVKPVALPSSCAPAGTMCKVSGWGNTMSSTADKNKLQCLDLPI  161
P. perryi BAJ12017              82  EQFISSSRVIRHPNYSSYNIDNDIMLIKLSKPATLNTYVQPVALPTSCAPAGTMCTVSGWGNTMSSTADKNKLQCLNIPI  161
S. salar CAA49677               55  EQFISSSRVIRHPNYSSYNIDNDIMLIKLSKPATLNTYVQPVALPTSCAPAGTMCTVSGWGNTMSSTADSNKLQCLNIPI  134
C. violaseus AAX83265           42  EQFIRSSRVIRHPNYSSYNINNDIMLIKLSEPATLNQYVKPVALPTSCAPAGTMCKVTGWGDTMSSTADGNKLQCLNIPI  121
X. atropurpureus AAX85688       43  EQFIQSSRVIRHPNYSSYNIDNDIMLIKLSKPATLNQYVQAVALPTSCAPAGTMCKVTGWGNTMSSTADKNKLQCLNIPI  122
O. masou BAG84558               82  EQFISSSRVIRHPNYSSYNIDNDIMLIKLSKPATLNTYVQPVALPSSCAPAGTMCTVSGWGNTMSSTADGDKLQCLNIPI  161
D. mawsoni AEA08589             81  EQFISSSRVIRHPNYSSYNIDNDIMLIKLSKPATLNQYVKPVALPRSCAPAGTMCTVSGWGSTQSSTADGNKLQCLNIPI  160

C. poeyi AKP92840               111  LSDSDCDNAYPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSW----------------------------  161
C. undecimalis AFP86440         128  LSDSDCKNAYPGMITQAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSW----------------------------  178
E. coioides ACM41844           165  LSKEDCDNAYPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWGYGCAEKDHPGVYARVCIFNDWLERTMAT  244
D. labrax CAA07315              128  LSFKDCDNSYPGMITDAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSW----------------------------  178
C. semilaevis NP 001281136      162  LSDSDCRNSYPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWGYGCAEKDHPGVYAKVCIFNDWLERTMAS  241
P. perryi BAJ12017              162  LSYSDCNNSYPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWGYGCAEPGNPGVYAKVCIFTNWLTSTMAS  241
S. salar CAA49677               135  LSYSDCNNSYPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWGYGCAEPGNPGVYAKVCIFNDWLTSTMAS  214
C. violaseus AAX83265           122  LSDDDCDNSYPGMITDAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWG--------------------------  173
X. atropurpureus AAX85688       123  LSFKDCDNSYPGMITDAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWGQRCA------------------------  178
O. masou BAG84558               162  LSYSDCNNSYPGMITNAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWGYGCAEPGNPGVYAKVCIFNDWLTSTMAT  241
D. mawsoni AEA08589             161  LSDRDCDNSYPGMITDAMFCAGYLEGGKDSCQGDSGGPVVCNGELQGVVSWGYGCAERDNPGVYAKVCLFNDWLETTMAS  240
```

Figure 2. Alignments of the amino acid sequence of TRY in *Centropomus poeyi* compared with other fish species (GeneBank access number or NCBI are indicated next to species name). The conserved amino acid residues that shape the catalytic triad of the active site of the trypsin like-serine proteases are marked in black bold (H: histidine, D: aspartic acid, S: serine, and residues). Two substrate binding sites residues (D and S) are marked in blue. The histidine active site and the serine active site for the trypsin Family of serine proteases are boxed.

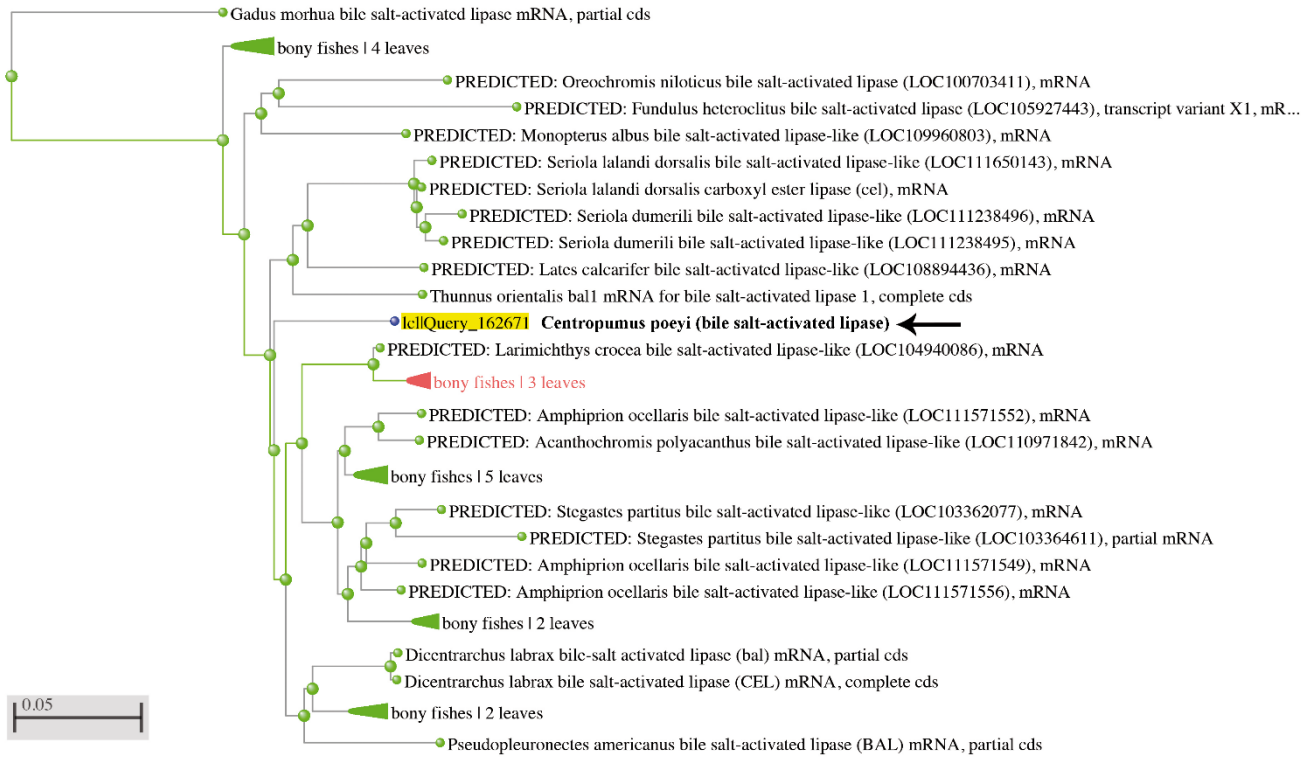

Figure 3. Similarity analysis of BAL gen in *Centropomus poeyi*.

