Latin American Journal of Aquatic Research, 47(1): 102-113, 2019
DOI: 10.3856/vol47-issue1-fulltext-11

Research Article

Changes of digestive enzymes in totoaba (*Totoaba macdonaldi* Gilbert, 1890) during early ontogeny

Mabelyn Córdova-Montejo[1], Carlos A. Álvarez-González[1], Lus M. López[2], Conal D. True[2] Carlos A. Frías-Quintana[1] & Mario A. Galaviz[2]

[1]Laboratorio de Acuicultura Tropical, División Académica de Ciencias Biológicas
Universidad Juárez Autónoma de Tabasco, Villahermosa, Tabasco, México
[2] Facultad de Ciencias Marinas, Universidad Autónoma de Baja California
Ensenada, B.C., México
Corresponding author: Mario A. Galaviz (mgalaviz@uabc.edu.mx)

ABSTRACT. *Totoaba macdonaldi* is an endemic species which has been overexploited causing its population to decrease and to be cataloged as endangered. Understanding the larval period is the most critical stage in the development of marine fish farming. Our study aims to determine the changes in digestive enzyme activities during the early ontogeny of totoaba, using biochemical and electrophoretic techniques as SDS-PAGE. The results show that the acid protease had maximum activity between days 28 and 32 days post-hatching (DPH) when the *Artemia* was replaced by formulated food. Alkaline protease activity was detected at 2 DPH with maximal activity between 20 and 32 DPH; trypsin activity became active from 2 DPH; chymotrypsin activity increased at 6 DPH; leucine aminopeptidase activity was detected at 3 DPH, showing its maximal level at 22 DPH; carboxypeptidase A activity increased at 3 DPH; and lipase and amylase activities were detected at 2 DPH. Acid zymogram showed only one isoform (0.72 rf) from 2 DPH and increased in intensity from 8 DPH. For alkaline proteases, four isoforms were detected from yolk absorption (1 DPH), increasing to six isoforms (77.8, 47.3, 43.5, 21.0, 19.2, and 17.5 kDa) from 8 DPH onwards. Our results demonstrate that larvae of *T. macdonaldi* present characteristics typical of carnivore marine fish, showing the presence of alkaline digestive enzymes prematurely and the presence of offset pepsin. According to the results obtained in the present study, *T. macdonaldi* can be a juvenile beginning between 24 and 28 DPH, being the most appropriate day to perform replacement by artificial food.

Keywords: *Totoaba macdonaldi*; ontogeny; nutrition; proteases; amylase; lipase; zymogram

INTRODUCTION

Ontogeny enzymatic studies can provide better knowledge of the physiology of nutrition during development, and they are essential for understanding larval nutritional needs. These studies are performed with biochemical, molecular, histological and electrophoretic techniques which can track the digestive enzymes throughout the larval development of cultivated species (Alarcón & Martínez, 1998; Murray *et al*., 2006; Darias *et al*., 2008; Srichanun *et al*., 2013; Galaviz *et al*., 2015). Such studies have been conducted in species such as California halibut (*Paralichthys californicus*, Álvarez-González *et al.*, 2006), tropical gar (*Atractosteus tropicus*, Frías-Quintana *et al.*, 2016), spotted sand bass (*Paralabrax maculatofasciatus*, Álvarez-González *et al*., 2010), white seabass (*Atractoscion nobilis*, Galaviz *et al.*, 2011), Mayan cichlid (*Cichlasoma urophtalmus*, López-Ramírez *et al.*, 2011), Bay snook (*Petenia splendida*, Uscanga-Martínez *et al.*, 2011), common snook (*Centropomus undecimalis*, Jiménez-Martínez *et al*., 2012), rose snapper (*Lutjanus guttatus*, Galaviz *et al.*, 2012) and three spotted cichlid (*Cichlasoma trimaculatum*, Toleto-Solís *et al.*, 2014). These authors, when assessing changes in the activities of various digestive enzymes, noted that the maturation of the digestive system in the species determined the activities of digestive enzymes and their relationship with changes in living foods. Further, the authors noted the

Corresponding editor: Fernando Vega-Villasante

weaning processes for the use of inert food in order to improve growth and survival.

Totoaba macdonaldi (Gilbert, 1890) is a marine species reaching two meters in length and over 100 kg. This species is found in shallow coastal waters down to depths of 25 m. It has a large annual breeding migration during the spring season to the Colorado River delta at the northern end of the Gulf of California. It feeds on fish and shrimp. Totoaba extraction during much of the 20th century supported an important commercial and sports fishing industry in the Gulf of California; however, mismanagement of the fishery was responsible for the decline of this species, and since 1975, it has been included in the endangered species list (Bobadilla *et al.*, 2011).

Consequently, the need for cultivation of the species is essential, either as a primary task of conservation through repopulation or as a commercial activity. Studies that investigate its digestive physiology support the development of foods that can set the basis for cultivation (Galaviz *et al.*, 2015; Mata-Sotres *et al.*, 2015). Therefore, in order to understand the digestive capacity during ontogeny of this species, this study aims to describe the development of the activity of digestive enzymes during the early ontogeny of totoaba.

MATERIALS AND METHODS

Eggs and larval rearing

The larval rearing in the present study was according to Galaviz *et al.* (2015). Fertilized totoaba gametes were obtained from a captive broodstock in the Unidad de Biotecnología en Piscicultura (UBP) of the Facultad de Ciencias Marinas, Universidad Autónoma de Baja California, Ensenada, México. Gonadal maturation in adult totoaba was induced using photothermal control to simulate natural seasonal cycles, and fish were induced to ovulate and spermiate using [des-Gly10, D-Ala6]-LHRH ethylamine acetate salt hydrate (SIGMA) according to True (2012). The hormone implant was applied to the dorsal sinus just below the caudal fin. This hormone is effective in both females and males as it stimulates normal production of gonadotropins, which are natural hormones controlling the reproduction process. In the case of males, it leads to increased production of sperm and hydration (more fluid semen), while it encourages females to enter the final phase of maturation in which the oocyte leaves her maturation process and proceeds to rehydrate and final ovulation. Two males and four females were selected and implanted with LHRHa and subsequently transferred to a tank with temperature control and photoperiod control (24ºC and 14:10 h light: darkness).

The collection of totoaba eggs was according to Galaviz *et al.* (2015). The reproduction was performed at 24-36 h after the hormone implant in the broodstock. The egg collection was performed by draining the tank whose drain was connected to a surge tank where an egg collector was installed with a 300 µm mesh. Neutrally buoyant, eggs were collected from the surge tank between 5 and 6 h after spawning. Eggs were collected with a 500 µm mesh and placed in a container with previously filtered seawater. The eggs then received a bath with 0.026% formaldehyde for 20 min to remove bacteria and protozoa that might be attached to the corium. This procedure was performed in buckets with enough air or the eggs to remain suspended. After receiving the treatment, the eggs were washed with previously filtered seawater and passed through ultraviolet rays in order to avoid the presence of pathogens before culturing. The eggs were placed in test tubes to separate the viable (with neutral buoyancy) from the non-viable (they fell to the bottom). In order to facilitate the separation, the density of seawater was increased. Several samples of one milliliter were collected and quantified under a stereoscopic microscope according to Kjørsvik *et al.* (1990) to estimate the amount of viable and nonviable eggs.

