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Original article

Changes in digestive enzymes activities during the initial ontogeny of wolf cichlid, *Parachromis dovii* (Perciformes: Cichlidae)

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Wolf cichlid, *Parachromis dovii*, is a species with a high potential for aquaculture in Central America; however, the knowledge of the digestive physiology in larvae period is limited. For these reason, this study evaluated the changes on digestive enzymes (alkaline and acid proteases, trypsin, chymotrypsin, aminopeptidase, carboxypeptidase, lipases, amylases, and phosphatases) during early ontogeny by biochemical analysis. All digestive enzymes were detected at first feeding (6 days after hatching, DAH, 9.49 mm, 168 degree-days DD). Afterwards all enzymes reached two main peaks in activity at 14 or 22 DAH (15.10 mm, 364 DD and 20.83 mm, 550 DD, respectively). Later, there was a gradual decrease in activity for trypsin and acid and alkaline phosphatases until reach the lowest values at 41 DAH. In the case of acid proteases, chymotrypsin, aminopeptidase, carboxypeptidase, lipase and amylase, all activities reached their maximum values at the end of the larval period, except for alkaline proteases, which showed the maximum value at 14 DAH (15.10 mm, 364 DD). *Parachromis dovii* larvae have an early capability to hydrolyze exogenous food, agreeing with other carnivorous neotropical cichlid species, for this reason we proposed that the weaning process could begin at 14 DAH.

Keywords: Larviculture, Proteases, Zymogram.

El guapote lagunero (*Parachromis dovii*) es una especie con un alto potencial para la acuicultura en la región de América Central; sin embargo, existe un conocimiento limitado sobre la capacidad digestiva en el periodo larval. Por este motivo, este estudio evaluó los cambios de las enzimas digestivas (proteasas alcalinas y ácidas, tripsina, quimotripsina, aminopeptidasa, carboxipeptidasa, lipasas, amilasas y fosfatasas) durante la ontogenia temprana mediante análisis bioquímico. Todas las enzimas digestivas analizadas se detectaron en la primera alimentación (6 días después de la eclosión, DAH, 9.49 mm, 168 día-grados DD). Después, todas las enzimas alcanzaron dos picos máximos a los 14 o 22 DAH (15.10 mm, 364 DD and 20.83 mm, 550 DD, respectivamente). Después las actividades tripsina, fosfatasas ácidas y alcalina disminuyeron a sus valores más bajos a los 41 DAH. En el caso de las proteasas ácidas y alcalinas, quimotripsina, aminopeptidasa, carboxipeptidasa, lipasa y amilasa, los niveles de actividad aumentaron y alcanzaron su máximo valor al final del período larvario, excepto las proteasas alcalinas, que mostraron su máximo valor a los 14 DAH (15.10 mm, 364 DD). Las larvas de *P. dovii* tienen una capacidad temprana para hidrolizar alimentos exógenos, lo que concuerda con otras especies de cíclidos neotropicales carnívoros, por lo que proponemos que el proceso de destete inicie a los 14 DAH.

Palavras-clave: Larvicultura, Proteasas, Zimograma.

Introduction

The Cichlidae family is composed of about 1,000 species and is widely distributed around the tropics. It has showed a remarkable commercial importance for fisheries, sport fishing and aquaculture (Luna-Figueroa, Figueroa-Torres, 2000). In Costa Rica and in most Central America countries, one of the most important species is the Wolf cichlid, guapote lagunero (*Parachromis dovii* Günther, 1864), which has a tasty flesh, good performance in earthen pond culture systems and in addition, it is used as biological control for undesired reproduction in tilapia culture (Nonell, Rojas, 1995; Hernández, 1992; Barrera, Paz, 2006). However, this species still has some production problems (*e.g*. low growth rate) related to malnutrition especially at early stages; which had affected negatively its production at commercial scale.

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For this reason, the knowledge of its digestive physiology is essential for maximizing its growth by knowing the fish capability to metabolize protein sources needed for tissue synthesis (Kolkovski, 2001). Further, studies on the digestive system development and enzymatic capacity should bring information to understand the nutritional status, the digestive enzyme activity and related processes to generate better strategies for control and improvement of survival at the earlier stages (larvae) (Moyano, 2006; Zambonino-Infante, Cahu, 2007).

In the past, several studies characterizing some digestive enzymes has been done with cichlids of commercial importance; Nile tilapia, *Oreochromis niloticus* (Linnaeus) (Tengjaroenkul *et al.*, 2002), blue disc, *Symphysodon aequifasciatus* (Pellegrin) (Chong *et al.*, 2002), Mayan cichlid, *Mayaheros urophthalmus* (Günther) (López-Ramírez *et al.*, 2011), bay snook, *Petenia splendida* (Günther) (Uscanga-Martínez *et al.*, 2011), three-spot cichlid, *Amphilophus trimaculatus* (Günther) (Toledo-Solís *et al.*, 2015), and other fish species such as sobaity sea bream, *Sparidentex hasta* (Valenciennes) (Nazemroaya *et al.*, 2015), zebrafish, *Danio rerio* (Hamilton) (Guerrera *et al.*, 2015), Caspian kutum, *Rutilus kutum* (Kamensky) (Khosravi Bakhtiarvandi, Abedian-Kenari, 2015), golden pompano, *Trachinotus ovatus* (Linnaeus) (Ma *et al*., 2015), thick-lipped grey mullet, orange-spotted grouper, *Epinephelus coioides* (Hamilton) (Guo *et al.*, 2016), starry flounder, *Platichthys stellatus* (Pallas) (Song *et al.*, 2016), *Chelon labrosus* (Risso) (Pujante *et al*., 2017), turbot, *Scophthalmus maximus* (Linnaeus) (Tong *et al*., 2017), crimson snapper, *Lutjanus erythropterus* (Bloch) (Cui *et al*., 2017), American shad, *Alosa sapidissima* (Wilson) (Xiao-Qiang *et al.*, 2017), clown knifefish*Chitala chitala* (Hamilton) (Mitra *et al.*, 2017), and barbel chub, *Squaliobarbus curriculus* (Richardson) (Shen *et al*., 2018).