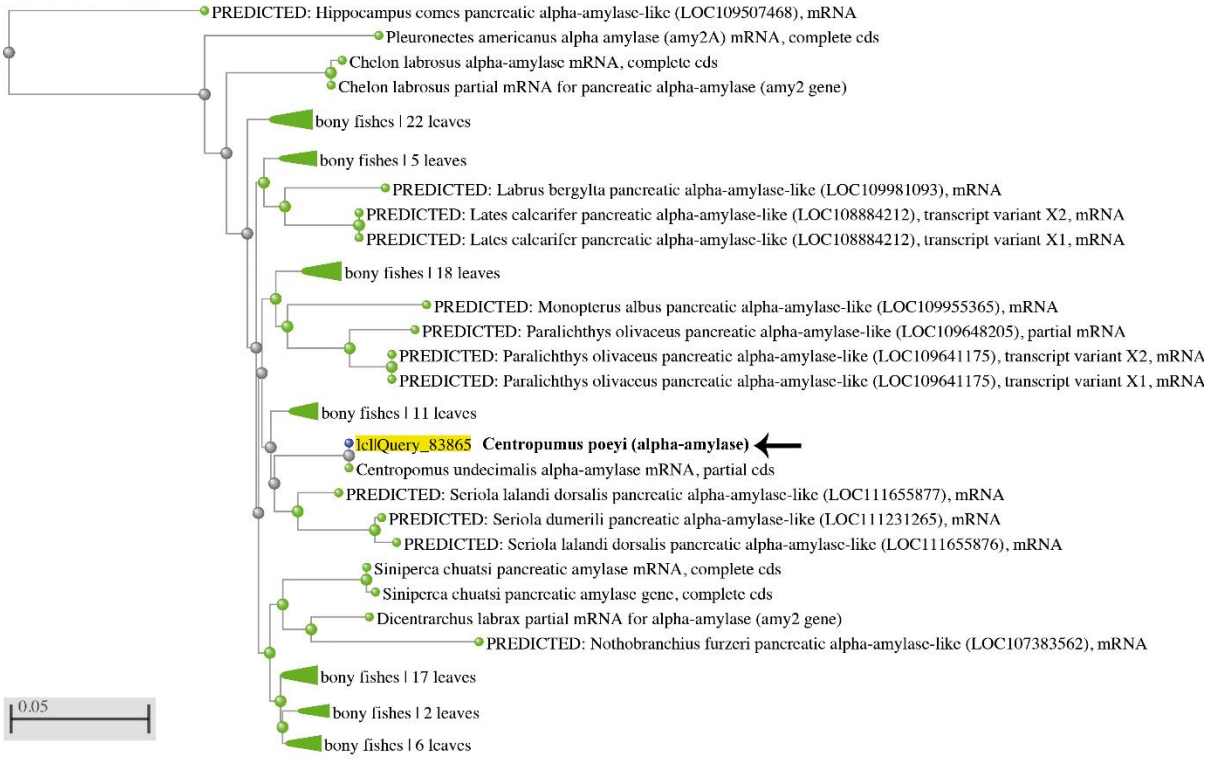

Figure 4. Similarity analysis of AMY gen in *Centropomus poeyi*.

Takao, 2017). In this way, the sequence similarity of the enzyme is relatively high, when compared to the isolated and purified tilapia (Yamada *et al*., 2005).

On the other hand, *C. poeyi* BAL sequence was a short sequence and was not possible to register in GeneBank; however, its sequence has high similarity with other fish BAL genes such as turbot *Scophthalmus maximus*; red seabream *Pagrus major* and rainbow trout *Oncorhynchus mykiss* (Hoehne-Reitan *et al*., 2005; Oku *et al*., 2006; Bouraoui *et al*., 2012). In this context, BAL is an essential digestive enzyme that cleaves ester bonds from triacyl glycerides releasing fatty acids. They are used as energy source, cell membrane recovery, hormones among other functions (Hide *et al*., 1992); however, the hydrolysis of lipids is highly complex by lipases due the interface water-lipid that implies the necessity for the enzyme to join to co-lipase and bile salt to break the ester bonds (Gjellesvik *et al*., 1992). In the case of partial α-amylase gene in *C. poeyi* was to short to be registered in GeneBank; however, this digestive enzyme has an essential function breaking glycosidic bond from glycogen and starch from food releasing α-glucose as a complementary energy source by glycolysis (Rønnestad *et al*., 2013). For other fish species, it has been proved that α-amylase in fish, such as *Sparus aurata*, *S. maximus*, redfish *Sebastes mentella*, and four species of prickleback fishes (Munilla-Morán & Sorbido-Rey, 1996a; Kim *et al*., 2014), is genetically programmed in the first days before hatching to use the glycogen in the yolk-sac, showing high activity and expression. Then reduce when larvae start to feeding, although fish still can be hydrolyzing carbohydrates for the action of the enzyme, but with some limitation for the utilization of disaccharides and monosaccharide, such as glucose, which has been detected in winter flounder *Pleuronectes americanus* and kutum *Rutilus frisiikutum* larvae (Douglas *et al.*, 2000; Hassanatabar *et al*., 2013).

