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

Diana Castro-Ruiz[1], Mansour Torfi Mozanzadeh[2], Christian Fernández-Méndez[1], Karl B. Andree[3], Carmen García-Dávila[1], Chantal Cahu[4], Enric Gisbert[3], Maria J. Darias[5*]

[1] Instituto de Investigaciones de la Amazonía Peruana (IIAP), Programa de Investigación para el Uso y Conservación del Agua y sus Recursos, Iquitos, Peru

[2] Department of Fisheries, South Iran Aquaculture Research Center, Marine Fish Aquaculture Research Station, Bandar Imam Khomeyni, Khozestan, Iran

[3] Institut de Recerca i Tecnologia Agroalimentaries (IRTA), Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Aqüícoles, Sant Carles de la Ràpita, Spain

[4] IFREMER, UMR Laboratoire des Sciences de l'Environnement Marin, Plouzané, France

[5] MARBEC, IRD, Univ. Montpellier, CNRS, Ifremer, Montpellier, France

*Author for correspondence: E-mail: maria.darias@ird.fr

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 0L : 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.

Keywords: Pseudoplatystoma punctifer; ontogeny; enzymatic activity; digestive system; brush border; pancreatic enzymes; gastric enzymes.

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;

96 Fernández-Méndez et al., 2015) and that it could be reduced using feeds having an
97 adequate texture and meeting the specific nutritional requirements of larvae and early
98 juveniles (Darias et al., 2015a; Fernández-Méndez et al., 2015). Further studies on the
99 digestive physiology and nutrition in this species are still needed for improving current
100 rearing strategies and removing bottlenecks associated to mass culture of fingerlings of
101 *P. punctifer*. According to Gisbert et al. (2014), the histological development of the
102 digestive system of *P. punctifer* followed the same general pattern reported in most
103 studied silurid species, such as African catfish (*Clarias garipinus*; Verreth et al., 1992),
104 silver catfish (*Rhamdia quelen*; De Amorim et al., 2009), slender walking catfish (*Clarias*
105 *nieuhohofii*; Saelee et al., 2011) or yellow catfish (*Pelteobagrus fulvidraco*; Yang et al.,
106 2010). However, different reproductive guilds, egg and larval size or even different larval
107 rearing practices might lead to some species-specific differences in the timing of
108 differentiation and functionality of various digestive structures among these species
109 (Gisbert et al., 2014). In addition, attention should be paid when extracting conclusions
110 about the functionality of the digestive system (*i.e.* beginning of acid digestion and the
111 onset of weaning of larvae) using only histological data, since morphology does not
112 always match functionality (Darias et al., 2005; Solovyev et al., 2016). Thus, in order to
113 complement the information about the histological development of the digestive system
114 of *P. punctifer* (Gisbert et al., 2014), the present study aimed to evaluate its functional
115 ontogeny through evaluating the activity of the main intestinal (alkaline phosphatase,
116 aminopeptidase N, maltase and leucine-alanine peptidase), pancreatic (trypsin,
117 chymotrypsin, total alkaline proteases, bile-salt activated lipase and amylase) and gastric
118 (pepsin) enzymes.
119
120 **2. Material and methods**

121

122 *2.1. Larval and early juvenile rearing and sampling*

123 Larvae were obtained by hormonally-induced spawning of a sexually mature pond-
124 reared pair of *P. punctifer* (♀: 3.60 kg; ♂: 1.85 kg) at the Instituto de Investigaciones de
125 la Amazonía Peruana (IIAP, Iquitos, Peru). Female and male were transferred to 500-L
126 tanks and injected intramuscularly with carp pituitary extract (Argent Chemical
127 Laboratories, Inc., Redmond, WA) at 5 mg kg^{-1} and 1 mg kg^{-1} of body weight,
128 respectively, according to Darias et al. (2015); then, eggs were fertilized according to
129 Nuñez et al. (2008). Fertilized eggs (fertilization rate ~99.9%) were incubated at 27.7 ±
130 0.6 °C in 40-L cylindroconical tanks connected to a freshwater recirculating system;
131 hatching took place after 18.0 ± 2.0 h (hatching rate ~96%). Mouth opening occurred at
132 day 1 post fertilization (dpf) and exogenous feeding started at 4 dpf. At 4 dpf (6.05 ± 0.28
133 mm total length-TL, n = 45; mean ± standard deviation) larvae were transferred into 40-
134 L square tanks made of fiberglass (functional volume = 30L), which were connected to a
135 water recirculating system with mechanical and biological filters. Water quality
136 parameters throughout the experimental period were as follows: temperature, 27.8 ± 0.7
137 °C; pH, 7.0 ± 0.5; dissolved oxygen, 7.4 ± 0.2 mg L^{-1}; NO_2-, 0.38 ± 0.27 mg L^{-1}, NH_4+,
138 0.26 ± 0.13 mg L^{-1} and water flow rate of 0.2 L min^{-1}. Larvae were reared in triplicate
139 (initial density = 90 larvae L^{-1}) under 0L:24D photoperiod (<0.001 Lx at the water
140 surface) and fed in slight excess with newly-hatched *Artemia* sp. nauplii (crude protein
141 content ~37%; crude fat content ~15%; crude carbohydrate content ~10%; 0.6–12.2
142 nauplii mL^{-1}; five times a day from 4 to 17 dpf). At 17 dpf, larvae were weaned onto an
143 experimentally formulated feed (crude protein content ~45%; crude fat content ~10%;
144 crude carbohydrate content ~8 %) within 3 days according to Darias et al. (2015), whereas
145 larvae were solely fed with the same formulated feed from 20 dpf to the end of the

146 experiment at 27 dpf. In order to monitor larval growth (total length, TL and wet body
147 weight, WW), triplicate groups of larvae (n = 15) were sampled from each rearing tank
148 at 4, 8, 12, 20 and 27 dpf and euthanized with an overdose of Eugenol (Moyco®, Moyco,
149 Lima, Peru). Then, larvae were placed in a Petri dish, photographed using a scale bar and
150 TL was measured on the digital images (300 dpi) using ImageJ software (Rasband, 1997-
151 2012). Individual WW was determined using an analytic microbalance (Sartorius BP 211
152 D, Data Weighing Systems, Inc., Elk Grove, IL, ± 0.01 mg). Specific growth rate (SGR,
153 in % day^{-1}) was calculated as SGR = (ln WW_f - ln WW_i) / (t_f - t_i) x 100; where WW_f,
154 WWi, t_f and t_i represented final and initial WW and time of the experiment, respectively.
155 Survival was calculated at 17 dpf (onset of weaning) and 27 dpf (end of the trial) by
156 counting the individuals surviving at each sampling point and taking into consideration
157 the number of individuals sampled throughout the experimental period. Larvae (n = 600
158 to 15, depending on their WW) were sampled from each tank (n = 3) at hatching and at
159 4, 12, 17, 20, 25 and 27 dpf in order to evaluate ontogenetic changes in digestive enzyme
160 activity patterns, and their relationship to diet and rearing protocol. Sampled larvae were
161 euthanized as previously described, rinsed in distilled water and frozen at -80 °C until
162 their analysis.
163
164 *2.2. Enzymatic assays*
165 Individuals younger than 12 dpf were completely homogenized for analytical
166 purposes, since they were too small for being dissected. Older fish were dissected in four
167 parts (head, pancreatic segment, intestinal segment and tail) and enzyme assays were
168 performed in the pancreatic and intestinal segments (Cahu and Zambonino-Infante,1994).
169 Samples were homogenized in 30 volumes (v/w) of Tris-Mannitol buffer (50 mM
170 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

196 homogenate at 27 °C and measured using 410 nm. Maltase (MAL, E.C.3.2.1.20) activity
197 was determined at 27 °C using D(+)-maltose as substrate in 100 mM sodium maleate
198 buffer (pH 6.0) (Dahkqvist, 1970); one unit of maltase (U) was defined as μmol of glucose
199 liberated min^{-1} mL^{-1} of homogenate and measured at 420 nm. Leucine-alanine peptidase
200 (LAP, E.C. 3.4.11) was performed using leucine-alanine as substrate in 50 mM Tris-HCl
201 buffer (pH 8.0); one unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed
202 substrate per min^{-1} $mL^{-1}$of homogenate at 27 °C and measured at 530 nm (Nicholson and
203 Kim, 1975).
204 Regarding pancreatic enzymes, trypsin (E.C. 3.4.21.4) activity was assayed at 27 °C
205 and measured using 407 nm using BAPNA (N-α-benzoyl-DL-arginine p-nitroanilide) as
206 substrate (Holm et al., 1988). One unit of trypsin per mL (U) was defined as 1 μmol
207 BAPNA hydrolyzed per min^{-1} mL^{-1} of enzyme. Chymotrypsin (EC. 3.4.21.1) activity was
208 determined at 27 °C and measured at 256 nm using BTEE (N-benzoyl-L-tyrosine ethyl
209 ester) as substrate in 80 mM Tris-HCl, 100 mM $CaCl_2$ buffer (pH 7.2). Chymotrypsin
210 activity (U) corresponded to the 1 μmol BTEE hydrolyzed per min^{-1} mL^{-1} of homogenate
211 (Worthington, 1991). The total activity of alkaline proteases was determined after 30 min
212 of incubation at 27 ºC, using 0.5 % (w/v) azocasein as substrate in 50 mM Tris–HCl buffer
213 (pH 8.0). One unit of total alkaline proteases per mL (U) was defined as 1 μmol azocasein
214 hydrolyzed per min^{-1} mL^{-1} of homogenate and measured at 366 nm (García-Careño and
215 Haard, 1993). Pepsin assays were performed at 27 °C and 280 nm using hemoglobin as
216 substrate (Worthington, 1991). Pepsin activity (U) was defined as 1 μmol of hemoglobin
217 liberated per min^{-1} mL^{-1} of homogenate after 10 min of incubation. The α-amylase (E.C.
218 3.2.1.1) activity was measured at 580 nm using soluble starch (0.3%) dissolved in
219 Na_2HPO_4 buffer (pH 7.4) as substrate (Métais and Bieth, 1968); and its activity (U) was
220 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} ± 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}; $P < 0.01$).

