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Ontogeny of the digestive enzyme activity of the Amazonian pimelodid catfish *Pseudoplatystoma punctifer* (Castelnau, 1855)

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Abstract :

The aim of the study was to evaluate the functional ontogeny of the digestive system of *Pseudoplatystoma punctifer* through the analysis of the activity of the main intestinal (alkaline phosphatase, aminopeptidase N, maltase and leucine-alanine peptidase), pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and amylase) and gastric (pepsin) enzymes. Larvae were raised in triplicate in a recirculation system from 4 to 27 days post fertilization (dpf) at an initial density of 90 larvae L^{-1}, 27.8 ± 0.7 °C and 0 L: 24D photoperiod. Larvae were fed from 4 to 17 dpf with *Artemia* nauplii and weaned onto an experimentally formulated feed (crude protein content ~ 45%; crude fat content ~ 10%; crude carbohydrate ~ 8%) within 3 days, then continued with the same diet until the end of the trial. *P. punctifer* showed an exponential growth pattern with two different growth rates: a slower one from hatching to 12 dpf followed by a faster one from 12 to 27 dpf. The specific and total activities of the pancreatic and intestinal enzymes were detected from hatching. The digestive system was functional at 12 dpf, indicating the transition from the larval to the juvenile stage (alkaline to acid digestion). Therefore individuals could be weaned from that day onwards. The variations observed in the enzymatic activity from 17 dpf reflected the adaptation of the enzymatic machinery to the new diet supplied. *P. punctifer* larvae showed a fast digestive system development with an enzymatic profile typical of a tropical and carnivorous species.

Highlights

► The ontogeny of the digestive enzymes activity was studied in *Pseudoplatystoma punctifer*, a tropical catfish species with high aquaculture potential in Amazonia. ► *P. punctifer* showed a fast digestive system development with an enzymatic profile typical of a tropical and carnivorous species. ► Results revealed that the digestive system of *P. punctifer* was fully functional at 12 days post fertilization, indicating that this species could be weaned from that day onwards. ► Results contribute to the elaboration of a feeding protocol adapted to the changing digestive capacities of developing fish. ► An adequate feeding protocol is needed to reduce the incidence of cannibalism at this stage of development, which is currently the main bottleneck for fingerling mass production.

1. Introduction

Over the last two decades, an increasing number of studies have been focused on the ontogeny of digestive enzyme activities in both marine and freshwater fishes considered as candidates for aquaculture. In fact, the knowledge of the digestive enzyme profiles during early life stages and their adaptation to the diet in a given species is essential for understanding its nutritional physiology. This is particularly important during ontogeny as the constantly changing digestive capacities determine the types of nutrients that can be digested and absorbed throughout its development (Gisbert et al., 2013; Hamre et al., 2013; Rønnestad et al., 2013). Moreover, considering the high cost of *Artemia* cysts and the associated costs of nauplii production, the replacement of live food by formulated feeds has become one of the main objectives in fish larviculture hence, identifying the type of digestive enzymes and the variation of their specific activities during ontogeny can be used as an indicator not only of larval development and gut maturation but also of survival and performance (Zambonino-Infante et al., 2008) when testing different feeding regimes. Notably, the transitions from endogenous to exogenous feeding as well as from the larval to the juvenile stage are considered key events during which feeding protocols should be adapted to the digestive capacities of a developing organism. Therefore, understanding of the ontogeny of digestive enzymes is critical for determining the most appropriate time for weaning and also for optimizing compound diets as well as feeding protocols, eventually leading to improve growth rates, survival and larval quality (Zambonino-Infante et al., 2008; Hamre et al., 2013; Rønnestad et al., 2013).

Among South American catfish species (Siluridae), the species of the pimelodid genus *Pseudoplatystoma* (Bleeker, 1862) are highly prized for human consumption due

to their excellent flesh quality and lack of intra-muscular spines. In Brazil, the country producing most of *Pseudoplatystoma* reared fish, commercial aquaculture is mostly centered on producing hybrids of *Pseudoplatystoma corruscans* and *Pseudoplatystoma reticulatum* (Valladão et al., 2018), which accept formulated feeds during early life stages more easily than pure species and hence present less cannibalism and a better growth performance (Porto-Foresti et al., 2013). Taking into consideration the risk of genetic variability loss through genetic introgression caused by the escape of interspecific hybrids to the natural environment (Vaini et al., 2014; Lima et al., 2018), research efforts have been made in Amazonia to develop the culture of *Pseudoplatystoma punctifer* (Castro-Ruiz, et al., 2009, 2016; Nuñez et al., 2011; Baras et al., 2011, 2012; Gilles et al., 2014; Gisbert et al., 2014; Darias et al., 2015a; Fernández-Méndez et al., 2015; among others). This carnivorous migratory tropical catfish species, native to the Amazon basin, can attain 140 cm of total length (Buitrago-Suárez & Burr, 2007). It is worth noting that the last taxonomic revision of the genus *Pseudoplatystoma* (Buitrago-Suárez & Burr, 2007) recognized eight species instead of the previous three species and redefined their geographic distribution, so attention should be paid when comparing studies done before and after this revision. However, inconsistencies between the taxonomy of the genus proposed by Buitrago-Suárez and Burr (2007) and subsequent molecular and morphological studies (e.g. García-Dávila et al., 2013; Estivals et al., 2015) highlight the need to reexamine the classification of the *Pseudoplatystoma* species.