Larval culture tanks

Totoaba eggs were placed at a rate of 100 eleuteroembryos L^{-1} in incubators with 2,200 L capacity with a controlled environment (24ºC, salinity of 34, 6 mg O_2 L^{-1}). The hatching occurred 20 h after seeding the incubators. After hatching, the eggs which did not hatch were extracted using a siphon to avoid contamination by decomposing organic matter within the system. Before the first exogenous feeding on the third day post-hatching (DPH), larvae were transferred to conical tanks with a capacity of 100 L^{-1} where the experimental cultivation was carried out. From these tanks, the samples for biochemical and electrophoretic analysis were obtained. Culture water conditions in experimental tanks were maintained at 24ºC, salinity of 34 and 6 mg O_2 L^{-1}.

Feeding

Larvae feeding were performed based on procedures from Galaviz *et al.* (2015), which started from 3 DPH once the yolk sac and the drop of oil were consumed entirely. The first live food provided to the larvae were rotifers enriched (ER) with fatty acids at five rotifers mL^{-1} for the first three days after starting exogenous feeding. From 8 DPH, larvae were fed three times a day with ten rotifers per mL^{-1} through 16 DPH. From that day the rotifers were combined with *Artemia* nauplii (AR) at 0.5 mL^{-1} (Salt Creek Inc., Salt Lake City, UT,

USA). The enriched of rotifers was done with a commercial emulsion (Bio-Marine Algamac 3050™) at a concentration of 0.6 g L^{-1}. The supply of rotifers was decreased until 20 DPH when it stopped being offered entirely to the culture tank. The feeding with *Artemia* was increased gradually to reach 5 nauplii mL until 24 DPH and was kept constant until the beginning of the change from live food to formulated food (micro diet) (Otohime Japanese Marine Weaning, Red Mariculture; protein 52.1%, lipids 16.3% and ashes 11.2%, particle size 200 to 1410 µm) performed from 28 DPH forward. The amount of *Artemia* nauplii decreased as the change of micro diet increased. It was eliminated from the culture tank at DPH 32 when the amount (g) of the micro diet increased to complete the weaning on this day. At the beginning of exogenous feeding, water flow increased to 4 L min^{-1} until the start of feeding with *Artemia* nauplii, then, water flow increased to 6 L min^{-1}. Incubators were siphoned once during the day to start the first feeding with enriched rotifers and twice during the day when feeding with *Artemia* sp. nauplii was started.

Collection of samples for biochemical analysis

Triplicate samples for each DPH were randomly collected depending on the size of totoaba larvae (n = 30-100) in triplicate tanks directly from experimental cultivation using a 300 µm mesh. The samples were collected before the first feeding (8:00 AM) in order to rule out possible effects of enzymes coming from the diet. Larvae samples were collected daily from 0 DPH to 6 DPH. The samples were then collected every two days until 20 DPH and every four days until the end of the study (DPH 32). After collection of larvae, they were anesthetized with tricaine methanesulfonate (MS 222), washed with distilled water to remove the excess of salts and frozen at -70°C until the analysis. In addition, samples (n = 10-30) were taken daily to record data size in total length and dry weight on days 0, 1, 2, 3, 5, 6, 8, 12, 16, 18, 22, 24, 28 and 32 DPH. The average total length (mm) was obtained by measuring a sample of 10 larvae under a microscope with digital camera and PAXcam2 software (Pax-it version 6, Mis Inc., USA). Dry weight (measured to the nearest 0.1 mg) was calculated by weighing the subsample of larvae using an analytical balance (Sartorius Gottingen, Germany, with the precision of 0.1 mg), then counting the larvae contained in the subsample. Individual larval weight was determined by dividing the subsample weight by the number of larvae in the sample.

Preparation of multienzyme extracts

Throughout the culture, larvae samples were homogenized in 200 mg mL^{-1} of the mix of 100 mmol L^{-1} of glycine-HCl buffer pH 2 for acid activity (pepsin) and with Tris-HCl 30 mmol L^{-1} buffer + $CaCl_2$ 12.5 mM pH 7.5 for alkaline enzyme activity. The homogenates were centrifuged at 16,000 *g* for 30 min at 4°C. The multienzyme extract was obtained and stored at -20°C for later analysis.

Biochemical analyses

The concentration of soluble protein in the multienzyme of stomach and intestine extracts was determined with the technique described by Bradford (1976). In the determination of acid protease enzymatic activity, the technique described by Anson (1938) was applied, using as the substrate 1% bovine hemoglobin with glycine-HCl buffer 100 mmol L^{-1} at pH 2. The mixture was incubated at 37°C for 30 min, and then, the reaction was stopped with the addition of 0.5 mL of trichloroacetic acid (TCA, 20%). The solution was centrifuged at 16,000 g for 15 min and the absorbance of the products reaction was measured at 280 nm. The unit of enzyme activity is defined as 1 µg of tyrosine released per minute, based on the molar extinction coefficient (0.005).

The enzyme activity of the total alkaline proteases was determined with the technique described by Kunitz (1947) modified by Walter (1984) using a casein-like substrate 1% Tris-HCl buffer 100 mmol L^{-1} + $CaCl_2$ 10 mmol L^{-1} at pH 9.

Trypsin activity was measured according to the method by Erlanger *et al.* (1961) at 37°C using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) dissolved in Tris-HCl 50 mmol L^{-1}, $CaCl_2$ 10 mmol L^{-1} as substrate at pH 8.2 and measured at 410 nm.

Chymotrypsin activity was determined using the Del Mar *et al.* (1961) method using the BTEE (N-benzoyl-L-tyrosine ethyl ester) 5 mmol L^{-1} substrate in Tris-HCl 44.4 mmol L^{-1} + $CaCl_2$ 55.5 mmol L^{-1} pH 7.8 buffer. The substrate was diluted in 200 µL of DMS; 623 µL of buffer were directly placed in the quartz cell of the spectrophotometer and zeroed, then 70 µL of the substrate was added. Absorbance was measured at 256 nm every 20 s for 2 min. After this time, 10 µL of the multi-enzyme extract was added, and the absorbance was measured every 20 s for 2 min again. The test was performed in triplicate; delta absorbance was calculated from the difference between the absorbance of the catalyzed reaction and the absorbance of the substrate once both were stabilized.