271 Maltase specific activity (Fig. 2) was detected at hatching (121.85 ± 3.11 mU mg
272 protein^{-1}), decreased abruptly at 4 dpf (11.51 ± 0.41 mU mg protein^{-1}) and remained
273 constant until the end of the study (22.81 ± 4.57 mU mg protein^{-1}). Total activity of MAL
274 remained statistically constant from hatching (0.19 ± 0.01 mU larva^{-1}) until 20 dpf (6.26
275 ± 0.43 mU larva^{-1}) and then gradually increased until 27 dpf (57.51 ± 13.20 mU larva^{-1},
276 $P < 0.01$).

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

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

290

291 3.3. *Activity of pancreatic enzymes*

292 Changes in the specific and total activities of pancreatic (trypsin, chymotrypsin, total
293 alkaline proteases, α-amylase and bile-salt activated lipase) enzymes are shown in Fig. 4.
294 Trypsin specific activity (Fig. 4) was detected from hatching (0.007 ± 0.002 mU mg
295 protein^{-1}) and remained constant until the end of the trial (0.012 ± 0.003 mU mg protein^{-}

296 $^{-1}$; $P > 0.05$). Total trypsin activity remained statistically constant from hatching (0.18 ±
297 0.005 µU larva^{-1}) until 20 dpf (0.02 ± 0.001 µU larva^{-1}) and sharply increased thereafter
298 until the end of the experiment (0.08 ± 0.02 mU larva^{-1}; $P < 0.001$).

299 Chymotrypsin specific activity (Fig. 4) remained constant from hatching (0.022 ± 0.04
300 mU mg protein^{-1}) until the end of the experiment (0.18 ± 0.072 mU mg protein^{-1}; $P >$
301 0.05). Chymotrypsin total activity was statistically constant from hatching (0.006 ± 0.001
302 mU larva^{-1}) to 4 dpf (0.024 ± 0.012 mU larva^{-1}), then gradually increased until 25 dpf
303 (0.046 ± 0.024 mU larva^{-1}) with an abrupt increase at 27 dpf (1.21 ± 0.544 mU larva^{-1}; P
304 < 0.05). Total alkaline proteases (Fig. 4) were detected at hatching and their specific
305 activity increased almost 2-fold from hatching (0.76 ± 0.02 mU mg protein^{-1}) to 12 dpf
306 (1.51 ± 0.004 mU mg protein^{-1}), but abruptly decreased 6 times at 17 dpf (0.23 ± 0.2 mU
307 mg protein^{-1}). However, the activity of total alkaline proteases increased from 20 dpf
308 (0.17 ± 0.02 mU mg protein^{-1}) until the end of the trial (0.38 ± 0.01 mU mg protein^{-1}; P
309 < 0.001). Total activity of total alkaline proteases increased from hatching (0.02 ± 0.004
310 µU larva^{-1}) to 12 dpf (2.11 ± 0.03 mU larva^{-1}) and gradually decreased thereafter until 20
311 dpf (0.47 ± 0.05 mU larva^{-1}). A 4-fold increase of total alkaline proteases activity was
312 observed 5 days later (1.95 ± 0.1 mU larva^{-1}), and continued to increase until the end of
313 the study (2.52 ± 0.1 mU larva^{-1}; $P < 0.001$).

314 Alpha-amylase activity (Fig 4) was detected at hatching (15.4 ± 3.2 mU mg protein^{-1})
315 and remained constant until the end of the study at 27 dpf (17.78 ± 0.7; $P > 0.05$). Total
316 α-amylase activity was statistically constant from hatching to 4 dpf (0.39 ± 0.08 mU larva^{-}
317 ^{1}and 3.76 ± 1.88 mU larva^{-1}, respectively), then increased from 12 to 17 dpf (11.8 ± 2.3
318 mU larva^{-1} and 31.23± 4.27 mU larva^{-1}), showing a 3-fold increase until the end of the
319 experiment (110.49 ± 2.09 mU larva^{-1}; $P < 0.001$).

320 The specific activity of bile-salt activated lipase (Fig. 4) was highest at hatching (7.50
321 ± 2.0 mU mg protein^{-1}), but its activity abruptly decreased at 4 dpf (1.28 ± 0.11 mU mg
322 protein^{-1}); however, lipase activity gradually increased from 12 dpf (1.81 ± 0.01 mU mg
323 protein^{-1}) until the end of the study (6.50 ± 0.41 mU mg protein^{-1}; $P < 0.05$). Total activity
324 of lipase remained statistically constant from hatching until 12 dpf (0.37 ± 0.15 mU larva^{-1}
325 ^{1}) and then sharply increased until 27 dpf (42.12 ± 2.17 mU larva^{-1}; $P < 0.001$).
326
327 3.4. *Activity of gastric enzyme*
328 Pepsin specific activity was first detected at 4 dpf (0.12 ± 0.02 μU mg protein^{-1}) and
329 its specific activity remained constant until 12 dpf (0.22 ± 0.08 μU mg protein^{-1}) (Fig. 5).
330 Then, the activity increased abruptly from 12 to 17 dpf (0.7 ± 0.19 μU mg protein^{-1}),
331 whereas it decreased sharply at 20 dpf (0.0003 ± 0.02 μU mg protein^{-1}). Pepsin specific
332 activity increased thereafter until the end of the trial (4.9 ± 0.7 μU mg protein^{-1}; $P < 0.05$).
333 Total pepsin activity followed a similar pattern to that of the pepsin specific activity ($P <$
334 0.05).
335
336 **4. Discussion**
337 Compared to temperate-water fish, tropical fish have a faster development due to the
338 effect of temperature on metabolic rate (Gillooly et al., 2002). Therefore, comparisons of
339 the present results are made with those obtained for other freshwater and marine
340 tropical fish species, but also for other catfish. Larvae and early juveniles of *P. punctifer*
341 followed a similar previously reported pattern of development and growth (Gisbert et al.,
342 2014, Darias et al., 2015a, Fernández-Méndez et al., 2015). The growth pattern of *P.*
343 *punctifer* larvae and early juveniles in terms of weight showed an initial slow growth
344 phase (SGR 0.19 ± 0.00) until 12 dpf (12.02 ± 0.18 mm TL), corresponding to the larval

345 stage, followed by a higher exponential growth rate (SGR 0.53 ± 0.10) from 12 dpf
346 onwards, coinciding with the beginning of the juvenile stage. Regardless of their different
347 reproductive guilds, this type of growth pattern has also been reported in both freshwater
348 and marine tropical fish species such as yellow catfish (*Pelteobagrus fulvidraco*; Yang et
349 al., 2010), green catfish (*Mystus nemurus*; Srichanun et al., 2012), common snook
350 (*Centropomus undecimalis*; Jimenez-Martinez et al., 2012), bay snook (*Petenia*
351 *splendida*; Uscanga-Martínez et al., 2011) and spotted rose snapper (*Lutjanus guttatus*;
352 Moguel-Hernández et al., 2014), among others. The extended low growth rate observed
353 from hatching to 12 dpf (12.02 ± 0.18 mm TL) in *P. punctifer* may be interpreted as an
354 evolutionary strategy for allocating the available energy from yolk-sac reserves and prey
355 items to promote larval physiological changes (*i.e.* gastrointestinal and other body system
356 development) rather than somatic growth, as it has also been reported in butter catfish
357 (*Ompok bimaculatus;* Pradhan et al., 2013), striped catfish, (*Pangasianodon*
358 *hypophthalmus;* Rangsin et al., 2012) and tropical gar (*Atractosteus tropicus*; Frías-
359 Quintana et al., 2015). Another reason for the low growth rate of *P. punctifer* could be
360 the fact that *Artemia* nauplii, offered during this developmental period, do not completely
361 satisfy the nutritional needs of larvae or let them fully exploit their growth potential
362 (Gisbert et al. 2014; Darias et al., 2015a). In fact, the nutritional composition of the diet
363 has been shown to modulate the SGR of *P. punctifer* during the early juvenile stage from
364 12 to 26 dpf (Darias et al., 2015a). Therefore, a higher growth rate during the larval phase
365 could be also expected if a feeding regime fitted to the specific nutritional needs of larvae
366 is offered. Survival rates observed under current experimental conditions were within the
367 range of values reported in other studies for the same species (Núñez et al., 2011; Gisbert
368 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

394 enzyme activities showed some fluctuations that could be attributed to the switch from
395 live food to the formulated feed. A similar increase and decrease (teeth saw profile) in
396 specific activities of BB and cytosolic enzymes during larval development has also been
397 reported in other fish species such as bay snook (Uscanga-Martínez et al., 2011) and
398 butter catfish (Pradhan et al., 2013), among others.