Consider the expression of digestive enzymes our study represents a significant advance for the understanding of the digestive physiology of the species; in this regard, during the larval development of *C. poeyi*, the digestive enzyme expression level was determined on the initial ontogeny, using the live preys-based scheme (rotifers and *Artemia*). According to our results, it must be highlighted that TRY expression was early detected from hatching, once the larvae absorbed the yolk, and similar patterns to those observed in other marine fish species such as *S. aurata*, *D. labrax*, and *Tatoaba macdonaldi* were obtained (Péres *et al*., 1998; Galaviz *et al*., 2015; Mata-Sostres *et al*., 2016). The observed pattern could be related to the yolk absorption process, pigmentation of the eye and opening of the mouth in *C. poeyi*, and the ossification process during

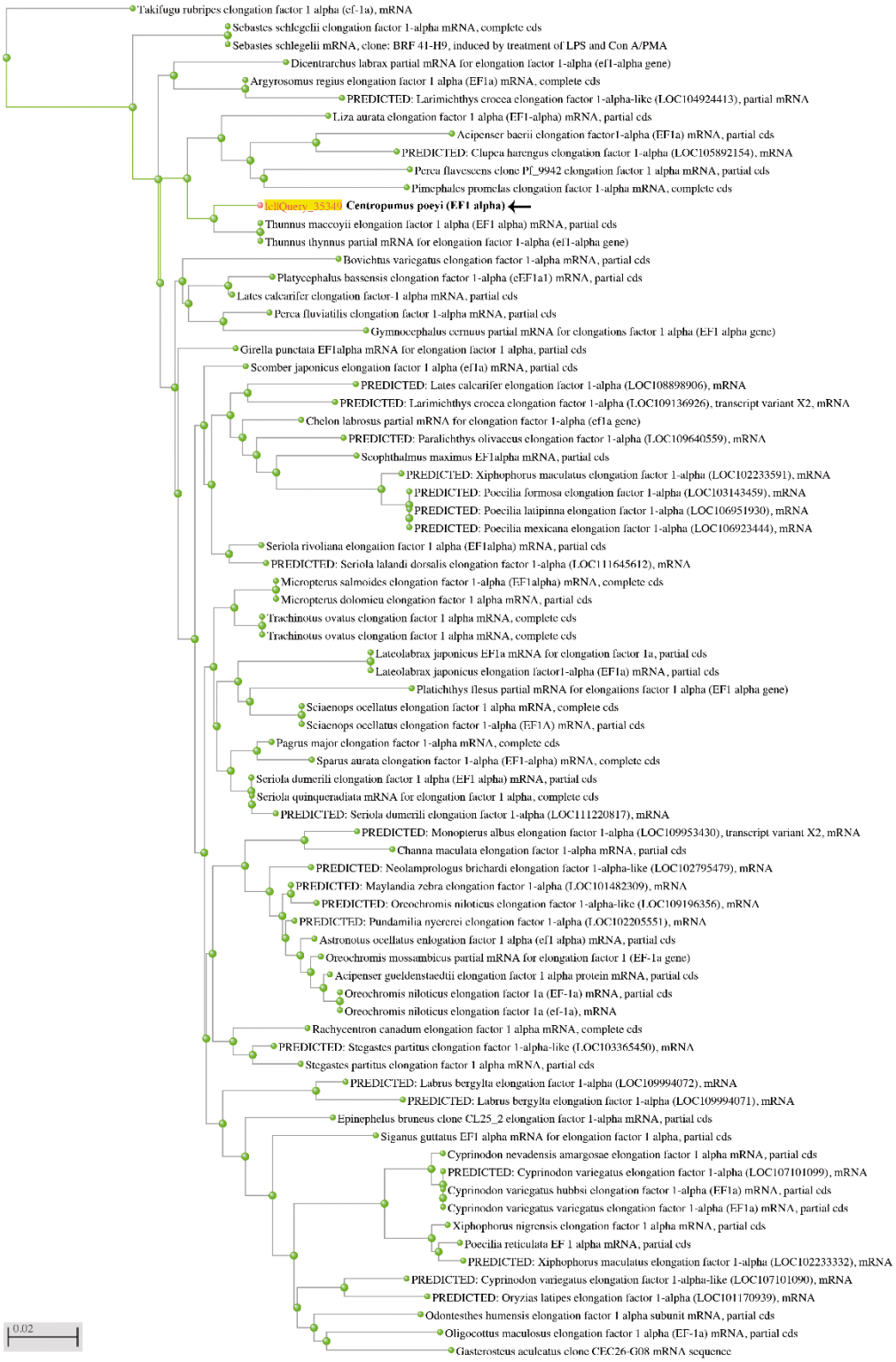

Figure 5. Similarity analysis of EF1-α gen in *Centropomus poeyi*.

the early ontogeny (Kubicek *et al*., 2018). So, its early expression allows the larvae to synthesize this enzyme *de Novo* from the primary cells of the exocrine pancreas (Kurokawa & Suzuki, 1996), which apart from hydrolyzing the proteins present during the first exogenous feed (rotifers), active to other digestive en-

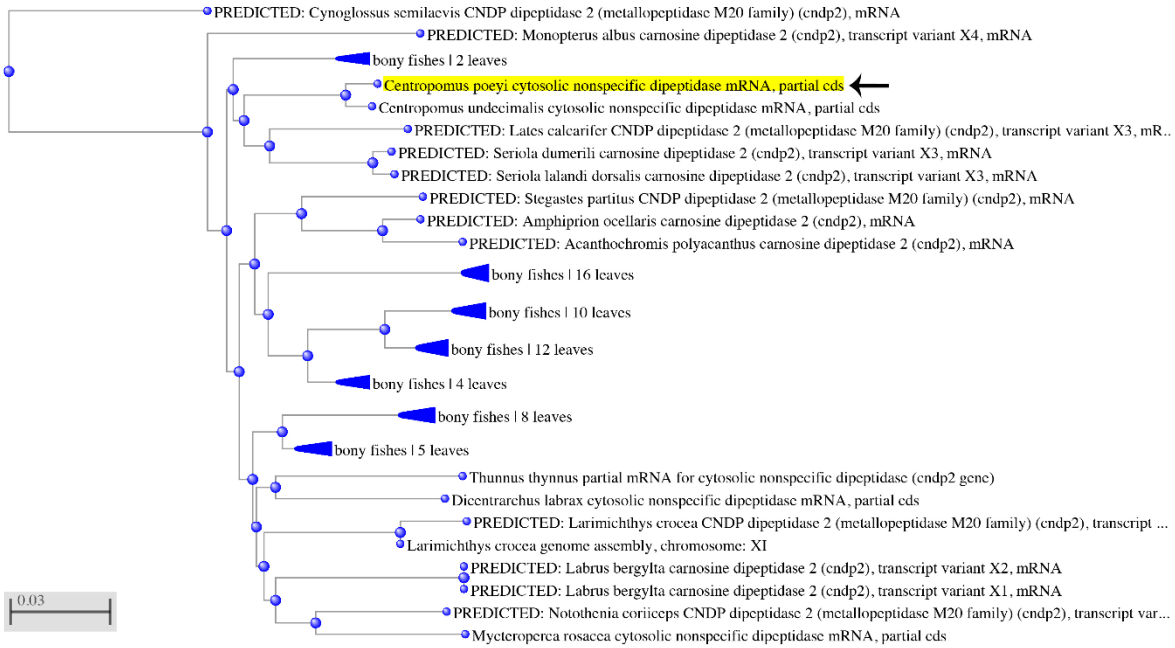

Figure 6. Similarity analysis of CNP gen in *Centropomus poeyi*.