These studies have allowed the understanding of digestive enzymes variability, which reflects changes in fish morpho-physiology and feed use during larvae period. Specifically, for the *P. dovii*, in Costa Rica only one study has been done related to the effect of feed on growth and proteolytic digestive activity in larvae (Quirós-Orlich *et al.*, 2014), evaluating the live feed substitution (*Artemia* Leach nauplii) by an inert diet. Further, studies related to the digestive physiology of wolf cichlid are needed to improve its growth by using specific diets, before to recommend its commercial scale culture. For this reason, this research focused on the digestive proteases, lipases and amylases of *P. dovii* during early ontogeny.

Material and methods

Larvae and sampling. The larvae were obtained from broodstock of *P. dovii* (1 male: 1 female) (adult specimens are included in Ichthyological collection ROM 84151), at the hatchery unit of the Escuela de Ciencias Biológicas of Universidad Nacional de Costa Rica, maintained in fiberglass tanks with a water recirculation system by air flow. Larvae were obtained from spontaneous spawns and after hatching; all were transferred and randomly distributed into aquaria (11 L each, 23 larvae L^{-1}) of a recirculation system. The water temperature was kept at 25-29 °C, dissolved oxygen at 6.0 g L^{-1} minimal and NH_3 lower than 0.06 g L^{-1}.

Larvae were fed to satiety with *Artemia* nauplii (Great Salt Lake, Artemia International®) four times daily (9:00, 11:30, 14:30 and 17:00 h) from the beginning of exogenous feeding (6 day after hatching, DAH) until the end of the larval period (30 DAH). The larvae were fed with a pelletized formulated feed SilverCup® (45% protein and 10% lipids) at the same larvae feeding frequency.After the programmed feedings, the surplus food was cleaned by suction with a hose to avoid excess nutrients and the subsequent contamination of the water.

Growth and survival. The specific growth rate (SGR) for weight and total length was calculated at the end of the trial by the formula: $SGR = (\ln W_f - \ln W_i / t_2 - t_1) * 100$ (% body length day^{-1} or % body weight day^{-1}), where: ln: natural logarithm, W_f: final length or weight (mm or mg), W_i: initial length or weight (mm or mg), t_2: final day, t_1: initial day. Survival rates were determined by counting remaining larvae at the end of the experiment and it was corrected for number of sampled larvae.

Degree-days calculation. For the determination of the degrees-days: DD = [Tmax-Tmin/2]-To, where Tmax and Tmin are the maximum and minimum daily ambient temperature, respectively, and To is the temperature below which growth or development does not occur (often referred to as the base or threshold temperature, Hazel, Prosser, 1974; Sharpe, DeMichele, 1977) multiplying for the number of days after hatching (DAH).

Sampling technique. Samples of 30 larvae were randomly taken on days: 6 (9.49 mm, 168 DD, 0.056 mm.ºC.days), 14 (15.10 mm, 364 DD, 0.041 mm.ºC.days), 22 (20.83 mm, 550 DD, 0.057 mm.ºC.days), 30 (23.42 mm, 810 DD, 0.028 mm.ºC.days) and 41 (27.66 mm, 1189 DD, 0.023 mm.ºC.days) DAH and DD respectively before feeding in the morning. Larvae were anesthetized until dead with an overdose of tricaine metansulfonate (MS-222, 0.15 mg L^{-1}), measured with a stereoscopy (Olympus 329345 with ocular micrometer: ±0.07 mm) and a caliper (±0.01 mm), and weighted with an analytical balance (Sartorius 2492 ± 0.1 mg). The growth in total length and weight was calculated as specific growth rate (SGR) and survival was monitored daily. Finally, larvae were washed with distilled water, frozen at -20 °C and lyophilized for further biochemical analysis.

Preparation of multi-enzymatic extracts. The viscera bulk was removed from each larva in cold conditions (kept on plates maintained in ice), by cutting off the tail, head and dorsal part of the body. Samples from the smallest larvae were taken by removing their head and tail. Viscera were split in two sections, to analyze stomach and intestine en-

zymatic activity separately. Samples for alkaline enzymes determinations were homogenized with buffer 50 mmol L^{-1} Tris-HCl, $CaCl_2$ 20 mmol L^{-1}, pH 7.5 (35 mg mL^{-1}) and for acid enzymes determinations with buffer 100 mmol L^{-1} glycine-HCl, pH 2.0. Next, samples were centrifuged at 16 000 g, 4 °C for 30 min (Hettich Mikro 200). The supernatant was kept at -20 °C f or further enzymatic analysis.

Biochemical analysis. The soluble protein concentration was determined by Bradford (1976), using serum bovine albumin as standard. Total alkaline proteolytic activity was measured according to Walter (1984), using casein (0.5%) as substrate in a Tris-HCl 50 mmol L^{-1}, pH 9.0 buffer solution. Total acid proteolytic activity was determined following Anson (1938), using hemoglobin (0.5%) in a glycine-HCl 100 mmol L^{-1}, pH 2.0 buffer solution. One unit of enzymatic activity was defined as 1 μg tyrosine released per minute, using the 0.005 molar extinction coefficient at 280 nm.