399 During the first stages of larval development and before the onset of acidic digestion,
400 the digestion of proteins occurs by the action of alkaline proteases, mainly trypsin and
401 chymotrypsin, in combination with intestinal cytosolic peptidases. In this phase, the
402 larvae have limited capacity of digesting macromolecules that are absorbed by
403 enterocytes (Cahu and Zambonino-Infante, 2001). According to Gisbert et al. (2014), the
404 pancreatic exocrine cells and zymogen granules (precursors of pancreatic digestive
405 enzymes) in *P. punctifer* were detected in the exocrine pancreas before the onset of
406 exogenous feeding. In line with this, the activity of pancreatic enzymes (total alkaline
407 proteases, trypsin, chymotrypsin amylase and lipase) in *P. punctifer* larvae was also
408 detected before the onset of exogenous feeding. The presence of pancreatic enzymes
409 activity before first feeding has also been reported in many other fish species (Cahu and
410 Zambonino-Infante, 2001; Zambonino-Infante et al., 2008) as it enables fish to be ready
411 to digest exogenous food at mouth opening. The early presence of alkaline proteases in
412 *P. punctifer* larvae coincides with the histological findings regarding the consumption of
413 the yolk sac and the morphogenesis of digestive organs, appearance of the liver and the
414 pancreas, as well as zymogen granules (between hatching and 4 dpf) (Gisbert et al., 2014).
415 These processes have also been noticed in other tropical carnivorous fish species such as
416 butter catfish (Pradhan et al., 2013), common snook (Jiménez-Martínez et al., 2012) and
417 tropical gar (Frías-Quintana et al., 2015). The increment in enzyme activities from 12 dpf
418 (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

469 optimal utilization (Uscanga-Martínez et al., 2011). The activity of lipase has been shown
470 to be greater in carnivorous than in omnivorous or herbivorous fish species (Chakrabarti
471 et al., 1995; Tengjaroenkul et al., 2000; Solovyev et al., 2014) and generally increases
472 during ontogeny (Zambonino-Infante et al., 2008). In the current study, lipase activity
473 was high at hatching and then decreased at first feeding and increased again from weaning
474 until the end of the study. The early lipase activity observed in *P. punctifer* is in line with
475 the previous histological findings that showed luminal digestion and absorption by the
476 first week of life of larvae, reflecting the functional development of the intestine, and the
477 vacuolization of the hepatocyte cytoplasm and the appearance of lipid droplets in the
478 intermediate regions of the intestine and the liver during the mixed nutritional period
479 (Gisbert et al., 2014). The early activity of lipase has also been reported in tropical gar
480 (Frías-Quintana et al., 2015) and spotted rose snapper (Moguel-Hernández et al., 2014).
481 The lower lipase activity level observed in *P. punctifer* larvae during the *Artemia* feeding
482 period (4-17 dpf) compared to that of the compound diet feeding phase (17-27 dpf) could
483 be related to the different lipid content and/or composition of the *Artemia* (~15 % DM)
484 and compound diet (~10 % DM) used in this study. The lower lipase activity may indicate
485 an inadequate lipid composition of *Artemia* for this species, since a better growth and
486 survival and a more balanced lipid metabolism of *P. punctifer* larvae and early juveniles
487 were observed when fed enriched *Artemia* and compound diets with an enriching product
488 containing high levels of DHA (Magris et al., 2013; Darias et al., 2015b). This would be
489 in accordance with the previous observation that *Artemia* nauplii do not fully cover the
490 nutritional needs of the larvae (Gisbert et al., 2014; Darias et al., 2015a) and, therefore,
491 this could be also accounting for the lower grow rate found at this developmental stage.
492 Further nutritional studies focused on the larval stage are needed in order to improve
493 growth. The changes in lipase activity after weaning might be indicative of changes in

494 the nutritional requirements, which are reflected in the rate of growth. Moreover, the
495 increase in specific activity of lipase at the end of the experiment (35.77 ± 1.11 mm TL)
496 may be related to the adaption of weaned early juveniles to formulated diet and to the
497 acquisition of the full digestive capacities of this species. The increase in the capacity for
498 digesting lipids during development has been also described for some other catfish
499 species like the sheatfish (*Silurus soldatovi*; Liu et al., 2010), striped catfish (Rangasin et
500 al., 2012) and butter catfish (Pradhan et al., 2013).

501 Although the appearance of gastric glands was observed at 8 dpf (7.31 ± 0.48 mm
502 TL) in this species (Gisbert et al. 2014), pepsin activity was surprisingly detected at 4 dpf
503 (6.05 ± 0.28 mm TL). However, this activity was not associated to the gastric function,
504 but rather to the presence of other acid proteases such as lysosomal cathepsins, which
505 might be involved in the digestion of yolk proteins (Carnevali et al., 2001). The increase
506 in acid protease activity from 4 dpf (6.05 ± 0.28 mm TL) to 12 dpf (12.02 ± 0.18 mm TL)
507 was related to the appearance of gastric glands as histological data indicated (Gisbert et
508 al., 2014). The onset of acid digestion occurred at the latest at 12 dpf (12.02 ± 0.18 mm
509 TL), after that, the stomach became morphologically complete and the glandular stomach
510 became functional, acquiring thus the adult-like mode of digestion. Therefore, 12 mm TL
511 may be considered as an optimal size for weaning of *P. punctifer* larvae. Fluctuations in
512 pepsin activity after the weaning period could indicate the adaptation of individuals to the
513 feed composition. Similar results were found in other silurid species with several
514 differences in the timing of organ differentiation and development, such is in butter
515 catfish (Pradhan et al., 2013), *Silurus glanis* (Kozaric et al., 2008) and yellow catfish
516 (Yang et al., 2010).

517

518 **5. Conclusions**

519 *Pseudoplatystoma punctifer* showed an exponential growth pattern with two different
520 growth rates: a slower one from hatching to 12 dpf (12.02 ± 0.18 mm TL) followed by a
521 faster one until the end of the study (27 dpf, 35.8 ± 1.11 mm TL). The growth was slow
522 in the initial phase because the energy was used for developing the digestive organs, while
523 after the completion of the digestive system ontogeny the energy was allocated for
524 growth. Also, the use of *Artemia* nauplii during the larval stage could account for this
525 slow growth as this prey has shown to not meet the nutritional requirements for this
526 species. *P. punctifer* larvae showed a fast digestive system development with an
527 enzymatic profile typical of a tropical and carnivorous species. The activity profiles of
528 the intestinal, pancreatic and gastric digestive enzymes reflected the functional changes
529 associated to their ontogeny and to the feeding regime. The digestive system was
530 functional at the latest at 12 dpf when larvae measured 12.02 ± 0.18 mm TL, indicating
531 the transition from the larval to the juvenile stage (alkaline to acid digestion) in this catfish
532 species. Present biochemical data on enzyme activity were in agreement with the previous
533 description of the histological organization of the digestive system. Thus, considering
534 both histological and functional data, it can be concluded that *P. punctifer* larvae can be
535 successfully weaned from 12 dpf onwards. The variations observed in the enzymatic
536 activity from 17 dpf (> 17 mm TL) onwards reflected the adaptation of the enzymatic
537 machinery to the new diet supplied.
538
539 **Acknowledgements**
540 The authors thank Marta Sastre (IRTA) for her contribution to enzyme activity analyses.
541 This work was funded by the International Joint Laboratory 'Evolution and
542 Domestication of the Amazonian Ichthyofauna' (LMI EDIA, IRD-IIAP-UAGRM) and

543 the IRTA. DCR benefited from a travel grant from the National Fund for Scientific,
544 Technological Development and Technological Innovation (FONDECYT, Peru).
545

546 **References**

547 Baras, E., Silva del Aguila, D.V., Montalvan Naranjos, G.V., Dugue, R., Chu-Koo, F.,
548 Duponchelle, F., Renno, J.-F., García-Dávila, C., Núñez, J., 2011. How many
549 meals a day to minimize cannibalism when rearing larvae of the Amazonian
550 catfish *Pseudoplatystoma punctifer*? The cannibal's point of view. Aquat. Living
551 Resour. 24, 379-390.

552 Baras, E., Montalvan Naranjos, G.V., Silva del Aguila, D.V., Chu Koo, F., Dugué, R.,
553 Chavez, C., Duponchelle, F., Renno, J.-F., García-Dávila, C., Núñez, J., 2012.
554 Ontogenetic variation of food intake and gut evacuation rate in larvae of the
555 doncella *Pseudoplatystoma punctifer*, as measured using a non-destructive
556 method. Aquac. Res. 43, 1764-1776.