Although captive-breeding methodology is well established under controlled conditions (Padilla et al., 2001; Nuñez et al., 2008), large-scale aquaculture of *P. punctifer* has not yet been implemented due to high rates of cannibalism resulting in increased mortality during early life stages. Although a genetic component cannot be

neglected, several studies have found a clear correlation between the feeding protocol and the incidence of cannibalism in *P. punctifer* (Gisbert et al., 2014; Darias et al., 2015a; Fernández-Méndez et al., 2015) and that it could be reduced using feeds having an adequate texture and meeting the specific nutritional requirements of larvae and early juveniles (Darias et al., 2015a; Fernández-Méndez et al., 2015). Further studies on the digestive physiology and nutrition in this species are still needed for improving current rearing strategies and removing bottlenecks associated to mass culture of fingerlings of *P. punctifer*. According to Gisbert et al. (2014), the histological development of the digestive system of *P. punctifer* followed the same general pattern reported in most studied silurid species, such as African catfish (*Clarias garipinus*; Verreth et al., 1992), silver catfish (*Rhamdia quelen*; De Amorim et al., 2009), slender walking catfish (*Clarias nieuhohofii*; Saelee et al., 2011) or yellow catfish (*Pelteobagrus fulvidraco*; Yang et al., 2010). However, different reproductive guilds, egg and larval size or even different larval rearing practices might lead to some species-specific differences in the timing of differentiation and functionality of various digestive structures among these species (Gisbert et al., 2014). In addition, attention should be paid when extracting conclusions about the functionality of the digestive system (*i.e.* beginning of acid digestion and the onset of weaning of larvae) using only histological data, since morphology does not always match functionality (Darias et al., 2005; Solovyev et al., 2016). Thus, in order to complement the information about the histological development of the digestive system of *P. punctifer* (Gisbert et al., 2014), the present study aimed to evaluate its functional ontogeny through evaluating the activity of the main intestinal (alkaline phosphatase, aminopeptidase N, maltase and leucine-alanine peptidase), pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and amylase) and gastric (pepsin) enzymes.

2. Material and methods

2.1. Larval and early juvenile rearing and sampling

Larvae were obtained by hormonally-induced spawning of a sexually mature pond-reared pair of *P. punctifer* (♀: 3.60 kg; ♂: 1.85 kg) at the Instituto de Investigaciones de la Amazonía Peruana (IIAP, Iquitos, Peru). Female and male were transferred to 500-L tanks and injected intramuscularly with carp pituitary extract (Argent Chemical Laboratories, Inc., Redmond, WA) at 5 mg kg^{-1} and 1 mg kg^{-1} of body weight, respectively, according to Darias et al. (2015); then, eggs were fertilized according to Nuñez et al. (2008). Fertilized eggs (fertilization rate ~99.9%) were incubated at 27.7 $\pm$ 0.6 °C in 40-L cylindroconical tanks connected to a freshwater recirculating system; hatching took place after 18.0 $\pm$ 2.0 h (hatching rate ~96%). Mouth opening occurred at day 1 post fertilization (dpf) and exogenous feeding started at 4 dpf. At 4 dpf (6.05 $\pm$ 0.28 mm total length-TL, n = 45; mean $\pm$ standard deviation) larvae were transferred into 40-L square tanks made of fiberglass (functional volume = 30L), which were connected to a water recirculating system with mechanical and biological filters. Water quality parameters throughout the experimental period were as follows: temperature, 27.8 $\pm$ 0.7 °C; pH, 7.0 $\pm$ 0.5; dissolved oxygen, 7.4 $\pm$ 0.2 mg L^{-1}; NO$_2$-, 0.38 $\pm$ 0.27 mg L^{-1}, NH$_4$+, 0.26 $\pm$ 0.13 mg L^{-1} and water flow rate of 0.2 L min^{-1}. Larvae were reared in triplicate (initial density = 90 larvae L^{-1}) under 0L:24D photoperiod (<0.001 Lx at the water surface) and fed in slight excess with newly-hatched *Artemia* sp. nauplii (crude protein content ~37%; crude fat content ~15%; crude carbohydrate content ~10%; 0.6–12.2 nauplii mL^{-1}; five times a day from 4 to 17 dpf). At 17 dpf, larvae were weaned onto an experimentally formulated feed (crude protein content ~45%; crude fat content

~10%; crude carbohydrate content ~8 %) within 3 days according to Darias et al. (2015), whereas larvae were solely fed with the same formulated feed from 20 dpf to the end of the experiment at 27 dpf. In order to monitor larval growth (total length, TL and wet body weight, WW), triplicate groups of larvae (n = 15) were sampled from each rearing tank at 4, 8, 12, 20 and 27 dpf and euthanized with an overdose of Eugenol (Moyco®, Moyco, Lima, Peru). Then, larvae were placed in a Petri dish, photographed using a scale bar and TL was measured on the digital images (300 dpi) using ImageJ software (Rasband, 1997-2012). Individual WW was determined using an analytic microbalance (Sartorius BP 211 D, Data Weighing Systems, Inc., Elk Grove, IL, ± 0.01 mg). Specific growth rate (SGR, in % day^{-1}) was calculated as SGR = (ln WW_f - ln WW_i) / (t_f - t_i) x 100; where WW_f, WW_i, t_f and t_i represented final and initial WW and time of the experiment, respectively. Survival was calculated at 17 dpf (onset of weaning) and 27 dpf (end of the trial) by counting the individuals surviving at each sampling point and taking into consideration the number of individuals sampled throughout the experimental period. Larvae (n = 600 to 15, depending on their WW) were sampled from each tank (n = 3) at hatching and at 4, 12, 17, 20, 25 and 27 dpf in order to evaluate ontogenetic changes in digestive enzyme activity patterns, and their relationship to diet and rearing protocol. Sampled larvae were euthanized as previously described, rinsed in distilled water and frozen at -80 °C until their analysis.

2.2. Enzymatic assays

Individuals younger than 12 dpf were completely homogenized for analytical purposes, since they were too small for being dissected. Older fish were dissected in four parts (head, pancreatic segment, intestinal segment and tail) and enzyme assays

were performed in the pancreatic and intestinal segments (Cahu and Zambonino-Infante,1994).

