



Larval digestive system ontogeny and early weaning in neon tetra *Paracheirodon innesi*

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Abstract The intensive culture of characid teleosts for ornamental trade is highly dependent on live feed organisms, particularly *Artemia* nauplii, to provide nutrition through the larval stage. Live feeds have inherent disadvantages relative to prepared microparticulate diets (MDs), specifically availability, labor and cost. In this research, the dependence of larval *Paracheirodon innesi* on live *Artemia* was confirmed via a nutritional trial. Next, digestive system ontogeny was characterized from the onset of exogenous feeding through metamorphosis. *P. innesi* exhibited an agastric larval stage, as well as low digestive enzyme activity at the onset of exogenous feeding followed by abrupt increases in trypsin, lipase and pepsin activity. Differentiation of the stomach, including gastric gland formation and production of neutral mucopolysaccharides, as well as the onset of pepsin activity, did not occur until 20 days post hatch (dph; 5.24 ± 0.20 mm). This shift from agastric to gastric digestive modes is indicative of a proliferation of digestive capacity and subsequent prey diversity in other fish species exhibiting similar altricial larval stages.

Based on this information, different schedules for weaning from *Artemia* to a MD were evaluated. For

P. innesi fed until 32 dph, weaning beginning at 12 dph and 17 dph resulted in similar survival to live *Artemia* (mean: $22.0 \pm 1.7\%$), and the MD resulted in the lowest survival ($0.8 \pm 0.3\%$). These results indicate that weaning is possible prior to gastric differentiation, potentially resulting in the reduction of *Artemia* use in the larval culture *P. innesi*.

Keywords Neon tetra · Larval nutrition · Weaning · Gastrointestinal development

Introduction

The neon tetra *Paracheirodon innesi* is a small, neotropical characid that is one of the most highly traded fish in the ornamental industry (Weitzman et al. 1983; Chapman et al. 1997, 1998). While an estimated 80% of the market demand, or 12–15 million individuals annually, is satisfied via import from wild collections originating in their native range, foreign and domestic aquaculture of *P. innesi* represents an economically significant endeavor with the potential for expansion (Chapman et al. 1998; Chao 2001; Kucharczyk et al. 2010). *P. innesi*, like many other species in the ornamental aquaculture trade, are characterized by having very small, poorly developed larvae at first feeding, which has led to the ubiquitous use of live zooplankters as first feed items (Lim et al. 2003; Sanaye et al. 2014). While rotifers (*Brachionus* spp.), *Paramecium* spp., and other live feed organisms have been used

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during the critical larval stage of *P. innesi* culture, newly hatched *Artemia franciscana* nauplii are the most widely used for this application (Chapman et al. 1998; Lim et al. 2003; Sanaye et al. 2014).

Commercial producers of freshwater ornamental fish rely heavily on newly-hatched 1–2 instar stage *A. franciscana* for early larval nutrition in intensive and semi-intensive aquaculture operations (Lim et al. 2003). This wild caught feed item, while convenient and nutritious relative to some other live feeds, is characterized by increasing costs, limited availability, and variable quality, with periods of shortage being a primary concern to ornamental fish producers. Commercially available microparticulate diets (MDs) can provide more complete nutrition than live feeds and are more convenient and reliable (Hamre et al. 2013; Kolkovski 2013; Lipscomb et al. 2020a). While total replacement of live feeds with MDs is ideal, this is not feasible for all species, particularly those with small, altricial larval stages (Rønnestad et al. 2003). In many marine fishes, as well as some freshwater species, first feeding larvae are physiologically incapable of utilizing complex nutrients found in MDs, therefore reduced reliance on live feeds requires optimizing the timing of weaning following critical developmental milestones (Cahu and Zambonino Infante 2001; Kolkovski 2001; Faulk and Holt 2009).

The gastrointestinal (GI) tract in teleosts begins as a straight and undifferentiated tube that generally resembles the intestine of a more developed juvenile (Govoni et al. 1986). At the earliest stages, collective digestive enzyme activities are generally low, and proteolytic hydrolysis is primarily catalyzed in the intestinal lumen by trypsin and other trypsin-like enzymes of pancreatic origin (Holt 2011). In gastric fishes, developing larvae exhibit incremental increases in activities of individual enzymes, as well as the differentiation of the anterior GI into a functioning stomach. Gastric glands, a pyloric valve, and folding and elongation of the GI, in conjunction with the production of hydrochloric acid, pepsin, and protective neutral mucopolysaccharides all characterize this critical developmental milestone (Holt 2011). The development of the stomach affords the larvae a greatly increased aptitude for digesting complex nutrients, particularly those present in MDs (Rønnestad et al. 2003; Thompson et al. 2019). Previous authors have used the timing of the differentiation of the stomach, as well as peaks in digestive enzyme

activities, to develop data-based weaning protocols for use in the production of fishes that exhibit this delayed mode of larval development (Faulk and Holt 2009; Thompson et al. 2019).