557 Bessey, O.A., Lowry, O.H., Brock, M.J., 1946. Rapid coloric method for determination
558 of alkaline phosphatase in five cubic millimeters of serum. J. Biol. Chem. 164,
559 321-329.

560 Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram
561 quantities of protein utilizing the principle of protein-dye binding. Anal Biochem.
562 72, 248-254.

563 Buitrago-Suárez, U.A., Burr, B.M., 2007. Taxonomy of the catfish genus
564 *Pseudoplatystoma Bleeker* (Siluriformes: Pimelodidae) with recognition of eight
565 species. Zootaxa 38, 1-38.

566 Cahu, C.L., Zambonino-Infante, J., 1994. Early weaning of sea bass (*Dicentrarchus*
567 *labrax*) larvae with a compound diet: effect on digestive enzymes. Comp.
568 Biochem. Physiol. 109A, 213–222.
569 Cahu, C., Zambonino Infante, J., 2001. Substitution of live food by formulated diets in
570 marine fish larvae. Aquaculture 200, 161-180.
571 Carnevali, O., Mosconi, G., Cambi, A., Ridolfi, S., Zanuy, S., Polzonetti-Magni, A.M.,
572 2001. Changes of lysosomal enzyme activities in sea bass (*Dicentrarchus labrax*)
573 eggs and developing embryos. Aquaculture 202, 249-256.
574 Castro-Ruiz, D., Querouil, S., Baras, E., Chota-Macuyama, W., Duponchelle, F., Núñez,
575 J., Renno, J.-F., García-Dávila, C., 2009. Determinación de parentesco en larvas
576 de *Pseudoplatystoma fasciatum* (Linnaeus, 1766) producidas en cautiverio. Folia
577 Amaz. 18, 33-40.
578 Castro-Ruiz, D., Baras, E., Fernández, C., Querouil, S., Chota-Macuyama, W.,
579 Duponchelle, F., Renno, J.-F., Darias, M., García-Dávila, C., Núñez, J., 2016.
580 Evaluación de la influencia parental en relación al crecimiento y supervencia
581 larval en familias de la doncella *Pseudoplatystoma punctifer*. Folia Amaz. 25, 77-
582 82.
583 Chakrabarti, I., Gani, M.A., Chaki, K.K., Sur, R., Misra, K.K., 1995. Digestive enzymes
584 in 11 freshwater teleost fish species in relation to food habit and niche segregation.
585 Comp. Biochem. Physiol. 112A, 167-177.
586 Crane, R.K., Boge, G., Rigal, A., 1979. Isolation of brush border membranes in vesicular
587 form from the intestinal spiral valve of the small dogfish (*Scyliorhinus canicula*).
588 Biochim. Biophys. Acta 554, 264-267.

589 Dabrowski, K., Portella, M.C., 2006. Feeding plasticity and nutritional physiology in
590 tropical fishes, in: Val, A.L., Almeida-Val, V.M.F., Randall, D.J. (Eds.), The
591 Physiology of Tropical Fishes. Elsevier, London, pp. 155-223.
592 Dahkqvist, A., 1970. Assay of intestinal disaccharidase. Enzym. Biol. Clin. 11,52–66.
593 Darias, M.J., Murray, H.M., Martínez-Rodríguez, G., Cárdenas, S., Yúfera, M., 2005.
594 Gene expression of pepsinogen during the larval development of red porgy
595 (*Pagrus pagrus*), Aquaculture 248, 245-252.
596 Darias, M.J., Castro-Ruiz, D., Estivals, G., Quazuguel, P., Fernández-Méndez, C., Núnez,
597 J., Clota, F., Gilles, S., García-Davila, C., Gisbert, E.; Cahu, C., 2015a. Influence
598 of dietary protein and lipid levels on growth performance and the incidence of
599 cannibalism in *Pseudoplatystoma punctifer* (Castelnau, 1855) larvae and early
600 juveniles. J. Appl. Ichthyol. 31, 74-82.
601 Darias, M.J., Castro-Ruiz D., García-Dávila, C., Gisbert, E., 2015b. Effects of Artemia
602 and inert diet enrichment with DHA on lipid deposition in the intestine and liver
603 of *Pseudoplatystoma punctifer* larvae and early juveniles, in: FENACAM &
604 LACQUA/SARA (WAS)'15: Abstract book. LACQUA, ABCC, WAS, Fortaleza,
605 p. 140.
606 de Amorim, M.P., Campos Gomes, B.V., Martins, Y.S., Sato, Y., Rizzo, E., Bazzoli, N.,
607 2009. Early development of the silver catfish *Rhamdia quelen* (Quoy and
608 Gaimard, 1824) (Pisces: Heptapteridae) from the Saõ Francisco River Basin,
609 Brazil. Aquac. Res. 40, 172-180.
610 Estivals, G., García-Dávila, C., Darias, M.J., 2015, Description of the skeletal anatomy
611 of reared juveniles of *Pseudoplatystoma punctifer* (Castelnau, 1855) with notes
612 on skeletal anomalies. J. Appl. Ichthyol. 31, 88-97.

613 Fernández-Méndez, C., David, F., Darias, M.J., Castro-Ruiz, D., Nuñez-Rodriguez, J.,
614 2015. Larval rearing of the Amazon catfish *Pseudoplatystoma punctifer*
615 (Castelnau, 1855): weaning with dry and moist diets. J. Appl. Ichthyol. 31, 83-87.
616 Frías-Quintana, C.A., Márquez-Couturier, G., Alvarez-González, C.A., Tovar-Ramírez,
617 D., Nolasco-Soria, H., Galaviz-Espinosa, M.A., Martínez-García, R., Camarillo-
618 Coop, S., Martínez-Yañez, R., Gisbert, E., 2015. Development of digestive tract
619 and enzyme activities during the early ontogeny of the tropical gar *Atractosteus*
620 *tropicus*. Fish Physiol. Biochem. 41, 1075-1091.
621 García-Careño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in
622 langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts.
623 J. Food Biochem. 17, 97-113.
624 García-Dávila, C., Duponchelle, F., Castro-Ruiz, D., Villacorta, J., Quérouil, S., Chota-
625 Macuyama, W., Núñez, J., Römer, U., Carvajal-Vallejos, F., Renno, J.-F., 2013.
626 Molecular identification of a cryptic species in the Amazonian predatory catfish
627 genus Pseudoplatystoma (Bleeker, 1962) from Peru. Genetica 141, 347-358.
628 Gilles, S., Ismiño, R., Sánchez, H., David, F., Núñez, J., Dugué, R., Darias, M.J., Römer,
629 U., 2014, An integrated closed system for fish-plankton aquaculture in Amazonian
630 fresh water. Animal 8, 1319-1328.
631 Gillooly, J.F., Charnov, E.L., West, G.B., Savage, V.M., Brown, J.H., 2002. Effects of
632 size and temperature on developmental time. Nature 417, 70-73.
633 Gisbert, E., Doroshov, S.I., 2006. Allometric growth in green sturgeon larvae. J. Appl.
634 Ichthyol. 22, 202-207.
635 Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y., Estévez, A., 2009. Development
636 of digestive enzymes in common dentex *Dentex dentex* during early ontogeny.
637 Aquaculture 287, 381-387.

638 Gisbert, E., Morais, S., Moyano, F.J., 2013. Feeding and Digestion, in: Qin J.G. (Ed.),
639 Larval fish aquaculture. Nova Science Publishers, New York, pp. 73-123.
640 Gisbert, E., Moreira, C., Castro-Ruiz, D., Ozturk, S., Fernández, C., Gilles, S., Nuñez, J.,
641 Duponchelle, F., Tello, S., Renno, J.-F., García-Davila, C., Darias, M.J., 2014.
642 Histological development of the digestive system of the Amazonian pimelodid
643 catfish *Pseudoplatystoma punctifer*. Animal 8, 1765-1776.
644 Gisbert, E., Nolasco, H., Solovyev, M., 2018. Towards the standardization of brush
645 border purification and intestinal alkaline phosphatase quantification in fish with
646 notes on other digestive enzymes. Aquaculture 487, 102-108.
647 Hamre, K., Yufera, M., Rønnestad, I., Boglione, C., Conceicao, L.E.C., Izquierdo, M.,
648 2013. Fish larval nutrition and feed formulation: knowledge gaps and bottlenecks
649 for advances in larval rearing. Rev. Aquacult. 5, S26-S58.
650 Holm, H., Hanssen, L.E., Krogdahl, A., Florholmen, J., 1988. High and low inhibitor
651 soybean meals affect human duodenal proteinase activity differently: In vivo
652 comparison with bovine serum albumin. J. Nutr. 118, 515-520.
653 Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile-salt
654 activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish
655 Physiol. Biochem. 18, 59-69.
656 Jiménez-Martínez, L.D., Álvarez-González, C.A., Tovar-Ramírez, D., Gaxiola, G.,
657 Sanchez-Zamora, A., Moyano, F.J., Alarcón, F.J., Márquez-Couturier, G.,
658 Gisbert, E., Contreras-Sánchez, W.M., Perales-García, N., Arias-Rodríguez, L.,
659 Indy, J.R., Páramo-Delgadillo, S., Palomino-Albarrán, I.G., 2012. Digestive
660 enzyme activities during early ontogeny in common snook (*Centropomus*
661 *undecimalis*). Fish Physiol. Biochem. 38, 441-454.