Carboxypeptidase A activity was determined using the method of Folk & Schirmer (1963) at 25°C using Hippuryl-L-phenylalanine (25 mmol L^{-1}) as a substrate in a buffer solution of Tris HCl 25 mmol L^{-1}, NaCl 50 mmol L^{-1}, a pH 7.5 and measured at 254 nm. The activity of Leucine aminopeptidase was determined

using the method of Maraux *et al.* (1973) using Leucine-p-Nitroanilide in DMSO 0.1 mmol L^{-1} as a substrate, with sodium phosphate buffer in 50 mmol solution L^{-1}, pH 7.2 at 37%, measured at 410 nm. The enzymatic reactions were stopped with 30% acetic acid where enzyme activity was defined as 1 µmol L^{-1} of nitroaniline released per minute, using a molar extinction coefficient of 8.8 for trypsin, chymotrypsin, and leucine aminopeptidase.

For the identification of α-amylase enzyme activity, 2% starch was used as a substrate in citrate-phosphate buffer 0.1 mol L^{-1} with NaCl 0.005 mol L^{-1} a pH 7.5. Absorbance was quantified at 600 nm, defining a unit as the amount of enzyme which liberates l µg maltose per minute (Robyt & Whelan, 1968).

Lipase activity was determined by Versaw *et al.* (1989) technique using β-naftil caprylate (200 mmol L^{-1}), dissolved in a buffer of Tris-HCl 50 mmol L^{-1} at pH 7.2 as a substrate with a sodium taurocholate solution (100 mmol L^{-1}). The enzyme extract and the substrate were incubated for 15 min and the reaction was stopped with TCA 0.72 N. For development of the activity, fast blue (100 mmol L^{-1}) solution was added clarifying the reaction with an ethanol: ethyl acetate mixture (1:1 v/v), and the absorbance was measured at 540 nm. The lipolytic activity was defined as 1 µg naphthol released per minute.

Calculation of specific activity of individual extracts was determined using the following equations: 1) units mL^{-1} = [Δabs × final reaction volume (mL)] × [CEM × time (min) × extract volume (mL)]; 2) mg protein $units^{-1}$ = [units per mL] × [mg of soluble protein]$^{-1}$; 3) larva $units^{-1}$ = [units per mL] × [number of larvae per mL^{-1}], where: Δabs is the absorbance increase at a determined wavelength; CEM is molar extinction coefficient for the reaction product (mL μg^{-1} cm^{-1}).

The characterization of acid proteases was used following the native PAGE technique according to the technique proposed by Díaz-López *et al.* (1998), using hemoglobin at pH 2 as revealing substrate. Analysis for alkaline isoforms along the initial ontogeny *T. macdonaldi* proteases were performed by discontinuous gels SDS-PAGE zymogram (Laemmli, 1970). The enzyme extracts were mixed with a sample buffer (Tris-HCI 0.5 mol L^{-1} pH 6.8, glycerol, SDS, Bromophenol Blue) at a v/v ratio of 1:1, and 20 mL of extract and sample buffer were loaded in gel wells (8.3×6.1×0.75 cm).

Native continuous zymogram was performed at a concentration of 10% polyacrylamide, while discontinuous gel Zymogrammes were performed at a concentration of a 4% storage gel and 10% separation gel and the run was carried out at 80 V for 15 min and at 100 V for 100 min with a 120 mA resistance, (using Mini-Protean III® Biorad®, California system), 4°C with lauryl (dodecyl) sulphate buffer. Gels were immersed in a 2% casein solution (in Tris-HCI 50 mmol L^{-1} pH 9) and hemoglobin at 0.25% (glycine HCl 100 mmol L^{-1} pH 2) at 4°C for 60 min to allow absorption of casein by the gel, followed by incubation at 37°C for 18 h for proteases to hydrolyze the substrate. The electrophoresis under native conditions was performed with an 80 V carried for 15 min at 120 mA and then at 80 V for 180 min at 24 mA. Incubation of proteases was first performed at 4°C for 30 min and then at 37°C for 90 min. Staining for both procedures was conducted with Coomassie R-250º (Biorad®) brilliant blue, 0.1 g for 100 mL methanol: distilled water: acetic acid (40:50:10 v/v), and it was bleached with a solution of methanol: acetic acid: distilled water (40:10:50 v/v).

For the determination of molecular weight of the alkaline proteases, a low-range molecular weight marker (LRMWM: Sigma, M-3913) was applied to each SDS-PAGE at 5 µL corresponding gel well. The LRMWM contains the following proteins used as markers: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and tripsic soybean inhibitor (20 kDa). The relative electromobility (Rf) was calculated for all Zymogrammes (Igbokwe & Downe, 1978) and molecular weight (MW) of each band with alkaline protease was calculated as the linear fit between Rf and the decimal logarithm of the molecular weights of the proteins used as markers using the Bio-Rad® Quality One 1-D Analyses Software calculating the molecular weight of each band.

Statistical analysis

Enzymatic activity data were analyzed using one-way ANOVA (data previously checked for normality and homogeneity of variance), and the Tukey test was used for multiple comparisons with a significance level of $P < 0.05$. To compare enzymes activities between DPH all measurements were carried out in triplicate. All statistics were conducted using Sigma-Stat 11.0 for Windows (Sigma-Plot® 11.0, USA).

RESULTS

Totoaba macdonaldi showed exponential growth for total length and dry weight from hatching until the end of the study (32 DPH) (Fig. 1).

Ontogeny of enzyme activities

The acid protease activity was observed from 2 DPH keeping a low level of activity until 8 DPH, when an increase in activity was observed that remained high

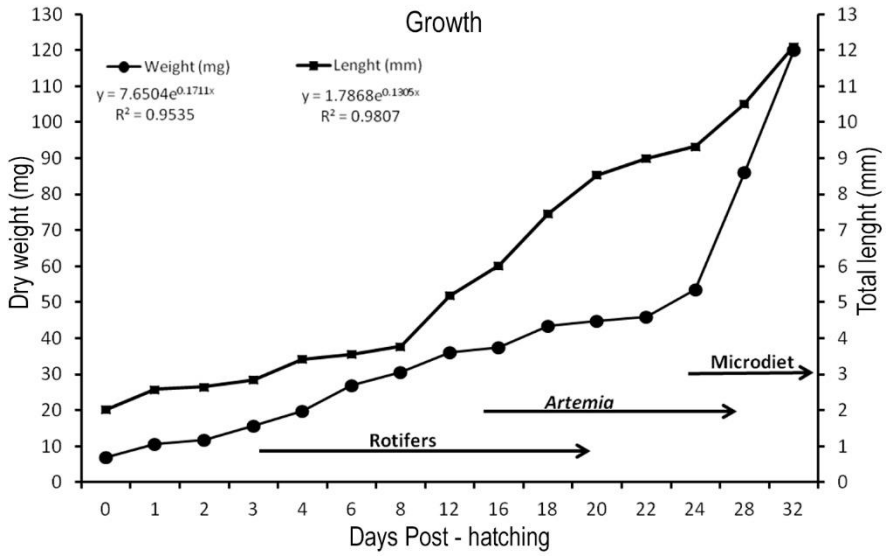

Figure 1. Mean total length and dry weight (three pooled samples of 30 larvae until 18 DPH, 10 larvae until 32 DPH) of *Totoaba macdonaldi* larvae.

until 16 DPH. It was significant from the early days post-hatching (P = 0.003) to decrease on 18 DPH, then increase its activity again on 20 DPH, continuing until 24 DPH, reached peak activity during 28 to 32 DPH. It was significantly higher on these later days than other days post-hatching (P < 0.05) (Fig. 2a).