Trypsin activity was done according to Erlanger *et al*. (1961), using as substrate BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) dissolved in dimetilsulfoxide (DMSO) 10 mmol L^{-1} and Tris-HCl 50 mmol L^{-1}, $CaCl_2$ 10 mmol L^{-1}, pH 8.2 buffer solution. Chymotrypsin activity was measured following Del Mar *et al.* (1979), with SAAPNA (N-succinyl-ala-ala-pro-phe p-nitroanilide) as specific substrate dissolved in DMSO 10 mmol L^{-1} and Tris-HCl 100 mmol L^{-1}, 1 mmol L^{-1} $CaCl_2$, pH 7.8 buffer solution. The activity of leucine-aminopeptidase was evaluated with the protocol of Maroux *et al.* (1973), using leucine *p*-nitroanilide (0.1 mmol L^{-1} dissolved in DMSO) as substrate and a sodium phosphate 50 mmol L^{-1}, pH 7.2 buffer solution. For these proteases, one unit of enzymatic activity was defined as 1μg of nitroanilide released per minute; using the following molar extinction coefficients: 8.8 for trypsin at 410 nm, 0.962 for chymotrypsin at 405 nm and 8.2 for leucine-aminopeptidase at 410 nm. The activity of carboxypeptidase A was evaluated according to Folk, Schirmer (1963), using hipuryl-L-phenylalanine 1 mmol L^{-1} as substrate in Tris-HCl 25 mmol L^{-1}, NaCl 50 mmol L^{-1}, pH 7.5 buffer solution. One unit of enzymatic activity was defined as 1 μg of hipuryl released per minute, using a 0.36 molar extinction coefficient at 254 nm.

The α-amylase analyses was realized following Somoyi-Nelson procedure (described by Robyt, Whelan, 1968) using soluble starch (2%) as substrate in a phosphate-citrate 100 mmol L^{-1}, NaCl 50 mmol L^{-1} pH 7.5 buffer solution. One unit of enzymatic activity was defined as 1μg of maltose released per minute, using a 0.0034 molar extinction coefficient at 600 nm. Lipase activity was quantified as described by Versaw *et al.* (1989), using β-naphthyl caprylate (200 mmol L^{-1}) as substrate, Tris-HCl 50 mmol L^{-1} pH 7.2 buffer solution and sodium taurocholate (100 mmol L^{-1}). A unit of enzymatic activity was defined as 1 μg of naphthyl released per minute, using a 0.02 molar extinction coefficient at 540 nm.

The acid and alkaline phosphatase activities were measured according to Bergmeyer (1974), incubating the extracts for the acid and alkaline phosphatase with 4-nitrophenyl phosphate 2% in citric/citrate (1:1 W/W) pH 5.5 and glycine-NaOH 50 mmol L^{-1} pH 10.1 buffer solutions, respectively. A unit of enzymatic activity was defined as 1μg of naphthyl released per minute, using a 0.0185 molar extinction coefficient at 405 nm.

Enzyme activity was calculated, by triplicate, following this equation: Total activity (Units mL^{-1}) = [Δabs*reaction final volume (mL)]/[MEC*time (min)*extract volume (mL)]. Specific activity (Units mg $prot^{-1}$) = Total activity/soluble protein (mg), where Δabs represent the increase in absorbance, and MEC represents the respective molar extinction coefficient.

Zymogram analysis. The classification of proteases was obtained by SDS-PAGE electrophoresis with discontinuous gels for alkaline proteases (Laemmli, 1970; García-Carreño *et al.*, 1993). The enzymatic extracts were mixed with the sample buffer (Tris-HCl 50 mmol L^{-1}, pH 6.8, glycerol, SDS, bromophenol blue) at a v/v ratio of 1:1, and 20 μL of this mixture was applied on the gel wells (8.3 cm 9 6.1 cm 9 0.75 cm). The discontinuous zymograms consisted of a storage gel at 4% and a separating gel at 10%. The gel was equilibrated at 80 V for 15 min, and the electrophoresis was done at 100 V and 120 mA for 100 min (Mini Protean III BIORAD Laboratories, CA, USA). The gels were submerged in a 2% casein solution in Tris-HCl 50 mmol L^{-1}, pH 9 at 4°C for 60 min to allow the gels to absorb the casein and then incubated at 37 °C during 18 h to allow the substrate hydrolysis. After development of enzyme activity, gels were stained by using the same Coomassie brilliant blue R-250 solution.

Reference front (Rf) and molecular weight calculations. A low molecular weight marker (LRMWM) from Pharmacia Biotech (Uppsala, Sweden) was applied to each SDS-PAGE adding 5 μl well. The LRMWM contained the following proteins: 1) phosphorylase b (97 kDa), 2) serum bovine albumin (66 kDa), 3) egg albumin (45 kDa), 4) carbonic anhydrase (29 kDa), 5) trypsinogen (24 kDa), and 6) soybean trypsin inhibitor (20 kDa). The relative electromobility (Rf) for all zymograms was calculated according to Igbokwe, Downe (1978), and the molecular weight (MW) of each band with alkaline protease activity was calculated as the linear fit between the Rf and the decimal logarithm of the molecular weights of the proteins used as markers, using the software Quality One V 4.6.5 (Hercules, CA, USA).

Results

At first feeding, *P. dovii* larvae weighed 7.56±0.39 mg and measured 9.49±0.28 mm (6 DAH, 168 DD, 0.056 mm.ºC.days), reaching 268.66±25.44 mg and 27.66±1.06 mm (41 DAH, 1189 DD, 0.023 mm.ºC.days) at the end of the study. The final SGR was 10.20% and 3.06% for wet weight and total length, respectively. The growth was exponential during larval development (Fig. 1). Survival was over 95% along experimental time.