zymes such as chymotrypsin which complements the hydrolysis of this substrate (Hjelmeland *et al*., 1983). Also, the first increase of gene expression at 9 DAH, coincides with the increase in size of the intestine and the elongation process (increase of brush border membranes), allows the larvae to enhance the hydrolysis in the lumen and absorption of peptides and amino acids in the enterocytes wall as was reported for species such as *C. undecimalis* (Jiménez-Martínez *et al.*, 2012). The increase of trypsin activity was detected at 11 DAH in *C. undecimalis*, which additionally matches with the morphological changes in the *C. parallellus* larvae at reported by Teles *et al*. (2015); these former authors show that once the exogenous feed starts, the size of the pancreas and zymogens increase, being these the precursors of the trypsin and chymotrypsin activity (Civera-Cerecedo *et al*., 2004). In the same way, the level of maximum expression detected at 17 DAH, might be due to two processes. The first, due to the change of feed of *Artemia* nauplii, while the second could also be related to the increase of brush border membranes and maturity of the enterocytes, which maximize the capacity to absorb not only for the peptides and amino acids but other nutrients such as lipids, carbohydrates, among others (Zambonino-Infante & Cahu, 1994). However, it must be mentioned that the action of trypsin, although fundamental to the larvae, is limited because the digestive system is not well developed yet. So, the formation of the stomach is required (secretion of hydrochloric acid and pepsin) to maximize the hydrolysis of the nutrients, and this process is reached during the transformation from the larvae into a juvenile (Zambonino-Infante & Cahu, 2001).

On the other hand, the low expression levels observed for the trypsin during the first days of *C. poeyi* development, were unexpected, since the activity of alkaline proteases usually are well represented in this period of the larval development and it was observed in other carnivore species such as the yellowtail amberjack (*Seriola lalandi*) (Chen *et al*., 2006), California halibut (*Paralichthys californicus*) (Alvarez-González *et al.*, 2006), red drum (*Sciaenops ocellatus*) (Lazo *et al*., 2007), and *Paralabrax maculatofasciatus* (Alvarez-González *et al*., 2008), before the acid digestion was established as occurs at the 25 DAH in *C. undecimalis* larvae (Jiménez-Martínez *et al*., 2012). This low expression pattern is similar to that found in some carnivore cyprinids (like the grass carp *Ctenopharyngodon idellus* and *Culter alburnus*), in which trypsinogen is detected, but at low levels, significantly increasing by 11 DAH, and with a second expression peak at 23 DAH. That could be related to morphological changes, even though it is worth mentioning that these are agastric fishes (Ruan *et al*., 2010). In this aspect, more research must be performed to describe the changes in the digestive enzymatic activity of the Mexican snook to correlate them with the expression at the transcriptional level, when we see the active protein. Nonetheless, there are reports on the trypsin type activity which is not necessarily correlated to the expression

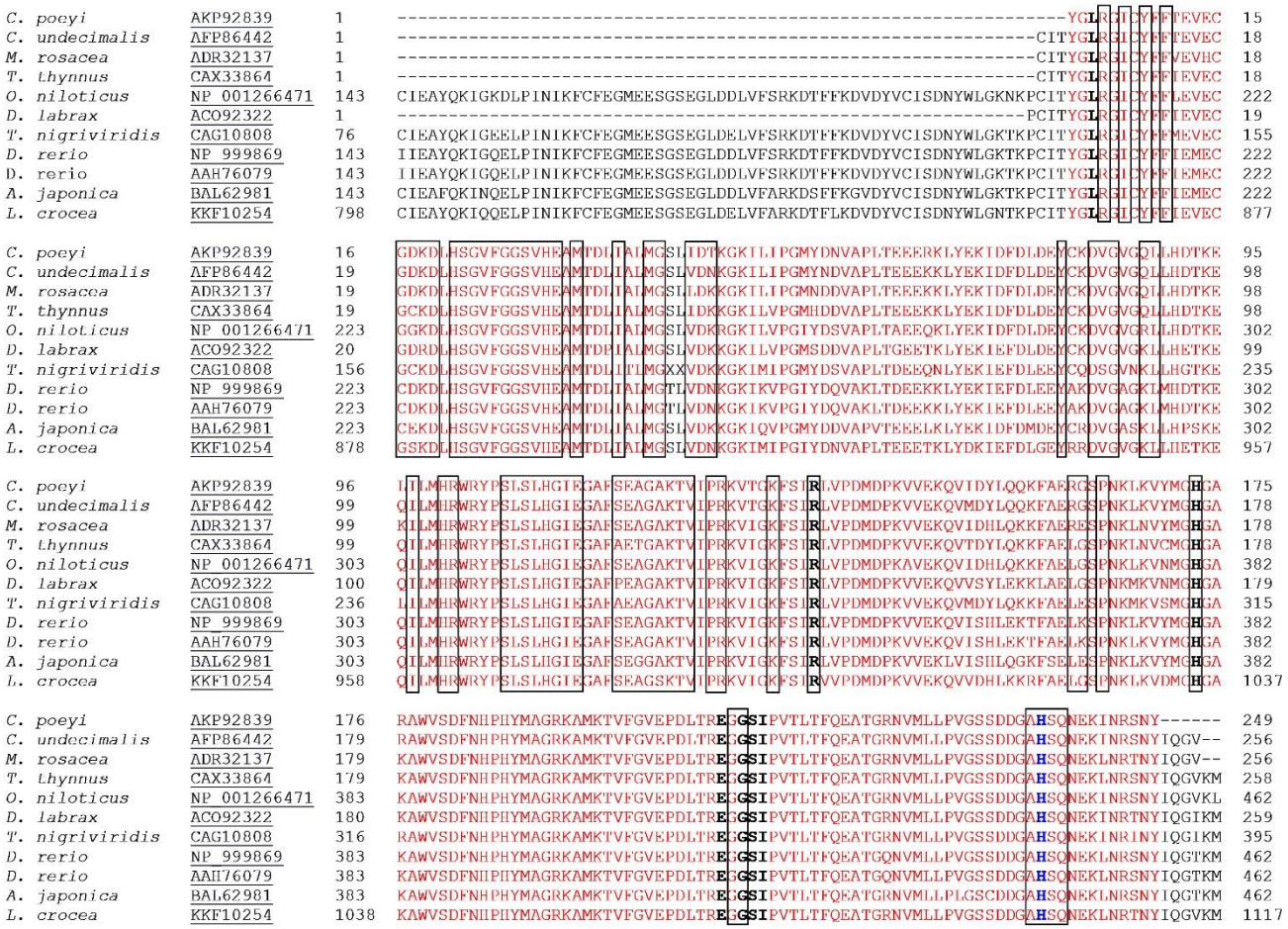

Figure 7. Alignments of the amino acid sequence of CNP in *C. poeyi* compared with other fish species (GeneBank access number or NCBI are indicated next to species name). Some of the conserved amino acid residues conforming the active site of the Carnosinase (CN2) which interacts with Mn^{+2} are marked in black bold. A conserved amino acid residue Histidine involved in the metal binding site is marked in blue bold. Also, amino acid residues involved in the dimer interface (polypeptide binding site) are boxed.