Samples were homogenized in 30 volumes (v/w) of Tris-Mannitol buffer (50 mM Mannitol, 2 mM Tris-HCl; pH 7.5) for 30 s (Ultra-Turrax T25, Germany), then 100 μL of 0.1M $CaCl_2$ was added to the homogenate and the extract was subjected to sonication (Vibra-cell[©], Sonics, Germany) for 1 minute. During the homogenizing process, samples were kept on ice (0-4 °C) for reducing the enzymatic activity. An aliquot of homogenate was stored at -80 °C until their analysis for determining activities of pancreatic (trypsin, chymotrypsin, total alkaline proteases, bile-salt activated lipase and amylase), gastric (pepsin) and intestinal cytosolic (leucine-alanine peptidase) enzymes. Processed samples were analyzed within the first two months after their homogenization in order to prevent a loss of activity of the pancreatic and gastric digestive enzymes (Solovyev and Gisbert, 2016). The remaining homogenate was processed for intestinal brush border purification according to the recommendations of Gisbert et al. (2018) in order to properly determine alkaline phosphatase, maltase and aminopeptdiase N activities. To initiate this process, the homogenate was centrifuged (9,000 g for 10 min at 4 °C), the precipitate discarded, then the supernatant centrifuged once again (34,000 g for 30 min at 4 °C). The pellet, containing the brush border (BB) of enterocytes, was re-suspended in 1 mL of buffer (0.1 M KCl, 5 mM Tris-Hepes, 1 mM DTT; pH 7.5) and stored at -80 °C until analysis (Crane et al., 1979).

The determination of the activity of pancreatic, gastric and intestinal digestive enzymes was conducted using spectrophotometric analyses as described by Gisbert et al. (2009). In brief, alkaline phosphatase (AP, E.C. 3.1.3.1) activity was quantified using PNPP (4-nitrophenyl phosphate) as substrate in 30 mM Na_2CO_3 buffer (pH 9.8). One unit (U) was defined as 1 μg nitrophenol released per min^{-1} mL^{-1} of BB homogenate at

27 °C and measured using 407 nm (Bessey et al., 1946). Aminopeptidase N (AN, E.C.3.4.11.2) activity was determined according to Maroux et al. (1973) using sodium phosphate buffer 80 mM (pH 7.0) and L-leucine p-nitroanilide as substrate (in 0.1 mM DMSO). One unit of enzyme activity (U) was defined as 1 μg nitroanilide released per min^{-1} mL^{-1} of BB homogenate at 27 °C and measured using 410 nm. Maltase (MAL, E.C.3.2.1.20) activity was determined at 27 °C using D(+)-maltose as substrate in 100 mM sodium maleate buffer (pH 6.0) (Dahkqvist, 1970); one unit of maltase (U) was defined as μmol of glucose liberated min^{-1} mL^{-1} of homogenate and measured at 420 nm. Leucine-alanine peptidase (LAP, E.C. 3.4.11) was performed using leucine-alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0); one unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min^{-1} mL^{-1} of homogenate at 27 °C and measured at 530 nm (Nicholson and Kim, 1975).

Regarding pancreatic enzymes, trypsin (E.C. 3.4.21.4) activity was assayed at 27 °C and measured using 407 nm using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as substrate (Holm et al., 1988). One unit of trypsin per mL (U) was defined as 1 μmol BAPNA hydrolyzed per min^{-1} mL^{-1} of enzyme. Chymotrypsin (EC. 3.4.21.1) activity was determined at 27 °C and measured at 256 nm using BTEE (N-benzoyl-L-tyrosine ethyl ester) as substrate in 80 mM Tris-HCl, 100 mM $CaCl_2$ buffer (pH 7.2). Chymotrypsin activity (U) corresponded to the 1 μmol BTEE hydrolyzed per min^{-1} mL^{-1} of homogenate (Worthington, 1991). The total activity of alkaline proteases was determined after 30 min of incubation at 27 ºC, using 0.5 % (w/v) azocasein as substrate in 50 mM Tris–HCl buffer (pH 8.0). One unit of total alkaline proteases per mL (U) was defined as 1 μmol azocasein hydrolyzed per min^{-1} mL^{-1} of homogenate and measured at 366 nm (García-Careño and Haard, 1993). Pepsin assays were performed at 27 °C and 280 nm using hemoglobin as substrate (Worthington, 1991). Pepsin activity (U) was

defined as 1 μmol of hemoglobin liberated per min^{-1} mL^{-1} of homogenate after 10 min of incubation. The α-amylase (E.C. 3.2.1.1) activity was measured at 580 nm using soluble starch (0.3%) dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate (Métais and Bieth, 1968); and its activity (U) was defined as the mg of starch hydrolyzed during 30 min per mL^{-1} of homogenate at 27 °C. Bile salt-activated lipase (E.C. 3.1.1) activity was measured using 4 p-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl (pH 9.0), 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone:n-heptane (5:2), the extract centrifuged (6,080 g, 2 min at 4 °C) and the absorbance of the supernatant read at room temperature and measured at 405 nm. Lipase activity (U) was defined as the μmol of substrate hydrolyzed per min^{-1} mL^{-1} of homogenate (Iijima et al., 1998). All enzymatic activities were measured using a microplate scanning spectrophotometer (Synergy HT, Bio-Tech, Germany) and expressed as specific (mU mg^{-1} protein) and total (mU larva^{-1}) enzyme activities. Soluble protein in enzyme extracts was quantified using the Bradford technique (1976) using bovine serum albumin as a standard. All the assays were made in triplicate (methodological replicates).

2.3. *Statistical analyses*

Activity of digestive enzymes was presented as a mean ± standard deviation of the mean (SD). Statistical analyses were conducted using SigmaStat 3.0 (Systat Software Inc., Richmond, USA). Data were checked for normality and heterogeneity of variance, and then analyzed by one-way ANOVA followed by the Holm–Sidak method when significant differences were found. The level of statistical significance was set at $P < 0.01$ and $P < 0.05$.