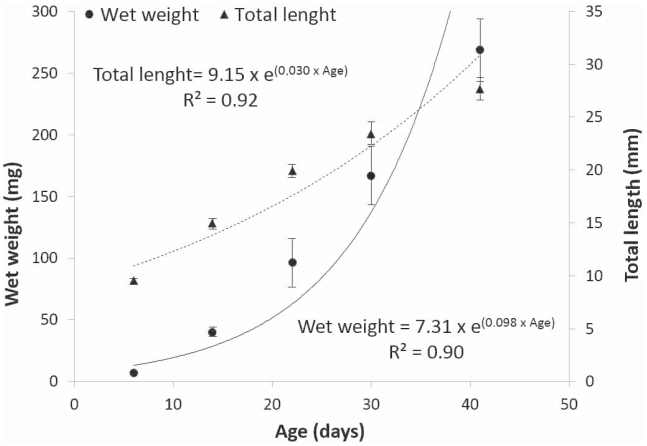

Fig. 1. Wet weight (mg) and total length (mm) of *Parachromis dovii* (mean ± SD, n=30) during experimental period.

All digestive enzymes tested were detected at first feeding (6 DAH, 9.49 mm, 168 DD, 0.056 mm.ºC.days). The specific acid proteolytic activity reached a peak at 22 DAH (20.83 mm, 550 DD, 0.057 mm.ºC.days), decreased at 30 DAH (23.42 mm, 810 DD, 0.028 mm.ºC.days), reaching to a maximum level at 41 DAH (27.66 mm, 1189 DD, 0.023 mm.ºC.days) (Fig. 2a). The specific alkaline proteolytic activity (Fig. 2b) reached the maximum level at 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days), decreased at day 22 after hatching (20.83 mm, 550 DD, 0.057 mm.ºC.days) and finally increased from day 30 to 41 DAH (23.42 mm, 810 DD, 0.028 mm.ºC.days and 27.66 mm, 1189 DD, 0.023 mm.ºC.days, respectively). The trypsin specific activity reached the highest level at 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days) and then, diminished gradually until 41 DAH (27.66 mm, 1189 DD, 0.023 mm.ºC.days) (Fig. 2c). Chymotrypsin,

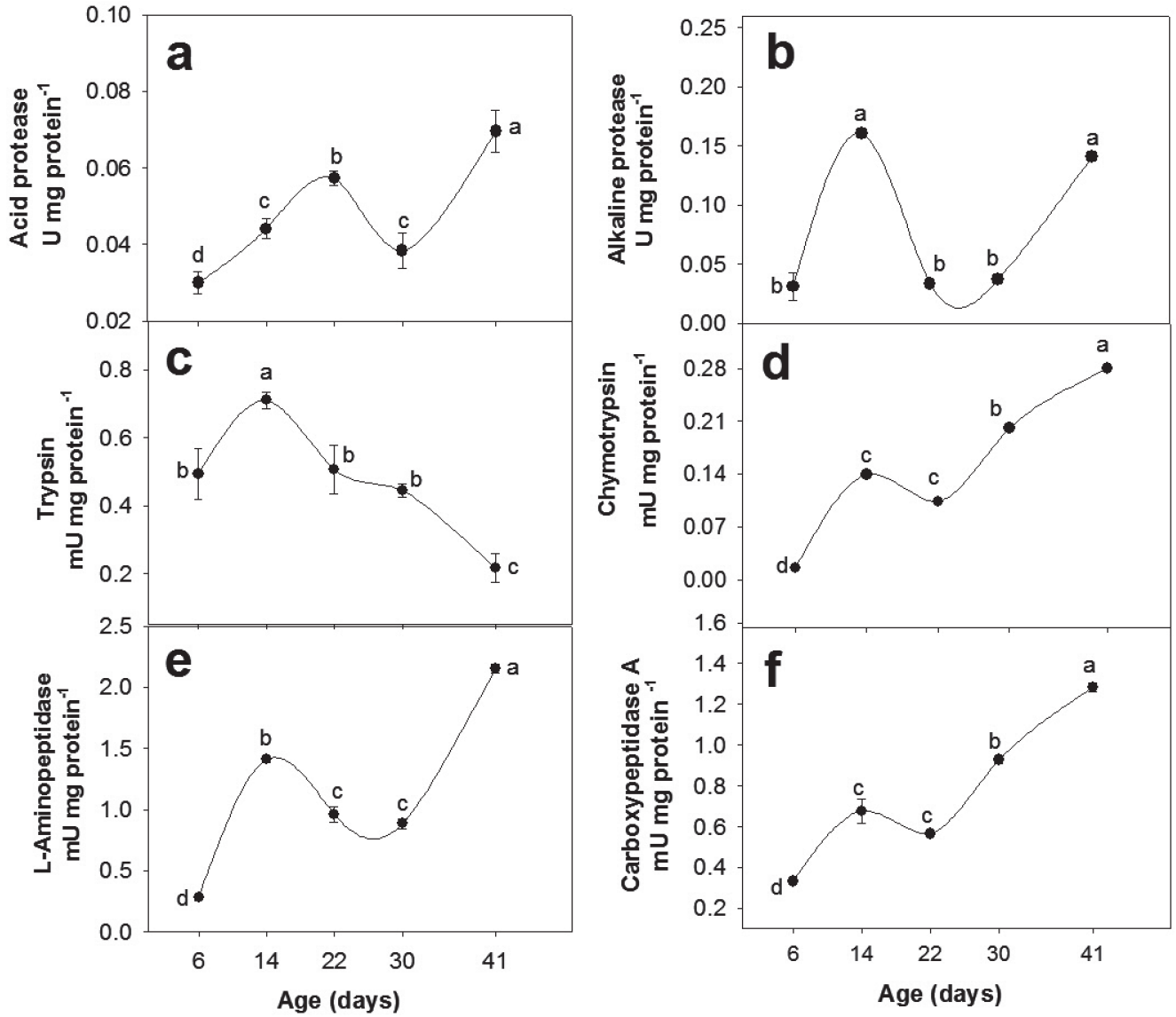

Fig. 2. Digestive proteolytic enzyme activity during ontogeny of *Parachromis dovii* larvae (means ± SD, n= 3 replicates). (a) specific acid proteolytic activity, (b) specific alkaline proteolytic activity, (c) specific trypsin activity, (d) specific chymotrypsin activity, (e) specific leucine-aminopeptidase activity, (f) specific carboxypeptidase A activity.