patterns, since there are different alkaline proteases (trypsin-like), using the same substrates resulting in higher activity during the development of the larvae (Kolkovski, 2001).

The expression of the bile salt depending lipase (BAL) was detected just the first day after hatching of *C. poeyi* which shows the capacity to use lipids from the diet and such as it has been quantified in other species like menhaden *Gadus morhua* (Sæle *et al*., 2010), *Lates calcarifer* (Srichanun *et al*., 2013) and *S. aurata* (Mata-Sotres *et al*., 2016). The use of lipids during the larval stage is essential for optimal growth and achieves the species makeup, so the quantification of this enzyme is of much greater importance during the first days of the development of candidate species for aquaculture. This enzyme has been considered the most important digestive enzyme in teleostean fish. It has also been identified as a carboxyl-ester hydrolase (BAL, EC: 3.1.1.3) (Sæle *et al*., 2010), which shows the capacity of the *C. poeyi* larvae to absorb lipids. It is also associated to the maturity of the digestive system (Zambonino-Infante *et al*., 1996), according to the yolk and oil globule absorption times, and later when the hydrolysis of the external lipids take place (Alvarez-González *et al.*, 2008). The expression pattern obtained in this study shows peaks corresponding to 10, 17 and 25, close to those obtained by the activity in *C. undecimalis* (5, 7, 25 y 36 DAH) (Jiménez-Martínez *et al*., 2012). These activity and expression fluctuations might represent the same changes in feeding schemes which were managed since the 6 and until 18 DAH with rotifer *Brachionus plicatilis* enriched with lipid emulsion, and from 15 DAH, co-fed with enriched *Artemia* nauplii up to 25 DAH. In this same sense, BAL

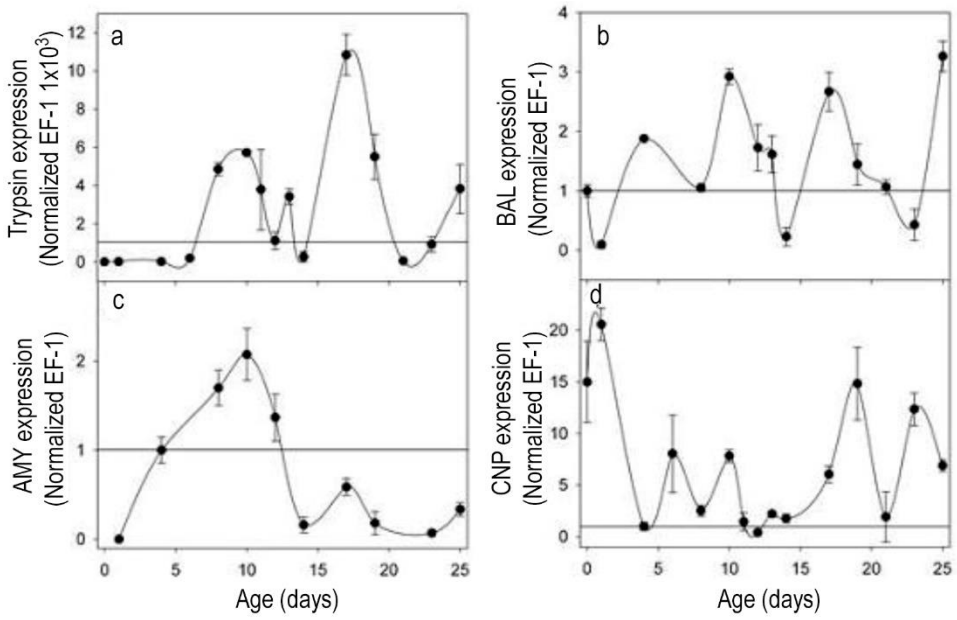

Figure 8. a) TRY, b) BAL, c) AMY, and d) CNP relative expression levels to Ef1 during early ontogeny of *Centropomus poeyi*. The qPCR data were normalized to the geometric mean of the expression efficiency-corrected data for EF1. The expression difference between samples (days after hatching) was tested by significant differences in Bio-Rad CFX Manager 3.1 Gene Study; Differences were considered statistically significant if $P < 0.05$.

expression is of vital importance for the development, and growth of the marine fish larvae, such as anchovy (*Engraulis mordax*), *D. labrax*, rainbow trout (*Oncorhynchus mykiss*) and *S. aurata*. Since the process starts from hatching, through the yolk absorption, this enzyme plays an essential role in the neutral lipid's digestion catalyzing the hydrolysis of the acyl glycerides and lower fats included in the diet such as cholesterol esters and vitamins (Izquierdo *et al*., 2000).

Amylase expression is reported from the first days, having as maximum expression peak on 10 DAH to then decrease the expression throughout the culture, as was observed in the red porgy (*Pagrus pagrus*) (Darias *et al.*, 2006), *D. labrax* (Darias *et al.*, 2008), *G. morhua* (Kortner *et al.*, 2011), *L. calcarifer* (Srichanun *et al.*, 2013), *Thunnus orientalis* (Murashita *et al.*, 2014) and *S. aurata* (Mata-Sotres *et al*., 2016). This enzyme has a typical pattern observed throughout the ontogeny in numerous fish studies, decreasing throughout the larval development to stay at low levels and has been considered as an indicator of digestive maturity in fish and other vertebrates. It is usually detected after hatching, under considerable expression level and subsequently decreases throughout the development, being a process that is regulated genetically (Zambonino-Infante & Cahu, 2001). This gene, despite having a tendency for a decreased expression can be induced by diet composition and feeding intensity and has been observed in other species such as *D. labrax*, by being fed with growing concentrations of starch (30 to 37% of carbohydrates) in its prepared food, where both the activity and expression of the amylase gene increase (Péres *et al*., 1998). On the other hand, the expression levels of the amylase in *C. poeyi*, after the administration of *Artemia*, and that reported by Jiménez-Martínez *et al*. (2012) with *C. undecimalis*, it does not seem to be stimulated by live prey. It is known that the mRNA and the amylase specific activity decrease in larvae fed with *Artemia* (Péres *et al*., 1998) since carbohydrates present in *Artemia* only represent 5% of the glycogen of the live prey composition (Dutrieu, 1960). A final expected pattern of amylase is to keep its low levels to use the carbohydrate from the food even in carnivore species (Munilla-Morán & Saborido-Rey, 1996b).