3. Results

3.1. *Larval and early juvenile growth and survival*

Larval and early juvenile growth in terms of WW (mg) and TL (mm) followed an exponential curve according to the following equations: $WW = 0.1655e^{0.2794T}$ ($r = 0.97$; $n = 45$) and $TL = 4.2914e^{0.0787T}$ ($r = 0.99$; $n = 45$), where T is the age of larvae in dpf (Fig. 1). Larvae at 4 dpf (WW = 0.22 ± 0.17 mg; TL = 6.05 ± 0.28 mm) grew up to 244.11 ± 4.36 mg and 35.77 ± 1.11 mm in weight and length, respectively, by the end of the study (27 dpf). Two phases of growth in WW were identified during *P. punctifer* development; during the first phase, a gradual growth was observed between 4 (0.22 ± 0.17 mg WW) and 12 dpf (6.56 ± 0.23 mg WW), whereas the second phase started from 12 dpf with a pronounced increase in WW, coinciding with the introduction of the formulated feed at 17 dpf (> 17 mm TL). The SGR of *P. punctifer* was 0.19 ± 0.00 from 4 to 12 dpf and 0.53 ± 0.10 from 12 to 27 dpf. Survival rates at 17 dpf and at the end of the experiment (27 dpf) were 82.7 ± 11.0 % and 16.0 ± 0.1 %, respectively.

3.2. *Activity of BB enzymes*

The specific and total activities of the assayed brush border intestinal (AP, AN and MAL) and cytosolic enzymes (LAP) are shown in Fig. 2. The specific activity of AP (Fig. 2) was detected at hatching (1.03 ± 0.83 mU mg protein^{-1}) and remained constant until 12 dpf (1.92 ± 0.53 mU mg protein^{-1}), then its activity gradually increased until 27 dpf (9.87 ± 2.27 mU mg protein^{-1}; $P < 0.001$). Total activity of AP remained constant from hatching to 25 dpf (0.001 ± 0.001 mU larva^{-1} at 0 dpf and 11.90 ± 1.42 mU larva^{-1} at 25 dpf), which was followed by a significant increase (24.92 mU larva$^{-1} \pm 6.43$) at the end of the experiment ($P < 0.001$).

The specific activity of AN (Fig. 2) remained constant from hatching (0.38 ± 0.31 mU mg protein^{-1}) to the end of the study (0.05 ± 0.004 mU mg protein^{-1}; $P > 0.05$). Moreover, total activity of AN was detected at hatching (0.001 ± 0.001 mU larva^{-1}) and remained constant until 12 dpf (0.08 ± 0.002 mU larva^{-1}), then increased from 17 dpf (0.04 ± 0.0001 mU larva^{-1}) until 27 dpf (0.13 ± 0.0002 mU larva^{-1}; $P < 0.01$).

Maltase specific activity (Fig. 2) was detected at hatching (121.85 ± 3.11 mU mg protein^{-1}), decreased abruptly at 4 dpf (11.51 ± 0.41 mU mg protein^{-1}) and remained constant until the end of the study (22.81 ± 4.57 mU mg protein^{-1}). Total activity of MAL remained statistically constant from hatching (0.19 ± 0.01 mU larva^{-1}) until 20 dpf (6.26 ± 0.43 mU larva^{-1}) and then gradually increased until 27 dpf (57.51 ± 13.20 mU larva^{-1}, $P < 0.01$).

The specific activity of LAP (Fig. 2) was highest at hatching (2438.96 ± 108.48 mU mg protein^{-1}) and it decreased ~3 times at 4 dpf (919.14 ± 27.98 mU mg protein^{-1}) and remained constant until 27 dpf (858.2 ± 38.0 mU mg protein^{-1}). Regarding total activity values for LAP, the lowest value was found at hatching (75.59 ± 11.0 mU larva^{-1}) and its activity gradually increased from 4 dpf to 17 dpf (3275.04 ± 98.62 mU larva^{-1}; $P < 0.01$). A decrease in LAP total activity was detected from 17 to 20 dpf (2142.79 ± 58.12 mU larva^{-1}), but it increased again from 20 dpf to the end of the trial (4868.68 ± 336.63 mU larva^{-1}; $P < 0.01$).

The level of intestinal maturation in *P. punctifer* larvae was determined as the ratio between the specific activities of BB enzymes and that of LAP. Differences among these ratios were found depending on the BB enzyme considered (Fig. 3). When considering the ratio MAL/LAP, there was a sharp increase at 4 dpf, whereas the AN/LAP and AP/LAP ratios showed an increase between 12 and 17 dpf ($P < 0.001$; Fig. 3).

3.3. *Activity of pancreatic enzymes*

Changes in the specific and total activities of pancreatic (trypsin, chymotrypsin, total alkaline proteases, α-amylase and bile-salt activated lipase) enzymes are shown in Fig. 4. Trypsin specific activity (Fig. 4) was detected from hatching (0.007 ± 0.002 mU mg protein^{-1}) and remained constant until the end of the trial (0.012 ± 0.003 mU mg protein^{-1}; $P > 0.05$). Total trypsin activity remained statistically constant from hatching (0.18 ± 0.005 µU larva^{-1}) until 20 dpf (0.02 ± 0.001 µU larva^{-1}) and sharply increased thereafter until the end of the experiment (0.08 ± 0.02 mU larva^{-1}; $P < 0.001$).

Chymotrypsin specific activity (Fig. 4) remained constant from hatching (0.022 ± 0.04 mU mg protein^{-1}) until the end of the experiment (0.18 ± 0.072 mU mg protein^{-1}; $P > 0.05$). Chymotrypsin total activity was statistically constant from hatching (0.006 ± 0.001 mU larva^{-1}) to 4 dpf (0.024 ± 0.012 mU larva^{-1}), then gradually increased until 25 dpf (0.046 ± 0.024 mU larva^{-1}) with an abrupt increase at 27 dpf (1.21 ± 0.544 mU larva^{-1}; $P < 0.05$). Total alkaline proteases (Fig. 4) were detected at hatching and their specific activity increased almost 2-fold from hatching (0.76 ± 0.02 mU mg protein^{-1}) to 12 dpf (1.51 ± 0.004 mU mg protein^{-1}), but abruptly decreased 6 times at 17 dpf (0.23 ± 0.2 mU mg protein^{-1}). However, the activity of total alkaline proteases increased from 20 dpf (0.17 ± 0.02 mU mg protein^{-1}) until the end of the trial (0.38 ± 0.01 mU mg protein^{-1}; $P < 0.001$). Total activity of total alkaline proteases increased from hatching (0.02 ± 0.004 µU larva^{-1}) to 12 dpf (2.11 ± 0.03 mU larva^{-1}) and gradually decreased thereafter until 20 dpf (0.47 ± 0.05 mU larva^{-1}). A 4-fold increase of total alkaline proteases activity was observed 5 days later (1.95 ± 0.1 mU larva^{-1}), and continued to increase until the end of the study (2.52 ± 0.1 mU larva^{-1}; $P < 0.001$).