leucine-aminopeptidase and carboxypeptidase specific activity showed a peak at 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days), diminished at 22 DAH (20.83 mm, 550 DD, 0.057 mm.ºC.days) and reached their highest levels at 41 DAH (27.66 mm, 1189 DD, 0.023 mm.ºC.days) (Fig. 2d-f).

Lipase specific activity showed a peak at 14 DAH and the highest level at 41 DAH (27.66 mm, 1189 DD, 0.023 mm.ºC.days) (Fig. 3a). The α-amylase specific activity was highly fluctuating with a peak at 22 DAH (20.83 mm, 550 DD, 0.057 mm.ºC.days) and reaching its highest value at the end of the period (Fig. 3b). Acid phosphatase specific activity was minimal at 6 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days), increased at its maximal level at 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days) and then, gradually diminished until 41 DAH (Fig. 3c). Finally, alkaline phosphatase specific activity was low at 6 and 14 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days and 15.10 mm, 364 DD, 0.041 mm.ºC.days, respectively), increased to its maximum at 22 DAH (20.83 mm, 550 DD, 0.057 mm.ºC.days) and then, sharply decreased until 41 DAH (27.66 mm, 1189 DD, 0.023 mm.ºC.days) (Fig. 3d).

The SDS–PAGE zymogram for alkaline proteases showed two bands that appeared at 6 DAH (19.2, 24.8 kDa), and a third band that was observed at 14 DAH (68 kDa), three bands were present until the end of the larviculture; however, the intensity of these bands increased gradually until 41 DAH (Fig. 4).

Discussion

The growth (in weight and total length average) and survival rate of *P. dovii* larvae was higher compared to that found by Quirós-Orlich *et al*. (2014), who reported a SGR of 12.2±0.1% and 3.4±0.1 % for weight and length, respectively, and a survival rate of 98.99% during a period of 20 days (from 8 to 28 DAH). In that study, *P. dovii* larvae were feeding with *Artemia* nauplii (at satiation) until 15 DAH, followed by *Artemia* nauplii substitution by formulated feed. The activity of digestive enzymes is used as an indicator of the larvae capability to hydrolyze nutrients and, to some extent, as a guide to elucidate the enzyme affinity to specific substrates according to feed types (Zambonino-Infante, Cahu, 2001).

Accordingly, our results show that *P. dovii* larvae had acid proteolytic activity (pepsin-like) since 6 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days), in agreement with the observation of a stomach with gastric glands at the time of first feeding (Valverde-Chavarría *et al.*, 2013). This early appearance of a stomach with gastric glands has also been reported for *Petenia splendida* (Treviño *et al.*, 2011). In addition, it is also possible that some of this early acid activity (6 DAH) corresponds to other acid enzymes, such as cathepsin, since the enzymatic extracts were made using the whole larvae (Moyano *et al.*, 1996).