Nonspecific cytosolic dipeptidase (CNP, E.C. 3.4.13.18) is a widely distributed enzyme among animals; it is not secreted and is also considered as a form of a glycyl-glycine dipeptidase [http://enzyme.expasy.org/EC/3.4.13.18], which preferentially hydrolyzes hydrophobic dipeptides (Bauer, 1998). CNP expression levels in *C. poeyi* show a pattern similar to that observed in the enzymatic activity of the leucine alanine peptidase, whose activity coefficient with brush border membrane enzymes, is used as a marker of the digestive maturity in fish (Zambonino-Infante *et al*., 1996). The decrease of the leucine alanine peptidase

expression throughout the larval development and the increase of the aminopeptidase, alkaline phosphatase, and maltase enzyme activities ar the brush border membranes, are indicators that the organism reached the digestive maturity such as the adults of its species (Zambonino-Infante *et al*., 1996). Peptidases play an important role during the larval development of the fish since they participate in the digestion of the hydrolyzed products of other more abundant digestive proteases such as trypsin and chymotrypsin. The role played by the CNP in the maturation of the *C. poeyi* digestive system and other species shall be further investigated to know if it could be used as a molecular marker, like the leucine alanine peptidase, related to the activity and expression of those enzymes from the brush border membranes. Among other fish species, CNP patterns along ontogeny are in current research.

In summary, we can mention that the changes in the expression patterns of digestive enzymes are associated to live preys and morpho-functional development of the larvae, which must necessarily complement this study with biochemical and RNA-seq techniques for a higher understanding of the digestive physiology of the species.

ACKNOWLEDGMENTS

The author thanks National Council of Science and Technology of Mexico (CONACYT), to the Programa Institucional de Superación Académica and the Programa Institucional de Movilidad Estudiantil from UJAT for grants. To the projects TAMU-CONACYT 2012-023, CONACYT-FOMIX 2014-03-245836 and CONACYT CB2010-157763 for the financial support. To Patricia Hinojosa Baltazar, Baudelio García García and Marcelo Burgoin Cota from CIBNOR, and María de Jesús Contreras García, Alejandro Macdonald Vera and Leonardo Cruz Rosado from UJAT for their helpful support in analytic techniques and larval production.

REFERENCES

Alvarez-González, C.A., M. Cervantes-Trujano, D. Tovar-Ramírez, D.E. Conklin, H. Nolasco, E. Gisbert & R. Piedrahita. 2006. Development of digestive enzymes in California halibut *Paralichthys californicus* larvae. Fish Physiol. Biochem., 31: 83-93.

Alvarez-González, C.A., F.J. Moyano-López, R. Civera-Cercedo, V. Carrasco-Chávez, J.L. Ortiz-Galindo & S. Dumas. 2008. Development of digestive enzyme activity in larvae of spotted sand bass (*Palabrax maculatofasciatus*). I. Biochemical analysis. Fish Physiol. Biochem., 34: 373-384. doi:10.1007/s10695-007-9197-7.

Álvarez-Lajonchère, L., R.V. Cequeira & M. Dos Reis. 2002. Desarrollo embrionario y primeros estadios larvales del róbalo chucumite, *Centropomus parallelus* Poey (Pices: Centropomidae) con interés para su cultivo. Hidrobiológica, 12(2): 89-100.

Avilés-Quevedo, A., U. McGregor-Pardo, R. Rodríguez-Ramos, O. Morales-Castro, M. Huerta-Bello & M. Lizawa. 1995. Biología y cultivo de la cabrilla arenera *Paralabrax maculatofasciatus* (Steindachner, 1868). Secretaría de Pesca. Instituto Nacional de la Pesca y JICA, México, 85 pp.

Bauer, K. 1998. Cytosol non-specific dipeptidase. In: A.J. Barrett, N.D. Rawlings & J.F. Woessner (eds.). Handbook of proteolytic enzymes. Academic Press, London, pp. 1520-1522.

Boldyrev, A., E. Bulygina, T. Leinsoo, I. Petrushanko, S. Tsubone & H. Abe. 2004. Protection of neuronal cells against reactive oxygen species by carnosine and related compounds. Comp. Biochem. Physiol. B, 137: 81-88.

Bouraoui, L., L. Cruz-Garcia, J. Gutiérrez, E. Capilla & I. Navarro. 2012. Regulation of lipoprotein lipase gene expression by insulin and troglitazone in rainbow trout (*Oncorhynchus mykiss*) adipocyte cells in culture. Comp. Biochem. Physiol. A, 161(1): 83-88.

Cara, B., F.J. Moyano, J.L. Zambonino & C. Fauvel. 2007. Trypsin and chymotrypsin as indicators of nutritional status of post-weaned sea bass larvae. J. Fish Biol., 70: 1798-1808.

Castro-Aguirre, J.L., H.E. Pérez & J.J. Schmitter-Soto. 1999. Ictiofauna estuarino-lagunar y vicaria de México, Editorial Limusa, México, 625 pp.

Cerqueira, R.V. & A.M. Brügger. 2001. Effect of light intensity on initial survival of fat snook (*Centropomus parallelus*, Pisces: Centropomidae) larvae. Braz. Archiv. Biol. Tech. Int. J., 44(4): 343-349.

Chakrabarti, R., R.M. Rathore, P. Mittal & S. Kumar. 2006. Functional changes in digestive enzymes and characterization of proteases of silver carp (♂) and bighead carp (♀) hybrid, during early ontogeny. Aquaculture, 253: 694-702.

Chen, B.N., G.Q. Jian, S.K. Martin, G.H. Wayne & M.C. Steven. 2006. Ontogenetic development of digestive enzymes in yellowtail kingfish *Seriola lalandi* larvae. Aquaculture, 256: 489-501.