Alpha-amylase activity (Fig 4) was detected at hatching (15.4 ± 3.2 mU mg protein^{-1}) and remained constant until the end of the study at 27 dpf (17.78 ± 0.7; $P > 0.05$). Total α-amylase activity was statistically constant from hatching to 4 dpf (0.39 ± 0.08 mU larva^{-1}and 3.76 ± 1.88 mU larva^{-1}, respectively), then increased from 12 to 17 dpf (11.8 ± 2.3 mU larva^{-1} and 31.23± 4.27 mU larva^{-1}), showing a 3-fold increase until the end of the experiment (110.49 ± 2.09 mU larva^{-1}; $P < 0.001$).

The specific activity of bile-salt activated lipase (Fig. 4) was highest at hatching (7.50 ± 2.0 mU mg protein^{-1}), but its activity abruptly decreased at 4 dpf (1.28 ± 0.11 mU mg protein^{-1}); however, lipase activity gradually increased from 12 dpf (1.81 ± 0.01 mU mg protein^{-1}) until the end of the study (6.50 ± 0.41 mU mg protein^{-1}; $P < 0.05$). Total activity of lipase remained statistically constant from hatching until 12 dpf (0.37 ± 0.15 mU larva^{-1}) and then sharply increased until 27 dpf (42.12 ± 2.17 mU larva^{-1}; $P < 0.001$).

3.4. *Activity of gastric enzyme*

Pepsin specific activity was first detected at 4 dpf (0.12 ± 0.02 μU mg protein^{-1}) and its specific activity remained constant until 12 dpf (0.22 ± 0.08 μU mg protein^{-1}) (Fig. 5). Then, the activity increased abruptly from 12 to 17 dpf (0.7 ± 0.19 μU mg protein^{-1}), whereas it decreased sharply at 20 dpf (0.0003 ± 0.02 μU mg protein^{-1}). Pepsin specific activity increased thereafter until the end of the trial (4.9 ± 0.7 μU mg protein^{-1}; $P < 0.05$). Total pepsin activity followed a similar pattern to that of the pepsin specific activity ($P < 0.05$).

4. Discussion

Compared to temperate-water fish, tropical fish have a faster development due to the effect of temperature on metabolic rate (Gillooly et al., 2002). Therefore, comparisons of the present results are made with those obtained for other freshwater and marine tropical fish species, but also for other catfish. Larvae and early juveniles of *P. punctifer* followed a similar previously reported pattern of development and growth (Gisbert et al., 2014, Darias et al., 2015a, Fernández-Méndez et al., 2015). The growth pattern of *P. punctifer* larvae and early juveniles in terms of weight showed an initial slow growth phase (SGR 0.19 ± 0.00) until 12 dpf (12.02 ± 0.18 mm TL), corresponding to the larval stage, followed by a higher exponential growth rate (SGR 0.53 ± 0.10) from 12 dpf onwards, coinciding with the beginning of the juvenile stage. Regardless of their different reproductive guilds, this type of growth pattern has also been reported in both freshwater and marine tropical fish species such as yellow catfish (*Pelteobagrus fulvidraco*; Yang et al., 2010), green catfish (*Mystus nemurus*; Srichanun et al., 2012), common snook (*Centropomus undecimalis*; Jimenez-Martinez et al., 2012), bay snook (*Petenia splendida*; Uscanga-Martínez et al., 2011) and spotted rose snapper (*Lutjanus guttatus*; Moguel-Hernández et al., 2014), among others. The extended low growth rate observed from hatching to 12 dpf (12.02 ± 0.18 mm TL) in *P. punctifer* may be interpreted as an evolutionary strategy for allocating the available energy from yolk-sac reserves and prey items to promote larval physiological changes (*i.e.* gastrointestinal and other body system development) rather than somatic growth, as it has also been reported in butter catfish (*Ompok bimaculatus;* Pradhan et al., 2013), striped catfish, (*Pangasianodon hypophthalmus;* Rangsin et al., 2012) and tropical gar (*Atractosteus tropicus*; Frías-Quintana et al., 2015). Another reason for the low growth rate of *P. punctifer* could be the fact that *Artemia* nauplii, offered during this developmental period, do not completely satisfy the nutritional needs of larvae or let them fully exploit

their growth potential (Gisbert et al. 2014; Darias et al., 2015a). In fact, the nutritional composition of the diet has been shown to modulate the SGR of *P. punctifer* during the early juvenile stage from 12 to 26 dpf (Darias et al., 2015a). Therefore, a higher growth rate during the larval phase could be also expected if a feeding regime fitted to the specific nutritional needs of larvae is offered. Survival rates observed under current experimental conditions were within the range of values reported in other studies for the same species (Núñez et al., 2011; Gisbert et al., 2014; Darias et al., 2015a) and may reflect larval adaptation to the weaning strategy since survival has shown to be affected by the composition (Darias et al., 2015a) and texture (Fernández-Méndez et al., 2015) of compound diets.