662 Kozarić, Z., Kuzir, S., Petrinec, Z., Gjurcvic, E., Bozic, M., 2008. The development of
663 the digestive tract in larval European catfish (*Silurus glanis* L). Anat. Histol.
664 Embryol. 37, 141–146.
665 Lazo, J.P., Holt, G.J., Arnold, C.R. 2000. Ontogeny of pancreatic enzymes in larval red
666 drum *Sciaenops ocellatus*. Aquacult. Nutr. 6, 183-192.
667 Lima, L.B., Oliveira, F.J., Giacomini, H.C. Lima-Junior, D.P. 2018. Expansion of
668 aquaculture parks and the increasing risk of non-native species invasions in Brazil.
669 Rev Aquacult. 10, 111-122.
670 Liu, W., Zhang, X.-M., Wang, L.-B., 2010. Digestive enzyme and alkaline phosphatase
671 activities during the early stages of *Silurus soldatovi* development. Zool. Res. 31,
672 627-632.
673 López-Ramírez, G., Cuenca-Soria, C.A., Alvarez-González, C.A., Tovar-Ramírez, D.,
674 Ortiz-Galindo, JL., Perales-García, N., Márquez-Couturier, G., Arias-Rodríguez,
675 L., Indy, J.R., Contreras-Sánchez, W.M., Gisbert, E., Moyano, F.J., 2010.
676 Development of digestive enzymes in larvae of Mayan cichlid *Cichlasoma*
677 *urophthalmus*. Fish Physiol. Biochem. 37, 197-208.
678 Magris, J., Sánchez, F.J., Gilles, S., Fernández, C., Núñez, J., Castro-Ruiz, D., García-
679 Dávila, C., Duponchelle, F., Tello, S., Renno J.-F., Gisbert, E., Darias, M.J., 2013.
680 Improving larval feeding protocols for Doncella*, Pseudoplatystoma punctifer*, by
681 enriching Artemia and compound diets, in: 4º Conferencia Latinoamericana sobre
682 Cultivo de Peces Nativos: Memorias. GRITOX, Villavicencio, p. 114.
683 Maroux, S., Louvard, D., Baratti, J., 1973. The aminopeptidase from hog-intestinal brush
684 border. Biochim. Biophys. Acta 321, 282-295.
685 Métais, P., Bieth, J., 1968. Détermination de l'α-amylase par une microtechnique. Ann.
686 Biol. Clin. 26, 133–142.

687 Moguel-Hernández, I., Peña, R., Nolasco-Soria, H., Dumas, S., Zavala-Leal, I., 2014.
688 Development of digestive enzyme activity in spotted rose snapper, *Lutjanus*
689 *guttatus* (Steindachner, 1869) larvae. Fish Physiol. Biochem. 40, 839-848.
690 Morais, S., Narciso, L., Dores, E., Pousao-Ferreira, P., 2004. Lipid enrichment for
691 Senegalese sole (*Solea senegalensis*) larvae: effect on larval growth, survival and
692 fatty acid profile. Aquac. Int. 12, 281-298.
693 Nicholson, J.A., Kim, Y.S., 1975. A one-step L-amino acid oxidase assay for intestinal
694 peptide hydrolase activity. Anal. Biochem. 63, 110-117.
695 Núñez, J., Dugué, R., Corcuy Arana, N., Duponchelle, F., Renno, J.-F., Raynaud, T.,
696 Hubert, N., Legendre, M., 2008. Induced breeding and larval rearing of Surubí,
697 *Pseudoplatystoma fasciatum* (Linnaeus, 1766), from the Bolivian Amazon.
698 Aquac. Res. 39, 764-776.
699 Nuñez, J., Castro, D., Fernández, C., Dugué, R., Chu-Koo, F., Duponchelle, F., García,
700 C., Renno, J.-F., 2011. Hatching rate and larval growth variations in
701 *Pseudoplatystoma punctifer*: maternal and paternal effects. Aquac. Res. 42, 764-
702 775.
703 Padilla, P., Alcantara, F., Ismiño, R., 2001. Reproducción inducida de la doncella
704 *Pseudoplatystoma fasciatum* y desarrollo embrionario-larval. Folia Amaz. 12,
705 141-154.
706 Porto-Foresti, F., Hashimoto, D.T., Prado, F.D., Senhorini, J.A., Foresti, F., 2013, Genetic
707 markers for the identification of hybrids among catfish species of the family
708 Pimelodidae. J. Appl. Ichthyol. 29, 643-647.
709 Pradhan, P.K., Jena, J., Mitra, G., Sood, N., Gisbert, E., 2013. Ontogeny of the digestive
710 enzymes in butter catfish *Ompok bimaculatus* (Bloch) larvae. Aquaculture 372-
711 375, 62–69.

712 Rangasin, W., Areechon, N., Yoonpundh, R., 2012. Digestive enzyme activities during
713 larval development of striped catfish, *Pangasianodon hypophthalmus* (Sauvage,
714 1878). Kasetsart J. (Nat. Sci.) 46, 217-228.
715 Rasband, W.S., 1997–2012. ImageJ. US National Institutes of Health, Bethesda.
716 Available at: http://imagej.nih.gov/ij/ (accessed on 4 September 2012).
717 Rønnestad, I., Yufera, M., Ueberschar, B., Ribeiro, L., Sæle, Ø., Boglione, C., 2013.
718 Feeding behaviour and digestive physiology in larval fish: Current knowledge,
719 and gaps and bottlenecks in research. Rev. Aquac. 5, S59-S98.
720 Saelee, T., Kiriratnikon, S., Suwanjarat, J., Thongboon, L., Pongsuwan, K., 2011. The
721 development of the digestive system in *Clarias nieuhofii* larvae: histology and
722 histochemical studies. J. Microsc. Soc. Thailand 4, 16-19.
723 Solovyev, M.M., Kashinskaya, E.N., Izvekova, G.I., Gisbert, E., Glupov, V.V., 2014.
724 Feeding habits and ontogenic changes in digestive enzyme patterns in five
725 freshwater teleosts. J. Fish Biol. 85, 1395-1412.
726 Solovyev, M.M., Campoverde, C., Öztürk, S., Moreira, C., Diaz, M., Moyano, F.J.,
727 Estévez, A., Gisbert, E., 2016. Morphological and functional description of the
728 development of the digestive system in meagre (*Argyrosomus regius*): An
729 integrative approach. Aquaculture 464, 381-391.
730 Solovyev, M., Gisbert, E., 2016. Influence of time, storage temperature and freeze/thaw
731 cycles on the activity of digestive enzymes from gilthead sea bream (*Sparus*
732 *aurata*). Fish Physiol. Biochem. 42, 1383-1394.
733 Srichanun, M., Tantikitti, C., Vatanakul, V., Musikarune, P., 2012. Digestive enzyme
734 activity during ontogenetic development and effect of live feed in green catfish
735 larvae (*Mystus emurus* Cuv. & Val.). Songlanakarin J. Sci. Technol. 34, 247-54.

736 Tengjaroenkul, B., Smith, B.J., Caceci, T., Smith, S.A., 2000. Distribution of intestinal
737 enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis*
738 *niloticus* L. Aquaculture 182, 317-327.
739 Uscanga-Martínez, A., Perales-García, N., Álvarez-González, C.A., Moyano, F.J., Tovar-
740 Ramírez, D., Gisbert, E., Márquez-Couturier, G., Contreras-Sánchez, W.M.,
741 Arias-Rodríguez, L., Indy, J.R., 2011. Changes in digestive enzyme activity
742 during initial ontogeny of bay snook *Petenia splendida*. Fish Physiol. Biochem.
743 37, 667-680.
744 Vaini, J.O., Grisolia, A.B., Prado, F.D., Porto-Foresti, F., 2014. Genetic identification of
745 interspecific hybrid of Neotropical catfish species (*Pseudoplatystoma corruscans*
746 vs. *Pseudoplatystoma reticulatum*) in rivers of Mato Grosso do Sul State, Brazil.
747 Neotrop. Ichthyol. 12, 635-64.
748 Valladão, G.M., Gallani, S.U., Pilarski, F., 2018. South American fish for continental
749 aquaculture. Rev. Aquacult. 10, 351-369.
750 Verreth, J.A., Torrelle, E., Spazier, E., Sluiszen, H.W., 1992. The development of a
751 functional digestive system in the African catfish, *Clarias garipinus*. J. World
752 Aquacult. Soc. 23, 286–298.
753 Worthington Biochemical Corporation, 1991. Worthington enzyme manual: Enzymes,
754 enzyme reagents, related biochemical. Worthington Biochemical Corp., Freehold,
755 New Jersey.
756 Yang, R., Xie, C., Fan, Q., Gao, C., Fang, L., 2010. Ontogeny of the digestive tract in
757 yellow catfish *Pelteobagrus fulvidraco* larvae. Aquaculture 302, 112-123.
758 Zambonino-Infante, J.L., Gisbert, E., Sarasquete, C., Navarro, I., Gutierrez, J., Cahu,
759 C.L., 2008. Ontogeny and physiology of the digestive system of marine fish

760 larvae, in: Cyrino, J.E.P., Bureau, D.P., Kapoor, B.G. (Eds.), Feeding and
761 digestive functions of fishes, Science Publishers, New Hamphire, pp. 281-348.