Civera-Cerecedo, R., C.A. Álvarez-González & F.J. Moyano-López. 2004. Nutrición y Alimentación de larvas de peces marinos. In: L.E. Cruz Suárez, D. Ricque-Marie, M.G. Nieto-López, D. Villarreal, U. Scholz & M. González (eds.). Avances en Nutrición Acuícola VII. Memorias del VII Simposium Internacional de Nutrición Acuícola. Hermosillo, Sonora, p. 8.

Darias, M.J., H.M. Murray, J.W. Gallant, A. Astola, S.E. Douglas, M. Yúfera & G. Martínez-Rodríguez. 2006. Characterization of a partial alpha-amylase clone from red porgy (*Pagrus pagrus*): expression during larval development. Comp. Biochem. Physiol. B, 143: 209-218.

Darias, M.J., J.L. Zambonino-Infante, K. Hugot, C.L. Cahu & D. Mazurais. 2008. Gene expression patterns during the larval development of European sea bass (*Dicentrarchus labrax*) by microarray analysis. Mar. Biotechnol., 10: 416-428.

Douglas, S.E., S. Mandla & J.W. Gallant. 2000. Molecular analysis of the amylase gene and its expression during development in the winter flounder, *Pleuronectes americanus*. Aquaculture, 190: 247-260.

Dutrieu, J. 1960. Observations biochimiques et physiologiques sur le développement d'*Artemia salina* Leach. Arch. Zool. Exp. Gén., 99: 1-134.

Galaviz, M., L.M. López, A. García-Casca, C.A. Álvarez-González, C. True & E. Gisbert. 2015. Digestive system development and study of acid and alkaline protease digestive capacities using biochemical and molecular approaches in totoaba (*Totoaba macdonaldi*) larvae. Fish Physiol. Biochem., 41: 1117-1130.

Galaviz, M.A., A. García-Gasca, M. Drawbridge, C.A. Álvarez-González & L.M. López. 2011. Ontogeny of the digestive tract and enzymatic activity in white sea bass, *Atractoscion nobilis*, larvae. Aquaculture, 138: 162-168. doi:10.1016/j. aquaculture.2011.05.014.

Gisbert, E., J.B. Ortiz-Delgado & C. Sarasquete. 2008. Nutritional cellular biomarkers in early life stages of fish. Histol. Histopathol., 23: 1525-1539.

Gjellesvik, D.R., D. Lombardo & B. Walther. 1992. Pancreatic bile salt-dependent lipase from cod (*Gadus morhua*): purification and properties. Biochem. Biophys. Acta, 1124(2): 123-134.

Hide, W.A., L. Chan & W.-H. Li. 1992. Structure and evolution of the lipase superfamily. J. Lipid Res., 33: 167-178.

Hjelmeland, K., I. Huse, T. Jorgensen, G. Molvik & J. Raa. 1983. Trypsin and trypsinogen as indices of growth and survival potential of cod (*Gadus morhua* L.) larvae. Flodevigen. Rapp., 3: 1-17.

Hoehne-Reitan, K., E. Kjørsvik & D.R. Gjellesvik. 2005. Development of bile salt-dependent lipase in larval turbot. J. Fish Biol., 58(3): 737-745.

Izquierdo, M.S., J. Socorro, L. Arantzamendi & C.M. Hernández-Cruz. 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem., 22: 97-107.

Jiménez-Martínez, L.D., C.A. Álvarez-González, D. Tovar-Ramírez, G. Gaxiola, A. Sánchez-Zamora, F.J. Moyano, F.J. Alarcón, G. Márquez-Couturier, E. Gisbert, W.M. Contreras-Sánchez, N. Perales-García, L. Arias-Rodríguez, J.R. Indy, S. Páramo-Delgadillo & I.G. Palomino-Albarrán. 2012. Digestive enzyme activities during early ontogeny in Common snook (*Centropomus undecimalis*) Fish Physiol. Biochem., 38: 441-454.

Kim, K.H., M.H. Horn, A.E. Sosa & D.P. German. 2014. Sequence and expression of an α-amylase gene in four related species of prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and species-level effects. J. Comp. Physiol., 184B(2): 221-34.

Kolkovski, S. 2001. Digestive enzymes in fish larvae and juveniles-implications and applications to formulated diets. Aquaculture, 200: 181-201.

Kortner, T.M., I. Overrein, G. Øie, E. Kjørsvik, T. Bardal, P.A. Wold & A. Arukwe. 2011. Molecular ontogenesis of digestive capability and associated endocrine control in Atlantic cod (*Gadus morhua*) larvae. Comp. Biochem. Physiol. A, 160: 190-199.

Kubicek, K.M., C.A. Alvarez-González, R. Martínez-García, W.M. Contreras-Sánchez, C. Pohlenz & K.W. Conway. 2018. Larval development of the Mexican Snook, *Centropomus poeyi* (Teleostei: Centropomidae). Neotrop. Ichthyol., 16(1): e170014.

Kurokawa, T. & T. Suzuki. 1996. Formation of the diffuse pancreas and the development of digestive enzyme synthesis in larvae of the Japanese flounder *Paralichthys olivaceus*. Aquaculture, 141: 267-276.

Lazo, J.P., M.J. Darias & E. Gisbert. 2011. Ontogeny of the digestive tract. In: G.J. Holt (ed.). Larval fish nutrition. Wiley-Blackwell, Hoboken, pp. 5-46.

Lazo, J.P., R. Mendoza, G.J. Holt, C. Aguilera & C.R. Arnold. 2007. Characterization of digestive enzymes during larval development of red drum (*Sciaenops ocellatus*). Aquaculture, 265: 194-205.

Liu, C.H., Y.L. Shiu & J.L. Hsu. 2012. Purification and characterization of trypsin from the pyloric ceca of orange-spotted grouper, *Epinephelus coioides*. Fish Physiol. Biochem., 38: 837-848.

Martínez-Burguete, T. 2013. Expresión de enzimas digestivas durante la ontogenia inicial del robalo blanco (*Centropomus undecimalis*). Dissertation, Univesidad Autónoma de Chiapas, Chiapas, 38 pp.

Mata-Sotres, J.A., A. Astola, M. Yúfera & G. Martínez-Rodríguez. 2016. Cloning and molecular ontogeny of digestive enzymes in fed and food-deprived developing gilthead seabream (*Sparus aurata*) larvae. Comp. Biochem. Physiol. B, 191: 53-65.

Moyano, F.J., M. Díaz, F.J. Alarcón & M. Sarasquete. 1996. Characterization of digestive enzyme activity during larval development of gilthead seabream *(Sparus aurata)*. Fish Physiol. Biochem., 15: 121-130.