Alkaline protease activity increased on 2 DPH, decreasing on days 3, 5, 6 and 12 DPH, they rapidly increased at 16 and 18 DPH to reach a statistically high peak at 20 DPH (P < 0.05). At 22, 24 and 28 DPH there was a slight decrease in the values to reach the statistically maximum peak at 32 DAH finally (P < 0.05) (Fig. 2b).

Trypsin activity showed the maximum significant peak on 2 DPH (P < 0.05), decreasing drastically on 3 DPH and increasing slightly on 5 DPH. From 5 DPH, constant activity continued until 22 DPH, decreasing slightly on 24 DPH and increasing its activity again to 28 and 32 DPH (Fig. 3a).

Chymotrypsin activity at baseline (0 DPH) had a low value and gradually increased until it obtained the maximum activity on 6 DPH (P = 0.03). Afterward, activity gradually decreased by 8 and 18 DPH, increased again and reached the second statistically high peak at 20 DPH. It decreased on 22 DPH and finally continually increased on 24, 28 and reach the significantly maximum at 32 DPH (P = 0.03) (Fig. 3b).

Leucine aminopeptidase activity was zero at hatching (0 DPH) and gradually increased by 3 DPH, then decreased at 5 and 6 DPH. Subsequently, activity sharply statistically increased at 8 DPH (P < 0.05) and showed slight fluctuations from 12 to 32 DPH, although

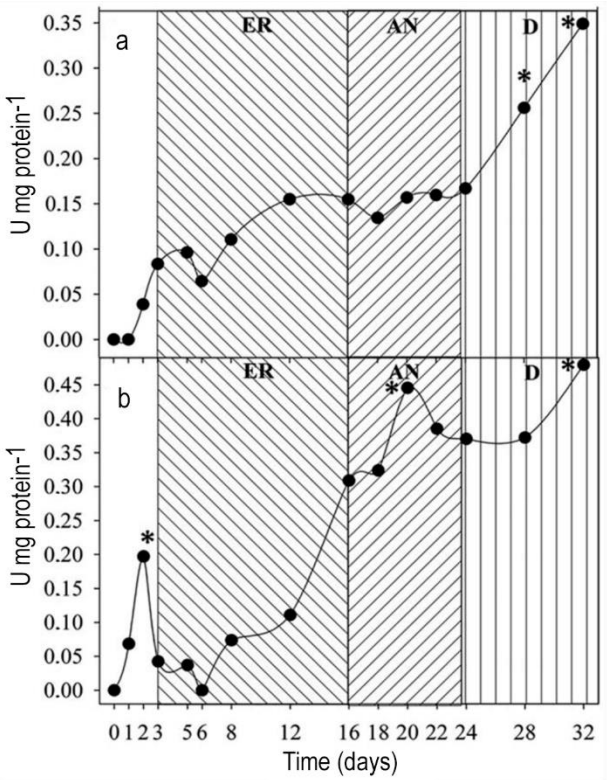

Figure 2. a) Enzymatic activity of acid protease, b) alkaline protease during initial ontogeny *T. macdonaldi* (average, n = 150). ER: enriched rotifers, AN: *Artemia* nauplii, D: microdiet. Asterisks indicate significant differences in activity levels of the two digestive enzymes between DPH.

at 22 DAH there was the highest activity peak (P < 0.05) (Fig. 3c).

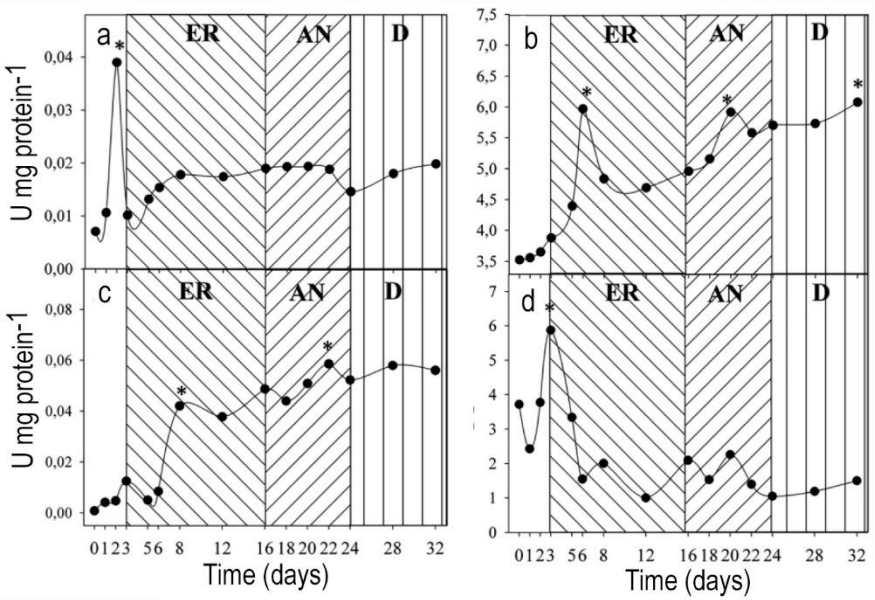

Figure 3. Alkaline digestive enzyme activities. a) Trypsin specific activity, b) chymotrypsin specific activity, c) leucine aminopeptidases specific activity, d) carboxypeptidase A specific activity. ER: enriched rotifers, AN: *Artemia* nauplii, D: microdiet. Asterisks indicate significant differences in activity levels of four digestive enzymes between DPH.

Carboxypeptidase A activity had a high value 0 DPH (embryo), decreasing on 1 DPH, and increasing on 2 DPH (eleuteroembryo) until reaching its maximum value on 3 DPH ($P < 0.05$). Next, the activity decreased quickly on 5 and 6 DPH and continued with light fluctuations from 8 to 32 DPH (Fig. 3d).

Lipase activity showed an increase from 2 DPH ($P < 0.05$), decreasing on 3, 5 and 6 DPH, and increasing again the second and third statistically maximum peaks at 8 and 10 DPH. Then the activity decreased gradually to 22 DPH, then slightly increased on 24 DPH and finally decreased on days 28 and 32 DPH (Fig. 4a).

The α-amylase activity showed only a peak of maximum activity on 2 DPH ($P < 0.05$), then quickly decreased on 3 DPH and maintained low levels of activity for the rest of the culture to 32 DPH (Fig. 4b).

The isoforms analysis using PAGE for acid proteases showed a single isoform (rf 0.72) from 2 DPH, albeit at a low level. This isoform was maintained for 32 DPH, although on 8 DPH, an increase in band intensity was detected (Fig. 5a). Moreover, the SDS-PAGE zymogram for alkaline proteases showed four isoforms (47.3, 43.5, 21.0 and 19.2 kDa) from 1 DPH, and from 8 DPH two more isoforms were detected (77.8 and 17.5 kDa) (Fig. 5b).