The appearance of a functional microvillus membrane in enterocytes constitutes a crucial step during larval development of fish, since this characterizes the maturation of the intestinal mucosa and the acquisition of an adult mode of digestion (Zambonino-Infante et al., 2008; Gisbert et al., 2018). The ratio of BB and cytosolic intestinal enzymes is considered as an indicator of the development of intestinal digestion and describes the change from the larval (intracellular) to the adult (intestinal lumen) mode of digestion. Values of this ratio are considered as important for readiness for introduction of formulated diet into larval rearing protocols (Cahu and Zambonino-Infante, 2001). In the present study, different intestinal maturation ratios were low at 4 dpf (6.05 ± 0.28 mm TL), which might be due to the presence of a straight, undifferentiated and unfolded intestine lined by a single layer of columnar epithelial cells (Gisbert et al., 2014). Then, the BB enzymes, and particularly AP and AN activities, increased at 12 dpf (12.02 ± 0.18 mm TL), which indicated the beginning of the enterocytes maturation as well as the increase of their proliferation that resulted in an increase in the intestinal luminal digestion and absorption surface (Gisbert et al.,

2014). The increase in activities of BB enzymes up to 20 dpf (20.53 ± 0.98 mm TL) also coincided with the formation of the intestinal loop for accommodating the increasing length of the intestine inside the abdominal cavity as well as the achievement of a morphologically complete digestive system during this stage (Gisbert et al., 2014). Previous histological data (Gisbert et al., 2014) coupled with present results of enzyme activities obtained by spectrophotometric methods indicate that *P. punctifer* larvae could be weaned as early as 12 dpf (12.02 ± 0.18 mm TL). These results explain the successful three-day weaning strategy starting at 12 dpf tested with this species (Darias et al., 2015a). After intestinal maturation, from 12 dpf onwards, BB enzyme activities showed some fluctuations that could be attributed to the switch from live food to the formulated feed. A similar increase and decrease (teeth saw profile) in specific activities of BB and cytosolic enzymes during larval development has also been reported in other fish species such as bay snook (Uscanga-Martínez et al., 2011) and butter catfish (Pradhan et al., 2013), among others.

During the first stages of larval development and before the onset of acidic digestion, the digestion of proteins occurs by the action of alkaline proteases, mainly trypsin and chymotrypsin, in combination with intestinal cytosolic peptidases. In this phase, the larvae have limited capacity of digesting macromolecules that are absorbed by enterocytes (Cahu and Zambonino-Infante, 2001). According to Gisbert et al. (2014), the pancreatic exocrine cells and zymogen granules (precursors of pancreatic digestive enzymes) in *P. punctifer* were detected in the exocrine pancreas before the onset of exogenous feeding. In line with this, the activity of pancreatic enzymes (total alkaline proteases, trypsin, chymotrypsin amylase and lipase) in *P. punctifer* larvae was also detected before the onset of exogenous feeding. The presence of pancreatic enzymes activity before first feeding has also been reported in many other fish species (Cahu and

Zambonino-Infante, 2001; Zambonino-Infante et al., 2008) as it enables fish to be ready to digest exogenous food at mouth opening. The early presence of alkaline proteases in *P. punctifer* larvae coincides with the histological findings regarding the consumption of the yolk sac and the morphogenesis of digestive organs, appearance of the liver and the pancreas, as well as zymogen granules (between hatching and 4 dpf) (Gisbert et al., 2014). These processes have also been noticed in other tropical carnivorous fish species such as butter catfish (Pradhan et al., 2013), common snook (Jiménez-Martínez et al., 2012) and tropical gar (Frías-Quintana et al., 2015). The increment in enzyme activities from 12 dpf (12.02 ± 0.18 mm TL) coincided with the complete morphological formation of the digestive system in *P. punctifer* (Gisbert et al., 2014). In addition, the variations in the level of digestive enzyme activities from 17 to 25 dpf coincided with the transition from live prey to compound diets, revealing a modulation of the activity of pancreatic enzymes (trypsin, chymotrypsin, total alkaline proteases, amylase and lipase) by food composition as has been typically observed for other species (Cahu and Zambonino-Infante, 2001; Uscanga-Martínez et al., 2011). In this study, the activity of trypsin and chymotrypsin was initially detected at hatching. The importance of these enzymes during the first stages of development might be explained by their involvement in the cleavage of proteins contained in the yolk (Zambonino-Infante et al., 2008) and/or digestion and breakage of the egg chorion during the hatching process (Gisbert et al., 2009). Then, the activity of trypsin and chymotrypsin in *P. punctifer* remained constant until the end of the study (27 dpf, 35.77 ± 1.11 mm TL). In a study made with *Pseudoplatystoma fasciatum*, a decrease in pancreatic alkaline protease activities was detected at 10 days after hatching (dah) concomitant with an increase in pepsin-like activity (Dabrowski and Portella, 2006). However, the re-description of the species of the genus *Pseudoplatystoma* (Buitrago-Suárez and Burr, 2007) as well as the

contradictions between this taxonomic revision and subsequent genetic and morphological studies (García-Dávila et al., 2013; Estivals et al., 2015) make drawing conclusions difficult at the species level. In any case, the aforementioned differences in digestive enzyme activity could be attributed to differences in the rearing conditions and feeding regimes. Similar profiles of trypsin and chymotrypsin activity to those of *P. punctifer* were observed in tropical gar (Frías-Quintana et al., 2015), both species fed a 45%-protein content diet. In other species, the activities of trypsin and chymotrypsin increased during the larval development and peaked at the transition from the larval to the juvenile stage, such as in the Mayan cichlid (*Cichlasoma urophthalmus*; López-Ramírez et al., 2010) and spotted rose snapper (Moguel-Hernández et al., 2014), which occurred at around 24-25 dah. It seems that a pattern of increasing trypsin and chymotrypsin activity is more easily detectable in species exhibiting a longer larval period and that this increase could be masked by changes in diet composition at weaning in faster-developing species.

In the present study, high values of α-amylase were detected from hatching, which may be due to the presence of glycogen deposits accumulated in the yolk sac (Gisbert and Doroshov, 2006). Moreover, the presence of α-amylase activity before exogenous feeding may be triggered by intrinsic mechanisms rather than by diet stimulation as it has also been reported in other fish species (Lazo et al., 2000; Zambonino-Infante et al., 2008). The α-amylase activity in carnivorous species is typically high at early stages and decreases during development (Cahu and Zambonino-Infante, 2001). Although differences were not statistically significant due to the high individual variability, α-amylase activity in *P. punctifer* tended to decrease during the larval period and to increase in response to changes in the diet composition at weaning. Such response may be related to the type of carbohydrate rather than the content, since the *Artemia* nauplii

and the compound diet used in the present study contained similar amounts of carbohydrates (~10 % and ~8 %, respectively). These results are in contrast with studies made in other tropical fish species like butter catfish (Pradhan et al. 2013) and tropical gar (Frías-Quintana et al., 2015), where α-amylase had low specific activity at hatching and gradually increased after exogenous feeding. Differences in α-amylase activity patterns between species might be linked to differences in their digestive physiology and feeding habits (Solovyev et al., 2014), but also due to variations in rearing protocols among species.