Munilla-Morán, R. & F. Sorbido-Rey. 1996a. Digestive enzymes in marine species. II. Amylase activities in the gut from seabream (*Sparus aurata*), turbot

(*Scophthalmus maximus*) and redfish (*Sebastes mentella*). Comp. Biochem Physiol. B, 113: 827-834.

Munilla-Morán, R. & F. Saborido-Rey. 1996b. Digestive enzymes in marine species. I. Proteinase activities in the gut from redfish (*Sebastes mentella*), seabream (*Sparus aurata*) and turbot (*Scophthalmus maximus*). Comp. Biochem. Physiol. B, 113: 395- 402.

Murashita, K., H. Matsunari, K. Kumon, Y. Tanaka, S. Shiozawa, H. Furuita, H. Oku & T. Yamamoto. 2014 Characterization and ontogenetic development of digestive enzymes in Pacific bluefin tuna *Thunnus orientalis* larvae. Fish Physiol. Biochem., 40: 1741-1755.

Okomura, N. & T. Takao. 2017. The zinc form of carnosine dipeptidase 2 (CN2) has dipeptidase activity, but its substrate specificity is different from that of the manganese form. Biochem. Biophys Res. Comm., 494(3-4): 484-490.

Oku, H., N. Koizumi, T. Okumura, T. Kobayashi & T. Umino. 2006. Molecular characterization of lipoprotein lipase, hepatic lipase, and pancreatic lipase genes: effects of fasting and refeeding on their gene expression in red sea bream *Pagrus major*. Comp. Biochem. Physiol. B, 145(2): 168-78.

Oku, T., S. Andob, T. Hayakawac, K. Babac, R. Nishic, K. Shiozakic & S. Yamada. 2011. Purification and identification of a novel primitive secretory enzyme catalyzing the hydrolysis of imidazole-related dipeptides in the jawless vertebrate *Lethenteron reissneri*. Peptides, 32: 648-655.

Péres, A., J.L. Zambonino-Infante & C.L. Cahu. 1998. Dietary regulation of activities and mRNA levels of trypsin and amylase in sea bass *Dicentrarchus labrax*. larvae. Fish Physiol. Biochem., 19: 145-152.

Rønnestad, I., M. Yúfera, B. Ueberschär, L. Ribeiro, Ø. Sæle & C. Boglione. 2013. Feeding behavior and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research. Rev. Aquacult., 5: S59-S98.

Ruan, G.L., Y. Li, Z.X. Gao, H.L. Wang & W.M. Wang. 2010. Molecular characterization of trypsinogens and development of trypsinogen gene expression and tryptic activities in grass carp (*Ctenopharyngodon idellus*) and topmouth culter (*Culter alburnus*), Comp Biochem. Physiol. B, 155: 77-85.

Rust, M.B. 2002. Nutritional physiology. In: J.E. Halver & R.W. Hardy (eds.). Fish Nutrition, Elsevier, Amsterdam, pp. 367-452.

Sæle, Ø., A. Nordgreen, P.A. Olsvik & K. Hamre. 2010 Characterization and expression of digestive neutral lipases during ontogeny of Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. A, 157: 252-259.

Sahlmann, C., J. Gu, T.M. Kortner, I. Lein, Å Krogdahl & A.M Bakke. 2015. Ontogeny of the digestive system of Atlantic salmon (*Salmo salar* L.) and effects of soybean meal from start-feeding. PLoS One, 10: 1371/0124179.

Srichanun, M., C. Tantikitti, P. Utarabhand & T. Kortner. 2013. Gene expression and activity of digestive enzymes during the larval development of Asian seabass (*Lates calcarifer*). Comp. Biochem. Physiol. B, 165: 1-9.

Stephen, R. & P. Shafland. 1982. Larval development of snook, *Centropomus undecimalis* (Pisces: Centropomidae). Copeia, 1982(3): 618-627.

Tamura, K., G. Stecher, D. Peterson, A. Filipski & S. Kumar. 2013. MEGA6: molecular 717 evolutionary genetics analysis version 6.0. Mol. Biol. Evol., 30: 2725-2729.

Tarcisio, T., R. Vinicius & J. Brown. 2005. Early weaning of fat snook *Centropomus parallelus* (Poey, 1864) larvae. Aquaculture, 253: 334-342.

Teles, A., W. Melo, D. Ammar, Y. Rauh, E. Nazari & V. Ronzani. 2015. Ontogeny of the digestive tract of *Centropomus parallelus* larvae. Fish Physiol. Biochem., 41: 549-559. doi: 10.1007/s10695-015-0028-y.

Teufel, M., V. Saudek, J.P. Ledig, A. Bernhardt, S. Boularand, A. Carreau, N.J. Cairns, C. Carter, D.J. Cowley, D. Duverger, A.J. Ganzhorn, C. Guenet, B. Heintzelmann, V. Laucher, C. Sauvage & T. Smirnova. 2003. Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. J. Biol. Chem., 278: 6521-6531.

Ueberschär, B. 1993. Measurement of proteolytic enzyme activity: significance and application in larval fish research. In: B.T. Walther & H.J. Fhyn (eds.). Physiological and biochemical aspects of fish development. University of Bergen, Norway, 233-239 pp.

Yamada, S., Y. Tanaka & S. Ando. 2005. Purification and sequence identification of anserinase. FEBS J., 272: 6001-6013.

Zambonino-Infante, J.L. & C.L. Cahu. 1994. Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. Fish Physiol. Biochem., 12: 399-408.

Zambonino-Infante, J.L. & C.L. Cahu. 2001. Ontogeny of the gastrointestinal tract of marine fish, Comp. Biochem. Physiol. C, 130: 477-487.

Zambonino-Infante, J.L., C.L. Cahu, A. Péres, P. Quazuguel & M.M. Le Gall. 1996. Sea bass (*Dicentrarchus labrax)* larvae fed different *Artemia* rations: growth, pancreas enzymatic response and development of digestive functions. Comp. Biochem. Physiol. C, 130: 477-487.

Zambonino-Infante, J.L., E. Gisbert, C. Sarasquete, I. Navarro, J. Gutierrez & C.L. Cahu. 2008. Ontogeny and physiology of the digestive system of marine fish larvae. In: J.E.O. Cyrino, D. Bureau & B.G. Kapoor (eds.). Feeding and digestive functions of fishes. Science Publishers, Enfield, 281-384 pp.

Received: 2 October 2017; Accepted: 11 April 2018