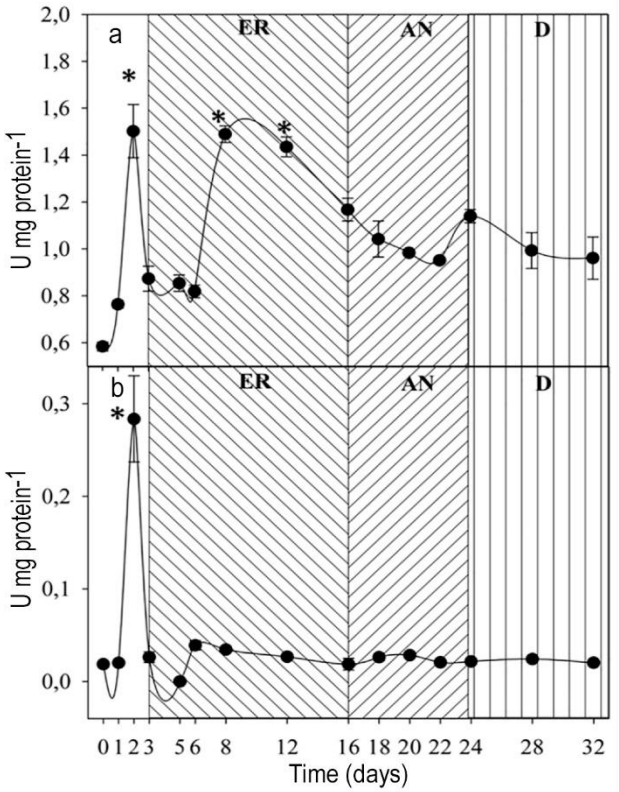

Figure 4. a) Lipase digestive enzyme activity during the initial ontogeny *T. macdonaldi* (Average ± SD, n = 150), b) amylase digestive enzyme activity during the initial ontogeny *T. macdonaldi* (average ± SD, n = 150). (ER) Enriched rotifers, (AN) *Artemia* nauplii, (D) microdiet. Asterisks indicate significant differences in activity levels of the two digestive enzymes between DPH.

DISCUSSION

Digestive proteases have a fundamental role in the hydrolysis of proteins as the key nutrient for feeding all species currently of interest in aquaculture, mainly ma-

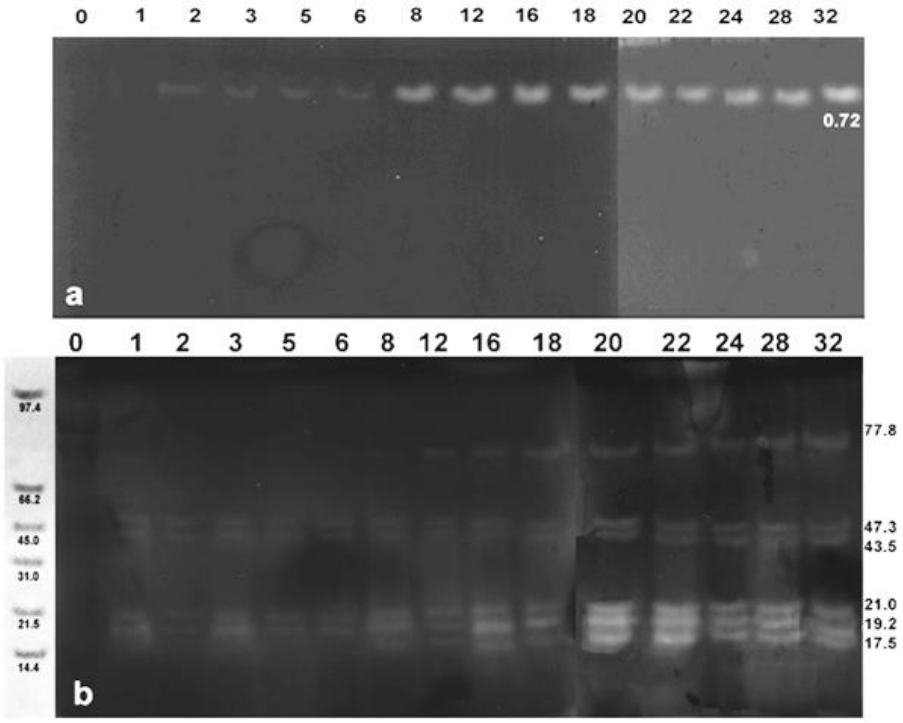

Figure 5. Zymograms a) PAGE of acid proteases, b) SDS-PAGE of alkaline proteases during the initial ontogeny of *T. macdonaldi*. M: Molecular weights marker: rabbit phosphorylase B 97.4 KD, serum bovine albumin 66.2 KD, ovalbumin 42.7 KD, carbonic anhydrase 31.0 KD and lysozyme 14.4 KD; higher numbers indicate days after hatching.

rine and freshwater fish (Moyano, 2006). Since protein in the form of free amino acids is found in higher amounts in the yolk, catabolic processes through the yolk provide free amino acids for tissue growth and as an energy source in addition o the supply of amino acids for protein synthesis, before the first exogenous feeding (Civera-Cerecedo *et al.,* 2004). In the present study, when live feed was replaced by formulated feed for totoaba larvae, the acid protease activity showed the highest enzyme activity between 28 and 32 DPH. In addition, hydrolysis of proteins by the action of acid proteases in the early days of the larvae was practically zero.

In contrast, alkaline proteases were present from hatching, perhaps to harness reserves of the yolk that had to be absorbed and then started exogenous feeding (rotifers). Overall, the digestive system presented very rapid development with a functional stomach, which allows the larvae to digest and process food more efficiently (Jiménez-Martínez *et al.,* 2012; Ahumada-Hernández *et al.,* 2014; Galaviz *et al.,* 2015). Corresponding with our results, similar findings include the larvae of common snook (*Centropomus undecimalis*), where the maximum activity of pepsin was reached between 25 and 36 DPH; and in hurta (*Pagrus auriga*), where the maximum values of activity for acid proteases were achieved between 35 and 40 DPH (Moyano *et al.,* 2005; Jiménez-Martínez *et al.,* 2012). Also, in a recent study with *T. macdonaldi* larvae, it was observed that activity levels of acid proteases start between 24 and 28 DPH, four days after the onset of gastric glands in the stomach (Galaviz *et al.,* 2015), coinciding with the results presented in this study. The activity of acid proteases such as pepsin is indicative of the start of the functionality of the stomach, which serves as the most appropriate time to make the change from feeding live prey to formulated foods (Lazo *et al.,* 2007; Galaviz *et al.,* 2011).