The activity of lipase is modulated by lipid composition (Cahu and Zambonino-Infante, 2001; Morais et al., 2004; Zambonino-Infante et al., 2008) and therefore its analysis is a valuable tool to determine the adequate lipid composition to include in the diet for an optimal utilization (Uscanga-Martínez et al., 2011). The activity of lipase has been shown to be greater in carnivorous than in omnivorous or herbivorous fish species (Chakrabarti et al., 1995; Tengjaroenkul et al., 2000; Solovyev et al., 2014) and generally increases during ontogeny (Zambonino-Infante et al., 2008). In the current study, lipase activity was high at hatching and then decreased at first feeding and increased again from weaning until the end of the study. The early lipase activity observed in *P. punctifer* is in line with the previous histological findings that showed luminal digestion and absorption by the first week of life of larvae, reflecting the functional development of the intestine, and the vacuolization of the hepatocyte cytoplasm and the appearance of lipid droplets in the intermediate regions of the intestine and the liver during the mixed nutritional period (Gisbert et al., 2014). The early activity of lipase has also been reported in tropical gar (Frías-Quintana et al., 2015) and spotted rose snapper (Moguel-Hernández et al., 2014). The lower lipase activity level observed in *P. punctifer* larvae during the *Artemia* feeding period (4-17

dpf) compared to that of the compound diet feeding phase (17-27 dpf) could be related to the different lipid content and/or composition of the *Artemia* (~15 % DM) and compound diet (~10 % DM) used in this study. The lower lipase activity may indicate an inadequate lipid composition of *Artemia* for this species, since a better growth and survival and a more balanced lipid metabolism of *P. punctifer* larvae and early juveniles were observed when fed enriched *Artemia* and compound diets with an enriching product containing high levels of DHA (Magris et al., 2013; Darias et al., 2015b). This would be in accordance with the previous observation that *Artemia* nauplii do not fully cover the nutritional needs of the larvae (Gisbert et al., 2014; Darias et al., 2015a) and, therefore, this could be also accounting for the lower grow rate found at this developmental stage. Further nutritional studies focused on the larval stage are needed in order to improve growth. The changes in lipase activity after weaning might be indicative of changes in the nutritional requirements, which are reflected in the rate of growth. Moreover, the increase in specific activity of lipase at the end of the experiment (35.77 ± 1.11 mm TL) may be related to the adaption of weaned early juveniles to formulated diet and to the acquisition of the full digestive capacities of this species. The increase in the capacity for digesting lipids during development has been also described for some other catfish species like the sheatfish (*Silurus soldatovi*; Liu et al., 2010), striped catfish (Rangasin et al., 2012) and butter catfish (Pradhan et al., 2013).

Although the appearance of gastric glands was observed at 8 dpf (7.31 ± 0.48 mm TL) in this species (Gisbert et al. 2014), pepsin activity was surprisingly detected at 4 dpf (6.05 ± 0.28 mm TL). However, this activity was not associated to the gastric function, but rather to the presence of other acid proteases such as lysosomal cathepsins, which might be involved in the digestion of yolk proteins (Carnevali et al., 2001). The increase in acid protease activity from 4 dpf (6.05 ± 0.28 mm TL) to 12 dpf (12.02 ±

0.18 mm TL) was related to the appearance of gastric glands as histological data indicated (Gisbert et al., 2014). The onset of acid digestion occurred at the latest at 12 dpf (12.02 ± 0.18 mm TL), after that, the stomach became morphologically complete and the glandular stomach became functional, acquiring thus the adult-like mode of digestion. Therefore, 12 mm TL may be considered as an optimal size for weaning of *P. punctifer* larvae. Fluctuations in pepsin activity after the weaning period could indicate the adaptation of individuals to the feed composition. Similar results were found in other silurid species with several differences in the timing of organ differentiation and development, such is in butter catfish (Pradhan et al., 2013), *Silurus glanis* (Kozaric et al., 2008) and yellow catfish (Yang et al., 2010).

5. Conclusions

Pseudoplatystoma punctifer showed an exponential growth pattern with two different growth rates: a slower one from hatching to 12 dpf (12.02 ± 0.18 mm TL) followed by a faster one until the end of the study (27 dpf, 35.8 ± 1.11 mm TL). The growth was slow in the initial phase because the energy was used for developing the digestive organs, while after the completion of the digestive system ontogeny the energy was allocated for growth. Also, the use of *Artemia* nauplii during the larval stage could account for this slow growth as this prey has shown to not meet the nutritional requirements for this species. *P. punctifer* larvae showed a fast digestive system development with an enzymatic profile typical of a tropical and carnivorous species. The activity profiles of the intestinal, pancreatic and gastric digestive enzymes reflected the functional changes associated to their ontogeny and to the feeding regime. The digestive system was functional at the latest at 12 dpf when larvae measured 12.02 ± 0.18 mm TL, indicating the transition from the larval to the juvenile stage (alkaline to acid digestion) in this

catfish species. Present biochemical data on enzyme activity were in agreement with the previous description of the histological organization of the digestive system. Thus, considering both histological and functional data, it can be concluded that *P. punctifer* larvae can be successfully weaned from 12 dpf onwards. The variations observed in the enzymatic activity from 17 dpf (> 17 mm TL) onwards reflected the adaptation of the enzymatic machinery to the new diet supplied.

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Figure 1. Larval growth of *P. punctifer* in terms of total length (TL, mm) and wet body weight (WW, mg) from 4 to 27 days post fertilization (dpf). Results are expressed as mean ± SD (n=45).

Figure 2. Specific (squares, mU mg^{-1} protein) and total (circles, mU larva^{-1}) activity of brush border (alkaline phosphatase, maltase and aminopeptidase N) and cytosolic (leucine-alanine pepidase) intestinal enzymes in *P. punctifer* from hatching to the juvenile stage (27 dpf). Results are expressed as mean ± SD (n = 3). Different letters indicate significant differences (ANOVA, $P < 0.05$).