762 **Figure captions**

763

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

767

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

773

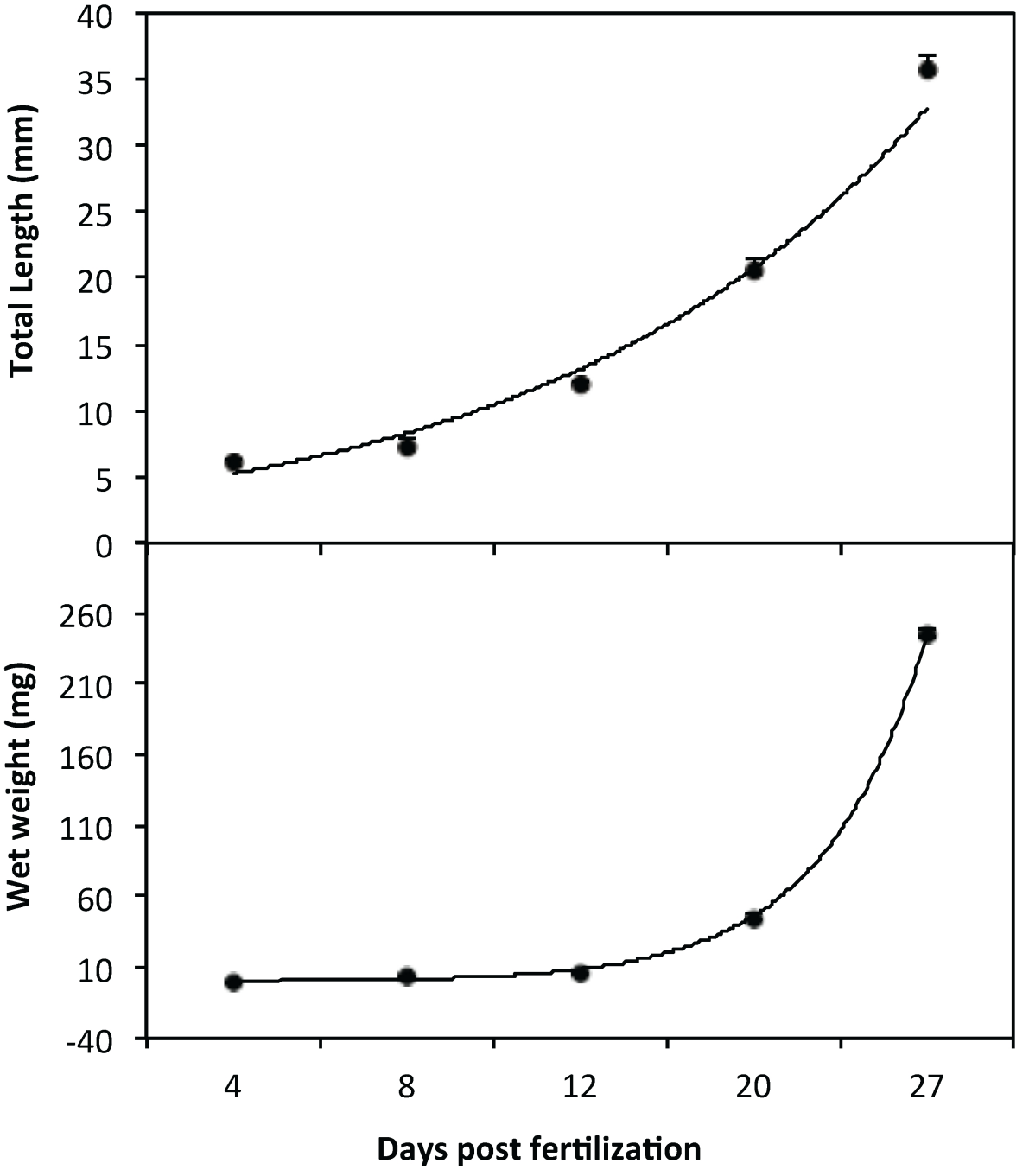
774 **Figure 3.** Intestinal maturation during the larval development of *P. punctifer* measured
775 as the ratio of specific activity of brush border membrane enzymes/cytosolic enzymes:
776 MAL/LAP, AP/LAP and AN/LAP. Results are expressed as mean ± SD (n = 3). Different
777 letters indicate significant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

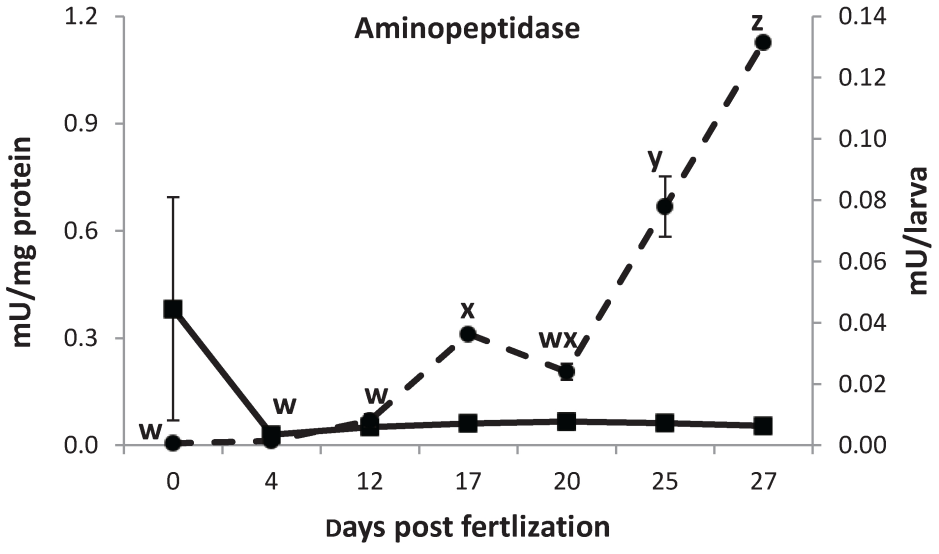
778

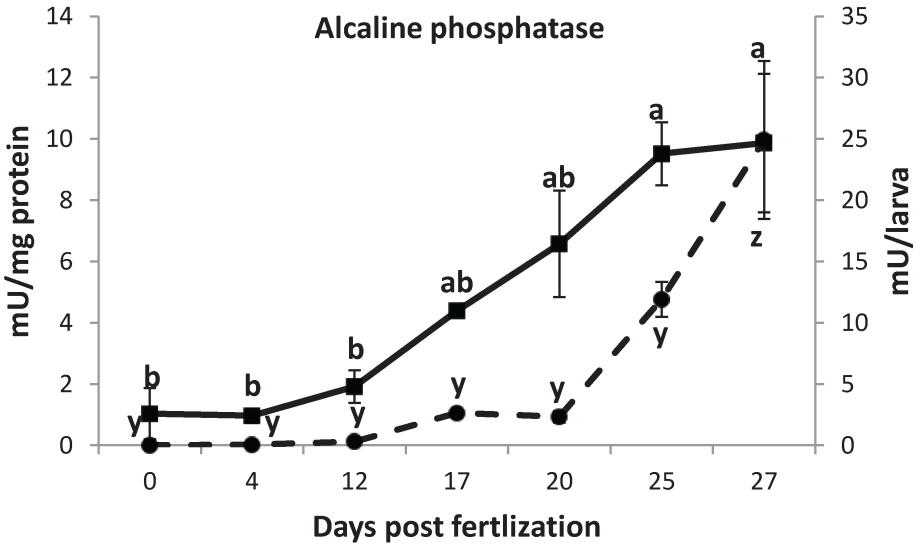
779 **Figure 4.** Specific (squares, mU mg^{-1} protein) and total (circles, mU $larva^{-1}$) activity of
780 pancreatic enzymes during the larval development of *P. punctifer.* Results are expressed
781 as mean ± SD (n=3). Different letters indicate significant differences (ANOVA, $P < 0.05$).