Alkaline protease activity was detected from two days after hatching, reaching a peak between 20 and 32 DPH, when feeding with *Artemia* nauplii and co-feeding with microdiet. Different levels of activities of alkaline proteases may be associated with morpho-physiological changes of the digestive system and annex organs because digestion in the intestine is performed by the action of different products secreted by the intestinal wall or attached organs such as the liver. The liver is one of the first organs to develop because it is involved in the absorption of the yolk sac, while the pancreas contributes in the secretion of proteases and lipases to the intestinal lumen (Lazo, 2000; Uscanga *et al.,* 2011; Jiménez-Martínez *et al.,*

2012). Similar results to those found in the present study were obtained in canane snapper (*Ocyurus chrysurus*), where the enzymatic activity of alkaline proteases started from 5 DPH, while activity of alkaline proteases was detected in species such as Mayan cichlid (*Cichlasoma urophthalmus*) on 13-36 DPH (López-Ramírez *et al.,* 2011; Ahumada-Hernández *et al.,* 2014).

In the present study, trypsin activity became active from 2 DPH, which is the time of opening of the mouth and before the first exogenous feeding. By being fed rotifers, trypsin activity decreased and was held until 32 DPH, which coincided with studies in *C. undecimalis* where the maximum activity was detected from 12 DPH, while in bream (*Pagrus pagrus*), trypsin was detected from 3 DPH (mouth opening day). Similarly, in species such as sea bream (*Dentex dentex*), the activity was detected at the time of hatching with a sharp increase at 3 DPH (Jiménez-Martínez *et al.,* 2012; Suzer *et al.,* 2007; Suzer *et al.,* 2014). In the case of chymotrypsin activity in our study, it increased after absorption of the yolk at 6 DPH during feeding with rotifers, even after trypsin activity. This behavior is primarily because rotifers, as the first live food, provide the larvae with nutrients that can be efficiently utilized.

Further, the late appearance of chymotrypsin seems to occur because the required levels of trypsin increased, in part, to hydrolyze proteins coming from food enabled zymogens of various enzymes, including chymotrypsinogen to be utilized (Jiménez-Martínez *et al.,* 2012). Chymotrypsin activity increased again when fed with *Artemia* nauplii, which might be caused by a compensation of the digestive system which must adapt to the food change. Also, when the intestine increases in size (villi increased) and mature enterocytes (microvilli increase) intensify, enzyme synthesis is required to optimize the protein hydrolysis. This process involves the amino acids such as tyrosine, phenylalanine or tryptophan to anchor the active site and carry out the breakdown of the peptide bond (Zambonino-Infante & Cahu, 2001). Consequently, since *Artemia* is the last living food provided to the larvae before cessation, it must be supplied at the right time and in adequate quantity and quality. If the timing, quantity, and quality are not considered, the protein cannot optimally be hydrolyzed, or, if hydrolyzed, will not be absorbed (Civera-Cerecedo *et al.,* 2004).

Interestingly, studies in *P. pagrus* larvae found that chymotrypsin activity was detected at 3 DPH, the day of the opening of the mouth, which is consistent with this study; however, in larvae of *C. undecimalis*, maximum chymotrypsin activity was detected between 25 and 34 DPH. This variation indicates that the appearance of chymotrypsin is differential: it is mainly related to the presence of trypsin and to maximizing activity exopeptidases when the digestive system has matured completely (Suzer *et al.,* 2007; Jiménez-Martínez *et al.,* 2012). Similar results to the present study were observed in larvae of *T. macdonaldi* where the activity of proteases such as trypsin and chymotrypsin were detected on 1 DPH, reaching a significant level of activity at 12 DPH, the moment at which the food supply switched from rotifer to *Artemia* nauplii (Galaviz *et al.,* 2015). Considering the above, most marine fish larvae including *T. macdonaldi* of indirect ontogeny at the time of hatching and the first days of life lack a functional stomach, so their intestinal tracts develop in a progressive manner (Zambonino-Infante & Cahu 2001).

In this study, the activity of leucine aminopeptidase showed activity at 3 DPH, with a maximum increase of enzymes at 22 DPH when they were fed *Artemia* nauplii. In contrast, studies in tahuina cichlid (*Cichlasoma trimaculatum*) showed activity from the moment of larvae hatching. In tenguayaca crappie (*Petenia splendida*), the activity of leucine aminopeptidase presented from the beginning of larviculture 0 DPH, reaching its peak between 20 and 40 DPH (Uscanga *et al.,* 2011; Toledo-Solís *et al.,* 2014). At the same time, the activity of carboxypeptidase A showed an increase at 3 DPH when being fed rotifers. In studies performed with *C. undecimalis*, an increase in carboxypeptidase A between 2 and 7 DPH was observed; in contrast, in *C. urophthalmus*, an increase occurred between 5 and 6 DPH (López-Ramírez *et al.,* 2011; Jiménez-Martínez *et al.,* 2012). Both the L-aminopeptidase and carboxy-peptidase A are considered exopeptidases produced in the acinar cells of the pancreas. The pancreas has action in the lumen to carry out the release of amino acids from the amino group and carboxyl-terminal peptides that hydrolyze. This implies that at the time the exopeptidases presence is detected, they actively participate in the hydrolysis of proteins, and by being parietal enzymes, they support nutrient uptake (Moyano 2006; Lazo *et al.,* 2007; Jiménez-Martínez *et al.,* 2012).

Lipase activity was detected at 2 DPH. This enzyme is related to the absorption of the yolk sac. It is rich in lipids and fatty acids; however, a decrease in enzyme activity of the larvae was seen when being fed rotifers, which has been detected in larvae of *C. undecimalis* where lipase activity appeared from the 3 DPH, compared with detected results in common carp (*Carassius auratus auratus*) which was detected on 34 DPH (Jiménez- Martínez *et al.,* 2012; Süzer *et al.,* 2014). Lipids have suitable fatty acids which are the most important reserve in the fish embryo's energy

since they are a crucial source of energy in the development of larvae (Zambonino-Infante & Cahu 2001). Further, lipids provide essential fatty acids and phospholipids needed for the formation of membranes of cells during growth. They must be emulsified by bile salts to facilitate their digestion; thus, lipases acting on lipid substrates release fatty acids which are absorbed by cells in the wall of the foregut and resynthesized intracellularly before being transported to the liver. The above is performed by two types of lipases: the non-specific neutral lipase which is activated by bile salts and the specific pancreatic lipase which is activated by co-lipase and bile salts (Lazo, 2000). It is likely that the lipid metabolism decreases affect the rate of growth and development of larvae in culture, so that when the level of lipids or the type of fatty acids is not adequate, they may accumulate in the liver and mesenteric tissues cavity. This accumulation consequently reduces the nutritional quality, which results in increased oxidation of tissues adversely affecting the health of the larvae (Álvarez-González *et al.*, 2008).