Figure 3. Intestinal maturation during the larval development of *P. punctifer* measured as the ratio of specific activity of brush border membrane enzymes/cytosolic enzymes: MAL/LAP, AP/LAP and AN/LAP. Results are expressed as mean ± SD (n = 3). Different letters indicate significant differences (ANOVA, $P < 0.05$).

Figure 4. Specific (squares, mU mg^{-1} protein) and total (circles, mU larva^{-1}) activity of pancreatic enzymes during the larval development of *P. punctifer*. Results are expressed

as mean $\pm$ SD (n=3). Different letters indicate significant differences (ANOVA, $P < 0.05$).

Figure 5. Specific (squares, mU mg^{-1} protein) and total (circles, mU larva^{-1}) activity of pepsin during the larval development of *P. punctifer*. Results are expressed as mean $\pm$ SD (n = 3). Different letters indicate significant differences (ANOVA, $P < 0.05$).

Highlights

- The ontogeny of the digestive enzymes activity was studied in *Pseudoplatystoma punctifer*, a tropical catfish species with high aquaculture potential in Amazonia.
- *P. punctifer* showed a fast digestive system development with an enzymatic profile typical of a tropical and carnivorous species.
- Results revealed that the digestive system of *P. punctifer* was fully functional at 12 days post fertilization, indicating that this species could be weaned from that day onwards.
- Results contribute to the elaboration of a feeding protocol adapted to the changing digestive capacities of developing fish.
- An adequate feeding protocol is needed to reduce the incidence of cannibalism at this stage of development, which is currently the main bottleneck for fingerling mass production.
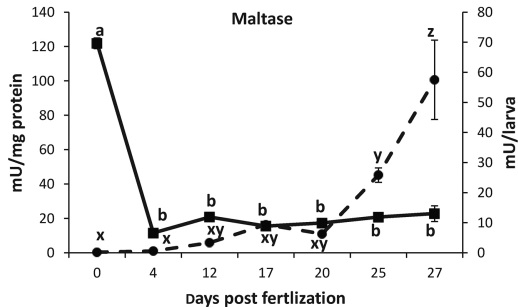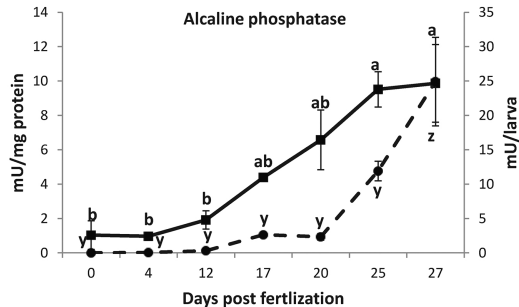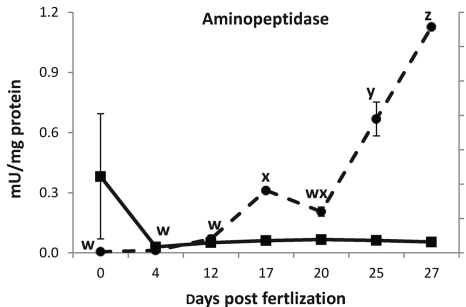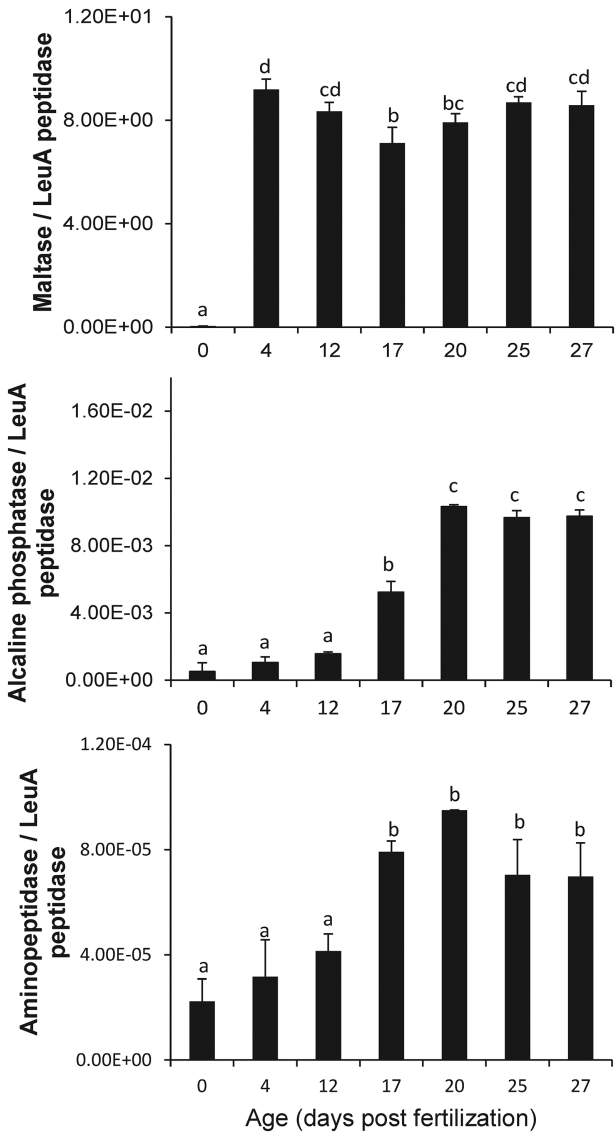
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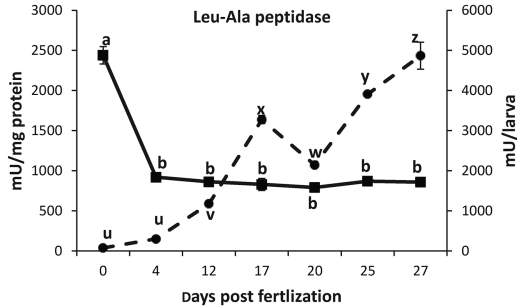
Figure 2

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