Many members of the order Characiformes have been identified as species that exhibit extended agastric larval phases, including pacu *Piaractus mesopotomicus*, bocón *Brycon amazonicus*, and dorado *Salminus brasiliensis*, as reviewed by Portella et al. (2014). Additionally, it has been shown that live-feed dependence of the black tetra *Gymnocorymbus ternetzi* larvae at first feeding was due to an agastric larval period and weaning to MD is possible shortly before stomach differentiation (Lipscomb et al. 2020b). By categorizing the GI development and digestive enzyme ontogeny of *P. innesi*, a reduction in weaning time and minimization of reliance on *Artemia* can be achieved.

In addition to utilizing biochemical assays to quantify digestive enzyme activities, histological and histochemical analysis of GI structures provides further evidence for timing of stomach differentiation, including the formation of gastric glands, which house oxynticopeptic cells, responsible in teleosts for producing both pepsinogen and hydrochloric acid. Additionally, the presence of neutral pH mucopolysaccharides, produced by the epithelial cells of the stomach to protect the tissues from hydrochloric acid and autolytic hydrolysis catalyzed by pepsin, indicate the presence of a functional stomach organ (Zambonino Infante and Cahu 2007; Rønnestad et al. 2013; Yúfera et al. 2018; Nolasco-Soria et al. 2020). The appearance of these milestones can be used to identify the timing of stomach differentiation and subsequently optimize weaning schedules.

Here, we used an applied nutritional trial to evaluate the effectiveness of feeding protocols associated with three commercially available MDs relative to *Artemia* nauplii for first feeding in *P. innesi*. We also characterized the development of the gastrointestinal tract using histology (H & E stain) and histochemistry (PAS), as well as the ontogeny of three critical digestive enzymes (trypsin, bile-salt dependent lipase, and pepsin) through 32 days post hatch (dph), a time period inclusive of stomach differentiation. Subsequently, the appearance of gastric glands, PAS positive neutral mucopolysaccharides, and initiation of pepsin activity were used to guide empirical evaluations of weaning from *Artemia* to a MD. Lastly, the

impacts of different weaning regimes on survival, growth, and digestive enzyme activity were assessed. The results of this study can be used to optimize the larval culture of *P. innesi*, reducing the overall reliance of commercial producers on live feeds during this critical production stage.

Materials and methods

General methods

Larval *P. innesi* for each trial were acquired from a commercial ornamental producer (VW Fish Hatcheries Inc, Lakeland, FL). At the hatchery, brood fish were placed in pairs in individual 10-L spawning tanks with spawning media and were allowed to volitionally spawn. Following spawning, brood fish were removed, and embryos hatched *in-situ*. At 1 dph, larvae were pooled and transferred to the University of Florida's Tropical Aquaculture Laboratory, Ruskin, FL where they were then counted by hand into experimental tanks. Dissolved oxygen (DO), pH, temperature, total ammonia nitrogen (TAN), and nitrite-N were monitored weekly for each tank in each trial. DO (7.40 ± 0.34 mg l⁻¹, average \pm standard deviation) and temperature (24.96 ± 0.60 °C) were measured using an optical dissolved oxygen meter (ProComm II, YSI Industries, Yellow Springs, OH). TAN (0.2 ± 0 mg l⁻¹), nitrite (0), and pH (8 ± 0) were measured using colorimetric assays according to manufacturer's protocols (Hach Company, Loveland, CO).

Nutritional trial

Two dph larval *P. innesi* were stocked into twenty-four 3 L tanks at a density of 200 larvae per tank. Tanks were maintained on flow-through, degassed well water for the duration of the feeding trial. Beginning at 4 dph, concurrent with yolk-sac depletion, larvae were offered one of four feeding treatments ($n=6$ per treatment) ad libitum to satiation, as indicated by the presence of feed in the tank following 15 min of feeding time. The treatments were as follows: newly hatched, 1st – 2nd instar stage *A. franciscana* nauplii twice per day, or one of three commercial MDs twice per day [MD1 (Larval AP-100, < 100 μ m, Ziegler Bros Inc., Gardners, PA; 50% protein, 12% fat), MD2 (Golden Pearls 50 -100 μ m, Brine Shrimp

Direct, Ogden, UT, USA; 60% protein, 8% fat), and MD3 (Otohime A1, 75–150 μ m, Marubeni Nishin Feed Co. LTD, Tokyo, Japan; 53% protein, 8% fat)]. *Artemia* cysts were decapsulated in 10% sodium hypochlorite and stored at 4 °C prior to hatching in a 30 g/L saline solution.

For evaluation of larval feeding, two feeding techniques were employed, congruent with established methods for live feed delivery and MD delivery, respectively (Siccardi et al. 2009). For feeding *Artemia*, nauplii were harvested using a small brine net and transferred to a 100 ml sample cup filled with degassed well water. Nauplii were then introduced to the experimental tanks to reach a concentration of 100 nauplii/L per feeding. MDs were simply added directly to the tank at a rate of ~13.3 mg/L per day, spread across two feedings. Feeding frequency was based established feeding protocols employed by commercial producers of *P. innesi* (personal communication Eric Cassiano, Assistant Extension Scientist, University of Florida). Following 10 days of feeding (14 dph; 4.71 ± 0.10 mm), all larvae remaining in each tank were harvested and enumerated.