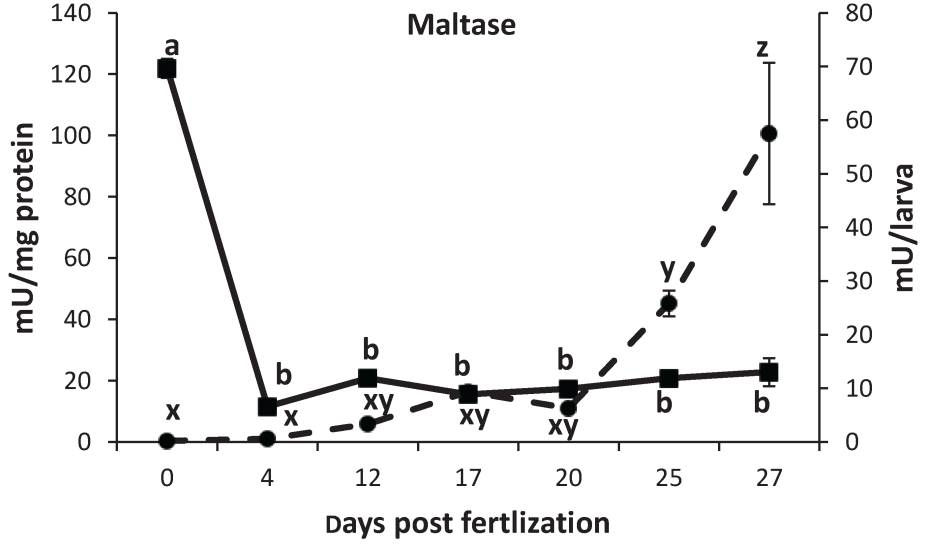
782

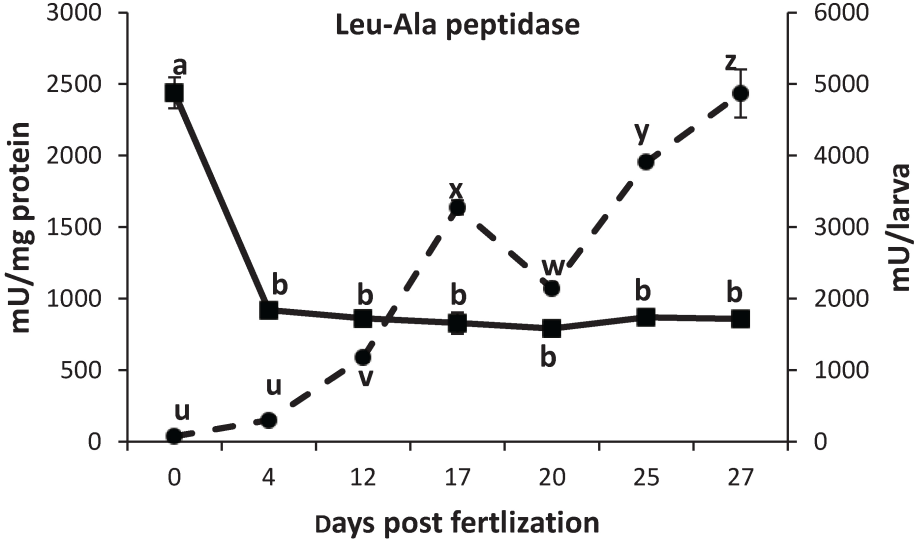
783 **Figure 5.** Specific (squares, mU mg^{-1} protein) and total (circles, mU $larva^{-1}$) activity of
784 pepsin during the larval development of *P. punctifer.* Results are expressed as mean ± SD
785 (n = 3). Different letters indicate significant differences (ANOVA, $P < 0.05$).

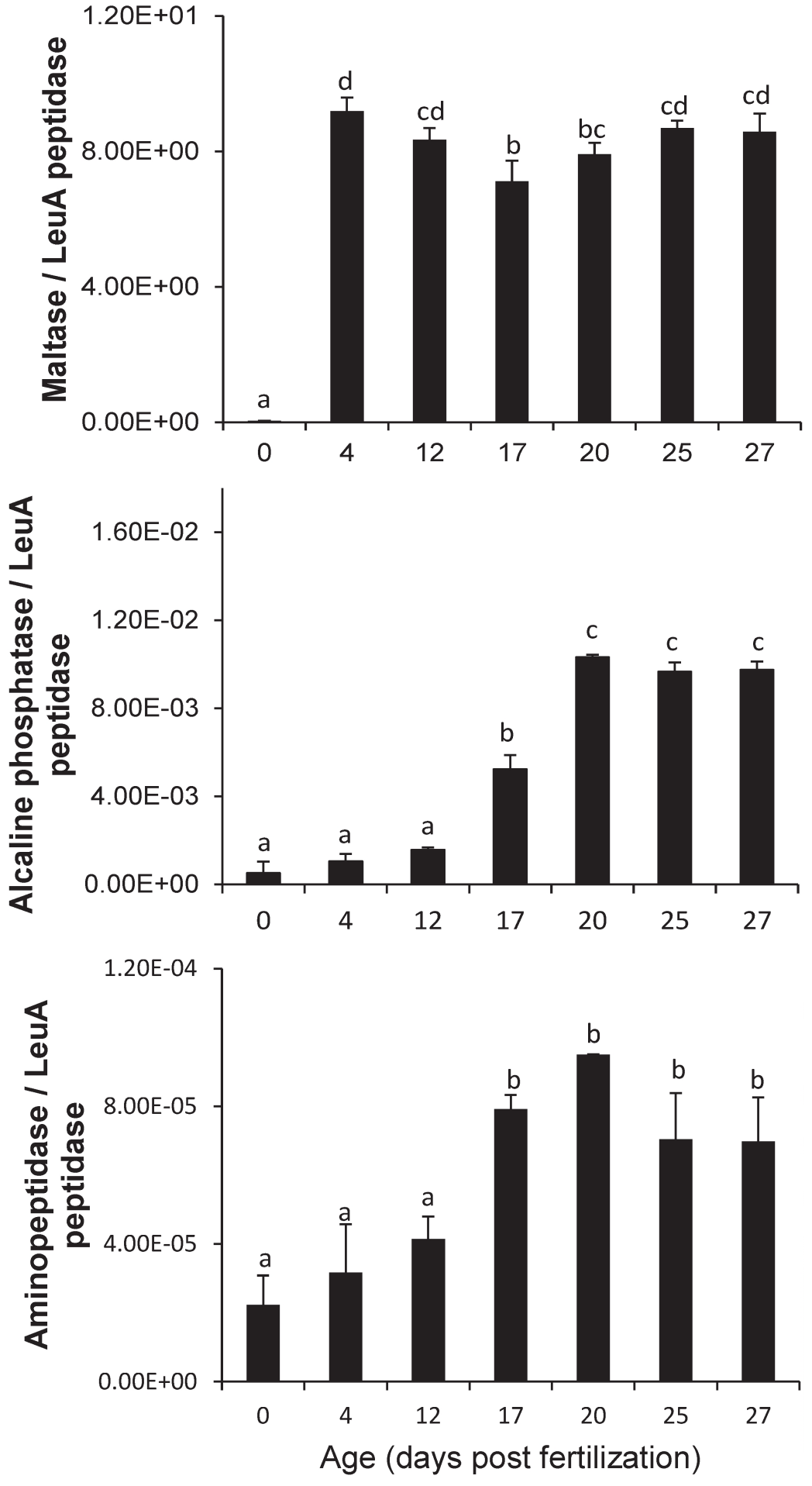

Total Length (mm)
40
35
30
25
20
15
10
5
0
Wet weight (mg)
260
210
160
110
60
10
-40
4
8
12
20
27
Days post fertilization

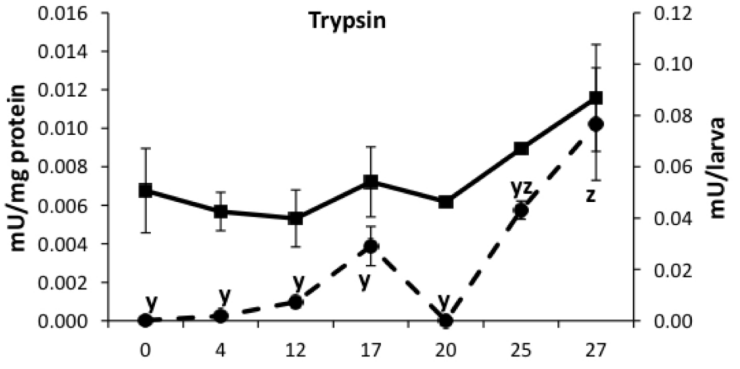

Aminopeptidase
mU/mg protein
mU/larva
Days post fertlization
1.2
0.9
0.6
0.3
0.0
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
4
12
17
20
25
27
w
w
w
x
wx
y
z

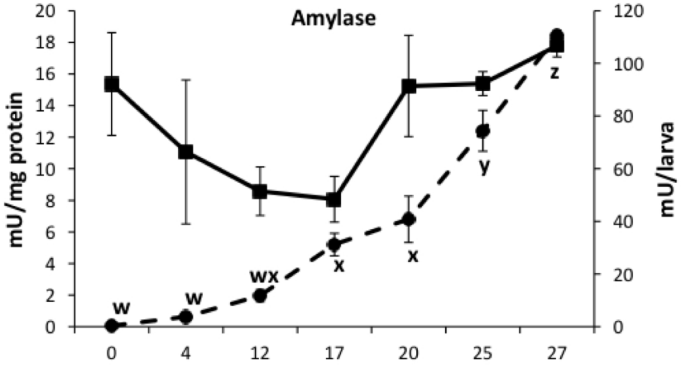

Alcaline phosphatase
mU/mg protein
mU/larva
Days post fertlization
14
12
10
8
6
4
2
0
35
30
25
20
15
10
5
0
0
4
12
17
20
25
27
b
b
b
ab
ab
a
a
y
y
y
y
y
z