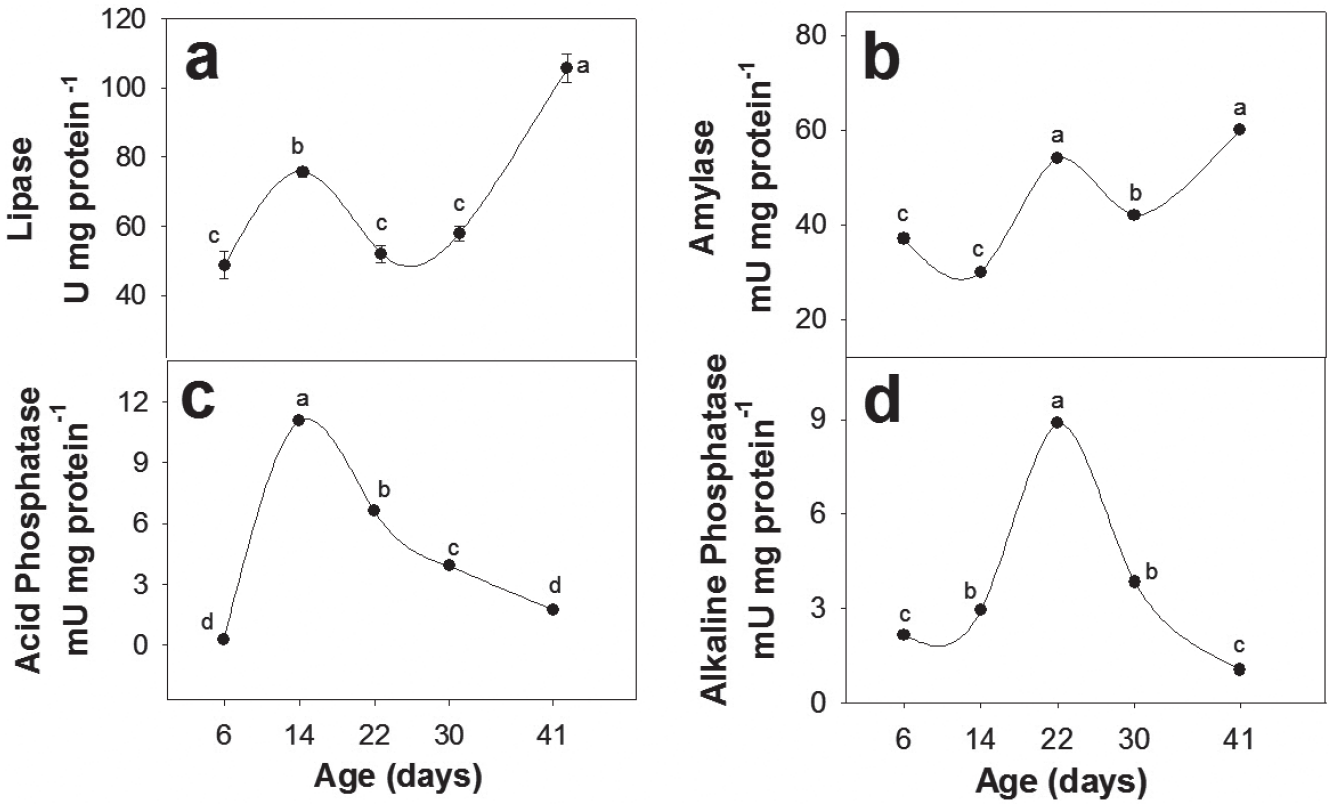

Fig. 3. Digestive enzymatic activity during ontogeny of *Parachromis dovii* larvae (means ± SD, n= 3 replicates). (a) specific lipase activity, (b) specific α-amylase activity, (c) specific acid phosphatase activity, (d) specific alkaline phosphatase activity.

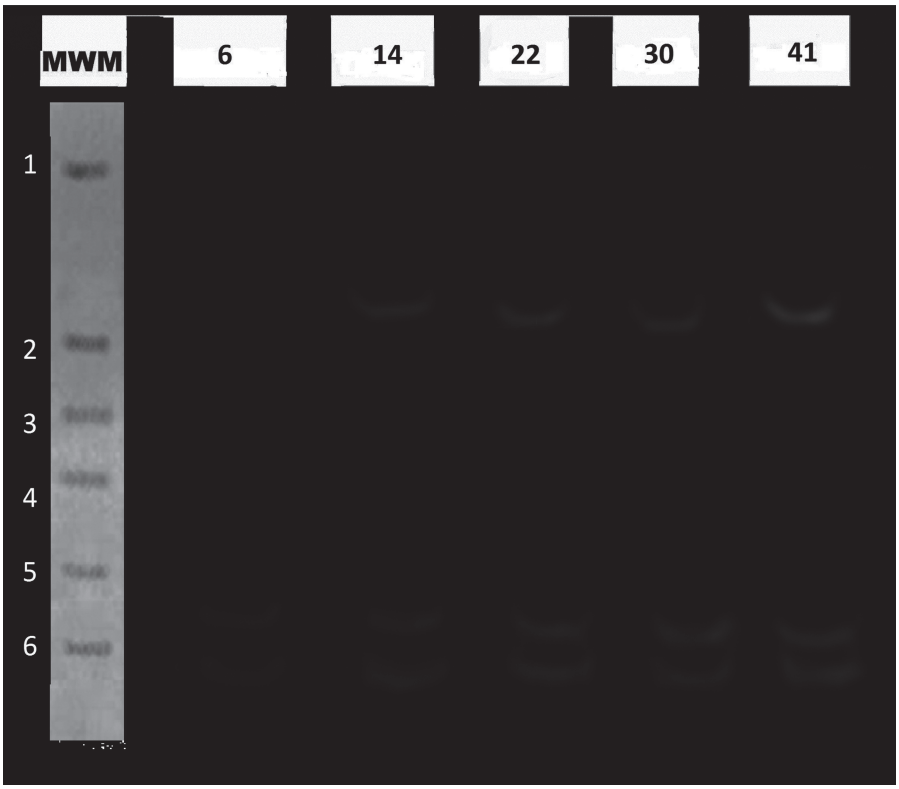

Fig. 4. Zymograms of digestive proteases during ontogeny of *Parachromis dovii* larvae a SDS–PAGE for alkaline proteases. MWM: Marker of molecular weights, 1) phosphorylase b (97 kDa), 2) serum bovine albumin (66 kDa), 3) egg albumin (45 kDa), 4) carbonic anhydrase (29 kDa), 5) trypsinogen (24 kDa), and 6) soybean trypsin inhibitor (20 kDa).

Further, the pepsin-like activity is also a good indicator of the transition from larvae to juvenile as it has been reported for perch, *Sander lucioperca* (Linnaeus), common pandora, *Pagellus erythrinus* (Linnaeus) (Suzer *et al.*, 2006; Hamza *et al.*, 2007) and freshwater species as *M. urouphthalmus* (López-Ramírez *et al*., 2011), *P. splendida* (Treviño *et al*., 2011; Uscanga-Martínez *et al*., 2011) and *O. niloticus* (Uscanga-Martínez *et al.*, 2010). The difference in the time of detection of pepsin activity and gastric glands may be due to the species variation. Recent study has demonstrated daily rhythms of digestive enzyme activity in fish larvae (Mata-Sotres *et al.*, 2016). To improve larval rearing and feeding practices, the functional development of digestive system in several species from the genus of *Lutjanus* has been evaluated in Pacific red snapper, *Lutjanus peru* (Nichols, Murphy), the appearance of the gastric glands and pyloric caeca was around 24 DAH (Peña *et al.*, 2017), and pepsin secretion was detected around 25 DAH (Moguel-Hernandez *et al.*, 2014).