The activity of the α-amylase in totoaba larvae increased on 2 DPH (embryonic period) just before providing rotifers, which is similar to that observed in other marine fish larvae, where high activity has been reported before hatching and yolk absorption, and a decrease in activity is presented when starting feeding rotifers (Ahumada *et al.*, 2014). Similarly, in a study performed on larvae of *C. undecimalis*, the maximum activity is shown between 5 and 12 DPH (Jiménez-Martínez *et al.*, 2012), with a peak reported before hatching and during absorption of the yolk. As reported, the α-amylase is a digestive enzyme influenced by age, so its activity is genetically programmed to take advantage of yolk glycogen and its decrease occurs automatically a few days after the start of exogenous feeding. This pattern seems to occur because of the amount of glycogen present in the reservoirs, while important, does not represent the fundamental energy needed to provide the larva with its metabolic processes nutrients (Zambonino-Infante & Cahu, 2001). Thus, glycogen is the most important polysaccharide during development in the embryonic period as a source of energy allowing, at the same time, the rapid development of fatty acids during early stages, which are present from fertilization to hatch (Civera-Cerecedo *et al.*, 2004). The activity of α-amylase tends to remain at relatively low levels, once exogenous feeding is started, which makes carbohydrates from live food (Jiménez-Martínez *et al.*, 2012).

In the present study, the overall analysis of isoforms used for acid proteases PAGE showed a single isoform (Rf 0.74), which has been found in species such as *P. splendida* where the appearance of an isoform (Rf 0.65) was detected; in grass carp (grass carp) a single band (28.5 kDa) was detected; in *Coryphaenoides pectoralis* two bands were detected (35 y 31 kDa); in *Paralabrax maculatofasciatus* one band was detected (Rf 0.72); and in *C. undecimalis* two isoforms were detected (Rf 0.32 and 0.51) (Klomklao *et al.*, 2007; Álvarez-González *et al.*, 2010; Uscanga *et al.*, 2011; Jiménez-Martínez *et al*., 2012). Although isoforms were detected early at 2 DPH, the zymogram and the colorimetric technique does not necessarily mean that it is pepsin. In our study, the homogenate of the whole body of the larva was performed, which could cause cathepsins to be detected, and although they are aspartic proteases dedicated to protein hydrolysis, they perform it intracellularly, so they do not belong to the group of digestive proteases (Moyano *et al.*, 2013). Further, concerning zymogram of alkaline proteases for *T. macdonaldi*, the larvae showed early onset (1 DPH) of four isoforms (47.3, 43.5, 21.0 and 19.2 kDa). These may correspond to the protease's trypsin and chymotrypsin, which were detected before the detection of enzymatic activities levels by biochemical analyzes.

Additionally, the appearance of two other isoforms was detected (77.8 and 17.5 kDa) from 8 DPH, which may be of the trypsin and aminopeptidase type. In this regard, our results are like those detected for *C. undecimalis* and *P. maculatofasciatus*, where the early presence of two isoforms was detected 21.1-21.4 and 51.6 kDa; in *P. maculatofasciatus* two isoforms of 23.8 and 21.8 were detected; and *Sparus aurata* presented 5 isoforms of 24.5 at 90 kDa (Moyano *et al.*, 2005; Álvarez-González *et al.*, 2010; Jiménez-Martínez *et al*., 2012). Thus, importantly, the presence of only six isoforms indicates that *T. macdonaldi* is a typical carnivore species. Apart from the early onset of alkaline proteases, this species shows the ability to hydrolyze proteins from live foods, which is consistent with what has been observed for *P. maculatofasciatus* (Álvarez-González *et al.*, 2010). This capacity to use nutrients from live foods is reflected in accelerated growth which is undoubtedly a desirable feature for the development of their larval culture. It is further indicated by the pepsin increase that occurred on 12 DPH which could indicate that this species has the potential to perform co-feed studies from an early age and see the possibility of bringing forward the time of weaning.

In this study, the enzyme activities of alkaline proteases, lipases, and amylases were detected at an early age. Fluctuations presented in these activities could be associated with morphophysiological changes of the digestive system, like changes in food. Perhaps that explains why the activity of acid protease (pepsin) showed higher enzyme activity between 28 and 32

DPH when live feed was replaced by formulated feed. At this time, the larvae had a fully-developed digestive system suggest that this age is appropriate to initiate the weaning process in this species.

ACKNOWLEDGMENTS

This work was partially supported by the Universidad Autónoma de Baja California (UABC, 401-5-C-91-170379) México, the National Council for Science and Technology (CONACyT) of México (INFR254946) and sectoral fund SAGARPA-CONACYT 2017-04-291837 no. 247698. The authors are grateful to M.C. Gerardo Sandoval Garibaldi for the assistance with the development of larvae rearing.

REFERENCES

Ahumada-Hernández, R.I., Alvarez-González, C.A., Guerrero-Zárate, R., Martínez-García, R., Camarillo-Coop, S., Sánchez-Zamora, A., Gaxiola-Cortés, M.G., Palomino-Albarrán, I.G., Tovar-Ramírez, D. & Gisbert, E. 2014. Changes of digestive enzymatic activity on yellowtail snapper (*Ocyurus chrysurus*) during initial ontogeny. International Journal of Biology, 6(4): 110-118.

Alarcón-López F.J. & Martínez-Díaz, M.I. 1998. Fisiología de la digestión en larvas de peces marinos y sus aplicaciones al cultivo larvario en masa. Revista AquaTIC, 5 pp.

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

Álvarez-González, C.A., Moyano-López, F.J., Civera-Cerecedo, R., Carrasco-Chávez, V., Ortiz-Galindo, J.L. & Dumas, S. 2008. Development of digestive enzyme activity in larvae of spotted sand bass *Paralabrax maculatofasciatus*. 1. Biochemical analysis. Fish Physiology and Biochemistry, 34: 373-384.

Álvarez-González, C.A., Moyano-López, F.J., Civera-Cerecedo, R., Carrasco-Chávez, V., Ortiz-Galindo, J.L., Nolasco-Soria, H., Tovar-Ramírez, D. & Dumas, S. 2010. Development of digestive enzyme activity in larvae of spotted sand bass *Paralabrax maculato-fasciatus* II: Electrophoretic analysis. Fish Physiology and Biochemistry, 36: 29-37.

Anson, M.L. 1938. The estimation of pepsin, trypsin, and cathepsin with hemoglobin. Journal of General Physiology, 22: 79-89.

Bobadilla, M., Álvarez-Borrego, S., Ávila-Foucat, S., Lara-Valencia, F. & Espejel, I. 2011. Evolution of environmental policy instruments implemented for the protection of totoaba and the vaquita porpoise in the Upper Gulf of California. Environmental Science & Policy, 14: 998-1007.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Civera-Cerecedo, R., Álvarez-Gonzáles, C.A. & Moyano-López, F.J. 2004. Nutrición y alimentación de larvas de peces marinos. In: Cruz, L.E., Ricque, D., Nieto, M.G., Villareal, D., Scholz, U. & González, M. (Eds.). Avances en nutrición acuícola VII. Memorias del VII Simposium Internacional de Nutrición Acuícola. 16-19 noviembre, 2004. Hermosillo, Sonora, México.

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

Del Mar, E.G., Largman, C., Brodrick, J. & Geokas, M. 1961. A sensitive new substrate for chymotrypsin. Analytical Biochemistry, 99: 316-20.