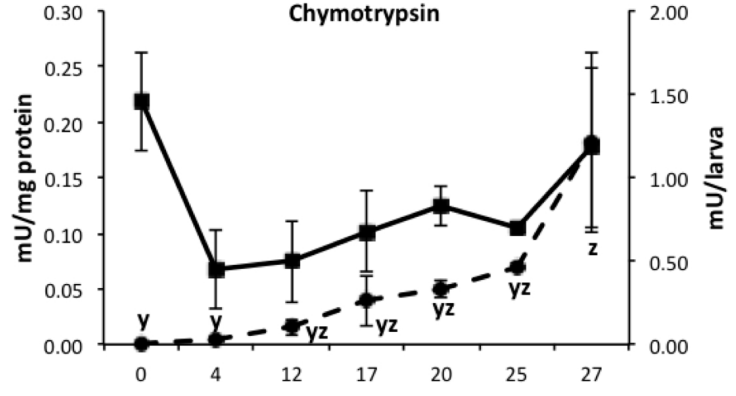

Maltase
mU/mg protein
mU/larva
Days post fertlization
140
120
100
80
60
40
20
0
80
70
60
50
40
30
20
10
0
0
4
12
17
20
25
27
a
x
b
x
b
xy
b
xy
b
xy
y
b
b
z

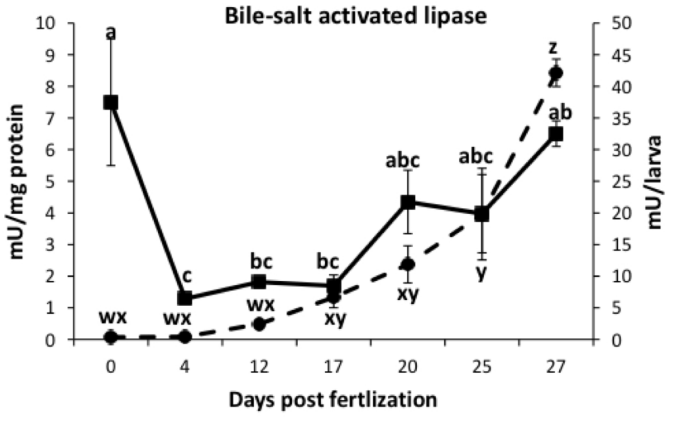

Leu-Ala peptidase
mU/mg protein
mU/larva
Days post fertlization
3000
2500
2000
1500
1000
500
0
6000
5000
4000
3000
2000
1000
0
0
4
12
17
20
25
27
a
u
b
u
b
v
b
x
b
w
b
y
b
z

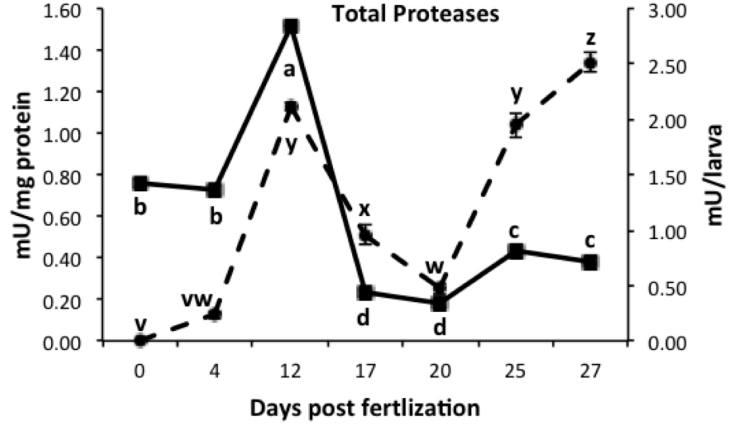

Maltase / LeuA peptidase
1.20E+01
8.00E+00
4.00E+00
0.00E+00
a
d
cd
b
bc
cd
cd
0
4
12
17
20
25
27
Alcaline phosphatase / LeuA peptidase
1.60E-02
1.20E-02
8.00E-03
4.00E-03
0.00E+00
a
a
a
b
c
c
c
0
4
12
17
20
25
27
Aminopeptidase / LeuA peptidase
1.20E-04
8.00E-05
4.00E-05
0.00E+00
a
a
a
b
b
b
b
0
4
12
17
20
25
27
Age (days post fertilization)

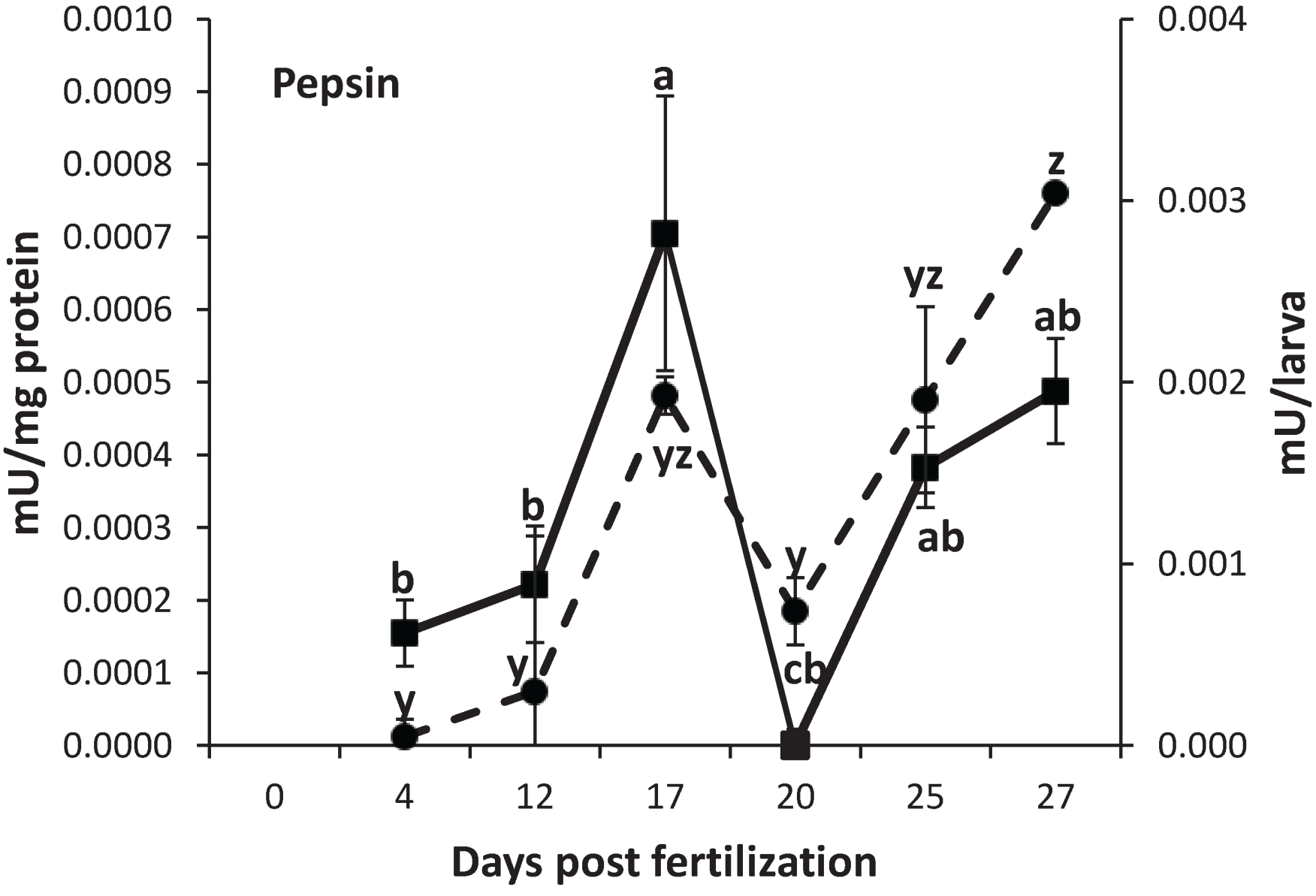

Trypsin
mU/mg protein
mU/larva
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0 4 12 17 20 25 27
y y y y y yz z


Amylase
mU/mg protein
mU/larva
20
18
16
14
12
10
8
6
4
2
0
120
100
80
60
40
20
0
0 4 12 17 20 25 27
w w wx x x y z


Chymotrypsin
mU/mg protein
mU/larva
0.30
0.25
0.20
0.15
0.10
0.05
0.00
2.00
1.50
1.00
0.50
0.00
0 4 12 17 20 25 27
y y yz yz yz yz z


Bile-salt activated lipase
mU/mg protein
mU/larva
10
9
8
7
6
5
4
3
2
1
0
50
45
40
35
30
25
20
15
10
5
0
0 4 12 17 20 25 27
Days post fertlization
a c bc bc abc abc z
wx wx wx xy xy y ab


Total Proteases
mU/mg protein
mU/larva
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0 4 12 17 20 25 27
Days post fertlization
b b a x d d c c
v vw y w z y


Pepsin
mU/mg protein
mU/larva
Days post fertilization
0.0010
0.0009
0.0008
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
0.004
0.003
0.002
0.001
0.000
0
4
12
17
20
25
27
a
b
b
y
y
yz
y
cb
yz
z
ab
ab