The result from the current study together with previous published data suggests that digestive enzymes of fish larvae are presented at a low level before the onset exogenous feeding and their activities increase to a high level during exogenous feeding. In contrast to a taxonomically similar species onset points of trypsin as *M. urophthalmus* (López--Ramirez *et al.*, 2011) and *A. trimaculatus* (Toledo-Solís *et al.*, 2015), amylase and lipase in pompano, *Trachinotus ovatus* (Linnaeus), are triggered by internal mechanisms, rather than dietary stimulation; for this reason, the fluctuations in specific enzyme activities covered the period of morphological differentiation in the digestive tract and the development of digestive glands (Ma *et al.,* 2015). According to this study, gastric glands were first observed on 14 DPH, but the pepsin activity of crimson snapper was not detected until 17 DPH. Such development may cause by the feeds shifting from rotifers to *Artemia* nauplii.

The alkaline proteolytic activity for *P. dovii* larvae shows a similar trend as that found with other freshwater species, specially related to the increasing in digestive enzyme activities during the first 10 to 15 DAH. This is the result of intestine digestion due to not only the secretion of different pancreatic proteases to the lumen (trypsin, chymotrypsin and carboxypeptidases) but also to the action of digestive enzymes from intestinal wall (aminopeptidases). The alkaline proteolytic activity observed from 6 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days) acts after protein hydrolysis by stomach acid proteases; agreeing with pattern described for *M. urouphthalmus* (López-Ramírez *et al*., 2011). Similarly, with *Solea senegalensis* Kaup, *Atractosteus tristoechus* (Bloch, Schneider), *Labeo catla* (Hamilton), *Hypophthalmichthys molitrix* (Valenciennes) and *Sciaenops ocellatus* (Linnaeus) larvae, the alkaline digestive enzyme activity was detected between 2 and 18 DAH, followed by a reduction (Ribeiro *et al.*, 1999; Comabella *et al.*, 2006; Kumar *et*

al., 2007; Lazo *et al.*, 2007; Khosravi Bakhtiarvandi, Abedian-Kenari, 2015; Xiao-Qiang *et al.*, 2017).

The reduction of the specific activity levels for most of the digestive enzymes tested at the transition from larvae to juvenile (between 22 and 30 DAH, 20.83 mm, 550 DD, 0.057 mm.ºC.days and 23.42 mm, 810 DD, 0.028 mm.ºC. days, respectively) has been reported for other species and it seems to be related to physiological changes that occur during larval development, such as the increment of other soluble proteins in the extracts, the appearance of other hormones or enzymes, or may be genetically programmed (Zambonino-Infante, Cahu, 2001; Lazo *et al*., 2007). It is well known that digestive enzymes play great roles in the catabolism of yolk, the processes of energy metabolism and growth regulation during embryonic developmental stages in fishes (Zhao *et al.*, 2016). Accordingly, enzymes represent important links between the nutrients and the basal metabolism and growth (Santos *et al.*, 2016). Furthermore, some specific enzymes correlate closely with gamete quality (Jia *et al*., 2013).

Larvae of *P. dovii* have a high capability to digest protein from different sources, because of the presence of trypsin, chymotrypsin, leucine aminopeptidase and carboxypeptidase A. These enzymes are capable to hydrolyze peptide bonds (functioning as endopeptidases or exopeptidases), releasing peptides and amino acids, which, in turn, are easily absorbed by the larvae to fulfill its requirements and complete the metamorphosis to juvenile (*e.g.* scale formation, spine growth). The latter is favored by the early appearance of a functional stomach (Valverde-Chavarría *et al*., 2013) with pepsin-like activity since 6 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days).

Trypsin specific activity reached its maximum during first 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days), decreasing days after, while the chymotrypsin specific activity was low at 6 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days) and gradually increased to reach its maximum at 41 DAH (27.66 mm, 1189 DD, 0.023 mm.ºC.days). Fluctuations in the specific activity of trypsin and chymotrypsin have also been reported in larvae of other species such as *S. ocellatus* (Lazo *et al.*, 2007), *S. hasta* (Nazemroaya *et al*., 2015), *E. coioides* (Guo *et al*., 2016), *L. erythopterus* (Cui *et al*., 2017) and *A. sapidissima* (Xiao-Qiang *et al.*, 2017), which has been attributed to genetically programmed changes and variations in the natural food organisms (Zambonino-Infante, Cahu, 2001). Furthermore, the different interactions between alkaline enzymes such as trypsin and chymotrypsin may be considered good indicators of nutritional quality of larvae stages (Cara *et al.*, 2003). The lack of trypsin in the first few days may be compensated by pinocytosis until the digestive tract becomes fully functional to digest protein in lumen itself (Sharma *et al.*, 2016).

The activity of leucine aminopeptidase and of carboxypeptidase A happens in the enterocyte microvilli (parietal digestion), being of relevance in the protein digestion to make available amino acids from the amino and carboxyl protein group. In this context, the aminopeptidase has been used as an indicator of digestive tract maturity. In European seabass, *Dicentrarchus labrax* (Linnaeus) and *O. niloticus* larvae, aminopeptidase was detected in the intestine just before first feeding, increasing its activity from 3 DAH when the appearance of mouth opening happens (Zambonino-Infante, Cahu, 1994; Tengjaroenkul *et al*., 2002). Similarly, yellowtail flounder, *Myzopsetta ferrugineus* (Storer), winter flounder, *Pseudopleuronectes americanus* (Walbaum), *M. urophthalmus* showed aminopeptidase activity during embryonic period and hatching (Baglole *et al.*, 1998; López-Ramírez *et al*., 2011), *R. kutum* (Khosravi Bakhtiarvandi, Abedian-Kenari, 2015) and *A. sapidissima* (Xiao-Qiang *et al.*, 2017) increased until 7-18 DAH.