Díaz-López M., Moyano, F.J., Alarcón, F.J., García-Carreño, F.L. & Navarrete del Toro, M.A. 1998. Characterization of fish acid proteases by substrate-gel electrophoresis. Comparative Biochemistry & Physiology B, 121(4): 369-377.

Erlanger, B., Kokowsky, N. & Cohen, W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. Archives of Biochemistry and Biphysics, 95: 271-278.

Folk, J. & Schirmer, E. 1963. The porcine pancreatic carboxypeptidase A system. I. Three forms of the active enzyme. Journal of Biological Chemistry, 238: 38-84.

Frías-Quintana, C.A., Domínguez, J., Álvarez-González, C.A., Tovar-Ramírez, D. & Martínez-García, R. 2016. Using cornstarch in microparticulate diets for larvicultured tropical gar (*Atractosteus tropicus*). Fish Physiology and Biochemistry, 42(2): 517-528.

Galaviz, M., García-Ortega, A., Gisbert, E., López, L.M. & García-Gasca, A. 2012. Expression and activity of trypsin and pepsin during larval development of the spotted rose snapper (*Lutjanus guttatus*). Comparative Biochemistry & Physiology B, 161(1): 9-16.

Galaviz, M.A., García-Gasca, A., Drawbridge, M., Álvarez-González, C.A. & López, L.M. 2011. Ontogeny of the digestive tract and enzymatic activity in white seabass, *Atractoscion nobilis*, larvae. Aquaculture, 318: 162-168.

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

Igbokwe, E.C. & Downe, A.E.R. 1978. Electrophoretic and histochemical comparison of three strains of *Aedes aegypti*. Comparative Biochemistry & Physiology B, 60: 131-136.

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

Kjørsvik, E., Hoehne-Reitan, K. & Reitan, K.I. 1990. Egg and larval quality criteria as predictive measures for juvenile production in turbot (*Scophthalmus maximus* L.) Aquaculture, 227: 9-20.

Klomklao, S., Kishimura, H., Yabe, M. & Benjakul, S. 2007. Purification and characterization of two pepsins from the stomach of pectoral rattail (*Coryphaenoides pectoralis*). Comparative Biochemistry and Physiology B, 147(4): 682-689.

Kunitz, M. 1947. Crystalline soybean trypsin inhibitor II. General properties. Journal of General Physiology, 30: 291-310.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Lazo, J. 2000. Conocimiento actual y nuevas perspectivas en el desarrollo de dietas para larvas de peces marinos. In: Cruz, L., Ricque, D., Tapia, M., Nieto, M., Villareal, D., Puello, A. & García, A. (Eds.). Avances en nutrición acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola. 19-22 noviembre, 2000. Mérida, Yucatán, México.

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

López-Ramírez, G., Cuenca-Soria, C.A., Álvarez-González, C.A., Tovar-Ramírez, D., Ortiz-Galindo, J.L., Perales-García, N., Márquez-Couturier, G., Arias-Rodríguez, L., Indy, J.R., Contreras-Sánchez, W.M., Gisbert, E. & Moyano, F.J. 2011. Development of digestive enzymes in larvae of mayan cichlid *Cichlasoma urophthalmus*. Fish Physiology and Biochemistry, 37: 197-208.

Maraux, S., Louvard, D. & Baratti, J. 1973. Then aminopeptidase from hog-intestinal brush border. Biochimica et Biophysica Acta, 321: 282-295.

Mata-Sotres, J.A., Lazo, J.P. & Barón-Sevilla, B. 2015. Effect of age on weaning success in totoaba (*Totoaba macdonaldi*) larval culture. Aquaculture, 437: 292-296.

Moyano, F.J. 2006. Bioquímica digestiva en especies acuicultivadas: aplicaciones en nutrición. In: Cruz, L., Ricque, D., Tapia, M., Nieto, M., Villareal, D., Puello, A. & García, A. (Eds.). Avances en Nutrición Acuícola VII. VIII Simposium Internacional de Nutrición Acuícola. 15-17 noviembre. Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, México.

Moyano, F.J., Barros, A.M., Prieto, A., Cañavate, J.P. & Cárdenas, S. 2005. Evaluación de la ontogenia de enzimas digestivas en larvas de hurta, *Pagrus auriga* (Pisces: Sparidae). Revista AquaTIC, 22: 39-47.

Murray, H.M., Gallant, J.W., Johnson, S.C. & Douglas, S.E. 2006. Cloning and expression analysis of three digestive enzymes from Atlantic halibut *(Hippoglossus hippoglossus*) during early development: predicting gastrointestinal functionality. Aquaculture, 252: 394-408.

Robyt, J.F. & Whelan, W.J. 1968. Starch and its derivates. In: Radley, J.A. (Ed.). Chapman and Hall, London, pp. 430-476.

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

Suzer, C., Coban, D., Yildirim, S., Hekimoglu, M., Kamaci, H.O., Firat, K. & Saka, S. 2014. Stage-specific ontogeny of digestive enzymes in the cultured common dentex (*Dentex dentex*) larvae. Turkish Journal of Fisheries and Aquatic Sciences, 14: 759-768.

Suzer, G., Kamaci, H.O., Coban, D., Saka, S., Firat, K., Ozkara, B. & Ozkara, A. 2007. Digestive enzyme activity of the red porgy (*Pagrus pagrus*, L.) during larval development under culture conditions. Aquaculture Research, 38: 1778-1785.

Toleto-Solís, F.J., Uscanga-Martínez, A., Guerrero-Zárate, R., Márquez-Couturier, G., Martínez-García, R., Camarillo-Coop, S., Perales-García, N., Rodríguez-Valencia, W., Gómez-Gómez, M.A. & Álvarez-González, C.A. 2014. Changes on digestive enzymes during initial ontogeny in the three-spot cichlid *Cichlasoma trimaculatum*. Fish Physiology and Biochemistry, 41: 267-279.

True, C.D. 2012. Desarrollo de la biotecnia de cultivo de *Totoaba macdonaldi*. Tesis de Doctorado, Universidad Autónoma de Baja California.

Uscanga-Martínez, A., Perales-García, N., Álvarez-González, C.A., Moyano, F.J., Tovar-Ramírez, D., Gisbert, G.E., Márquez-Couturier, G., Contreras-Sánchez, W.M., Arias-Rodríguez, L. & Indy, J.R. 2011. Changes in digestive enzyme activity during initial ontogeny of bay snook *Petenia splendida*. Fish Physiology and Biochemistry, 37: 667-680.

Versaw, W., Cuppett, S.L., Winters, D.D. & Williams, L.E. 1989. An improved colorimetric assay for bacterial lipase in nonfat dry milk. Journal of Food Science, 54: 232-254.

Received: 14 November 2017; Accepted: 20 August 2018

Walter, H.E. 1984. Proteinases: methods with hemoglobin, casein and azocoll as substrates. In: Bergmeyern, H.J. (Ed.). Methods of enzymatic analysis. Verlag Chemie, Weinham, pp. 270- 458.

Zambonino-Infante, J.L. & Cahu, C.L. 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. Comparative Biochemistry and Physiology C, 130(4): 477-487.