The increment of alkaline phosphatases activity reflects the enterocytes brush border development, which in turn, it is associated with the diminishing of cytosolic enzymes, agreeing with *S. senegalensis* larvae (Ribeiro *et al.*, 2002). The latter is an indication of the digestive system maturation (which it is faster than skeletal system development) and, from the digestive physiology viewpoint, of the transition from larvae to juvenile stage (Balon, 1984; Kendall *et al.*, 1984). The acid phosphatase was also present from 6 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days), with a maximal activity at 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days), which may indicate that not only the enterocytes are mature but also that nutrient absorption processes are more efficient. Similar trends for activity increments and microvilli development were found with *D. labrax, S. aurata*, *R. kutum* and *A. sapidissima* (Zambonino-Infante, Cahu, 1994; Moyano *et al*., 1996; KhosraviBakhtiarvandi, Abedian-Kenari,2015; Xiao-Qiang *et al.*, 2017). The increment in activity of these enzymes is not only correlated to enterocyte maturation but also to genetic modulation; however, it may also vary due to composition of feed used (Zambonino-Infante, Cahu, 1994; 2001; Gawlicka *et al.*, 1995; Hakim *et al.*, 2007).

Lipase study during larvae ontogeny has great importance because they are related to dietary lipids utilization (López-López*et al.*, 2008). As larvae can't synthesize *de novo* n-6 linoleic and linolenic n-3 fatty acids series, they must be added in diets taking into consideration that type and concentration of required polyunsaturated fatty acids (PUFA) vary between fish species and digestive larvae capability. In this context, PUFA's play a key role at cellular membrane, increasing its fluidity, flexibility and permeability, reason to incorporate them in the diets. Their utilization by the fish is modulated by esterases, phospholipases and true lipases (Evans *et al.,* 1998). *Parachromis dovii* larvae showed high lipases activity since 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days), reaching the highest activity at 41 DAH (at juvenile stage, 27.66 mm, 1189 DD, 0.023 mm.ºC.days) agreeing with yellowtail kingfish, *Seriola lalandi* Valenciennes, *L. catla*,*R. kutum*, *E. coloides*, *L. erythropterus*, *C. chitala*, *A. sapidissima* and Barbel chub, *Squaliobarbus curriculus* (Chen *et al.*, 2006; Kumar *et al*., 2007; Khosravi Bakhtiarvandi, Abedian-Kenari, 2015; Guo *et al.*, 2016; Cui *et al*.,

2017; Mitra *et al.*, 2017; Xiao-Qiang *et al.*, 2017; Shen *et al.*, 2018).

The α-amylase activity in *P. dovii* was founded from 6 DAH (9.49 mm, 168 DD, 0.056 mm.ºC.days), fluctuating with low levels, during ontogeny as found for *O. niloticus* (Tengjaroenkul *et al*., 2002). This finding differs from other fish species, especially marine, such as white seabream, *Diplodus sargus* (Linnaeus), *D. labrax*, *R. kutum*, *E. coloides*, *L. erythropterus*, *C. chitala*, *A. sapidissima* and *S. curriculus* (Cara *et al*., 2003; Cahu *et al.*, 2004; Khosravi Bakhtiarvandi, Abedian-Kenari, 2015; Guo *et al.*, 2016; Cui *et al*., 2017; Mitra *et al.*, 2017; Xiao-Qiang *et al.*, 2017; Shen *et al.*, 2018), in which amylase activity decreased with fish age. In monogastric fish, amylase activity has low impact but in agastric ones it is relevant in carbohydrate digestion, *e.g*. in *L. catla* (Kumar *et al*., 2007), an herbivorous species. In *P. dovii*, the amylase activity presented should improve the use of carbohydrates from the glycogen hydrolysis from its prey during larvae-juvenile transition.

The number of digestive enzyme isoforms expressed on the different species it is related with feeding habits of the species, so when counting with six bands of proteases activities, *P. dovii* may be considered a species of omnivore habits similar to *M. urophthalmus* (López-Ramirez *et al.*, 2011); besides, this type and number of isoforms have been detected in other fish species such as Senegal sole, *Solea senegalensis* (Kaup) with seven types of bands (Sáenz-de Rodrigáñez *et al*., 2005), while in carnivore species, the number of bands is low as Atlantic bluefin tuna, *Thunnus thynnus* (Linnaeus) with three isoforms (Essed *et al.*, 2002), spotted sand bass, *Paralabrax maculatofasciatus* (Steindachner) with two isoforms (Alvarez-González *et al*., 2010), common snook, *Centropomus undecimalis* (Bloch) with two isoforms (Jimenez-Martínez *et al*., 2012) and *C. trimaculatus* with six bands of proteases activities (Toledo-Solís *et al.*, 2015).

According to the results of this study, *P. dovii* larvae have a complete digestive enzyme activity from 6 DAH (first feeding, 9.49 mm, 168 DD, 0.056 mm.ºC.days), that maximize after 14 DAH (15.10 mm, 364 DD, 0.041 mm.ºC.days) when larvae were fed with live food, which indicates that the exogenous feeding with a formulated diet can be started by this time. From the nutritional point of view, it is needed to search for the nutritional requirements of the larval stage and to evaluate the *in vitro* digestibility of diets and ingredients for this specie.

In conclusion the digestive enzymes, alkaline phosphatase, amylase, lipase, chymotrypsin, and trypsin in our study were present before the onset of exogenous feeding in *P. dovii* larvae. The relatively high specific activity of chymotrypsin and trypsin facilitate digestion of protein at an early stage. The pattern of primary digestive enzyme activity indicates early functional development of the digestive system. This study on digestive enzyme patterns will provide valuable information on the nutritional requirements of larvae and help establish feeding protocols for optimizing larval mass-rearing production.

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