Digestive ontogeny characterization study

Two dph larvae were stocked into each of three 10 L tanks maintained on flow-through, degassed well water at a density of 600 per tank. Prior to the first feeding of the day at 3, 5, 7, 9, 11, 14, 17, 20, 23, 26, and 28 dph, 10 larvae from each tank were harvested and comingled in the same sampling cup ($n=30$ larvae per sampling day). All 30 larvae were photographed on a Sedgewick rafter cell and measured for notochord length (NL) prior to hypural plate formation (20 dph, 5.23 ± 0.20 mm) or standard length (SL) thereafter (mm). After photographs were taken, 20 larvae were placed on a Kimwipe to absorb excess moisture, placed in 1.5 ml microcentrifuge tubes, and stored at -80°C for future processing for pancreatic and gastric digestive enzyme assays (10 larvae for each category of enzyme). The remaining 10 larvae were placed in a 1.5 ml microcentrifuge tube filled with 1 ml of Trump's fixative (Electron Microscopy Sciences, Hatfield, PA) and stored at 4°C for future histological and histochemical processing.

All histological and histochemical processing was completed at the Fish and Wildlife Research Institute, operated by the Florida Fish and Wildlife

Conservation Commission, St. Petersburg, FL. Samples were embedded in epoxy, longitudinally sectioned, and stained with either routine H & E (all sampling days), or periodic acid Schiff's (PAS)/iron-hematoxylin with metanil yellow for contrast (14, 17, 20, and 23 dph; 4.71 ± 0.10 , 5.56 ± 0.12 , 5.24 ± 0.20 , and 5.87 ± 0.13 mm, Quintero-Hunter et al. 1991) to identify neutral mucopolysaccharides produced in the functioning, glandular stomach (Çinar and Şenol 2006; Ghosh and Chakrabarti 2015).

Weaning study

Following characterization of GI development and digestive enzyme ontogeny, a study was conducted to evaluate the effect of different weaning schedules on survival, growth and digestive enzyme activity. Twenty 10 L tanks maintained on flow-through, degassed well water were each stocked with 500 *P. innesi* larvae at 1 dph. Fish were fed twice daily beginning at 4 dph using one of five different feeding regimes: *Artemia* nauplii for the entirety of the trial, MD3 for the entirety of the trial, or one of three weaning schedules ($n=4$, Fig. 1). During periods of cofeeding, half rations of each MD3 and *Artemia* were given. MD3 was chosen for the weaning trial based on having the highest numerical survival following the nutritional trial. Weaning schedules consisted of a period of feeding exclusively *Artemia* from

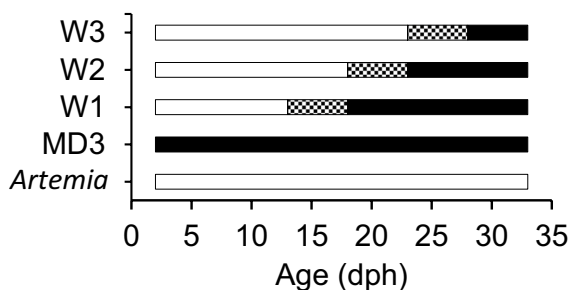


Fig. 1 Experimental feeding regimes for weaning optimization trial. Exclusive feeding with *Artemia* nauplii is indicated by black sections, while exclusive feeding with MD3 is indicated by white. Checkered sections indicate periods of cofeeding. Weaning schedules were as follows: W1 (*Artemia* 4 – 12 dph; cofeed 13 – 17 dph; MD1 18 – 32 dph), W2 (*Artemia* 4 – 17 dph; cofeed 18 – 22 dph; MD3 23 – 32 dph), and W3 (*Artemia* 4 – 22 dph; cofeed 23 – 27 dph; MD3 28 – 32 dph). Dph=days post hatch

first feeding, followed by a 5-day period of cofeeding with *Artemia* and MD3, and finally a transition to exclusively MD3. Schedules were as follows: W1 (*Artemia* 4 – 12 dph; cofeed 13 – 17 dph; MD1 18 – 32 dph), W2 (*Artemia* 4 – 17 dph; cofeed 18 – 22 dph; MD3 23 – 32 dph), and W3 (*Artemia* 4 – 22 dph; cofeed 23 – 27 dph; MD3 28 – 32 dph). At 32 dph, each tank was harvested, fish were enumerated, and 20 fish were photographed, measured and frozen at -80°C for future processing for pancreatic and gastric digestive enzyme assays as previously described. Tanks fed exclusively MD3 exhibited low survival and were thus excluded from subsequent enzymatic assays.

Digestive enzyme activity analysis

Digestive enzyme activity assays were performed according to Zambonino Infante and Cahu (1994, pepsin), and Faulk et al. (2007, trypsin and bilesalt-dependent lipase). On the morning of each assay, samples were thawed in an aluminum cooling chamber nested in ice. For measuring pepsin activity, pooled samples of 10 larvae were homogenized with a motorized micro-pestle in 400 μl reagent grade deionized water. Samples were then centrifuged for 10 min at 1700 G at 4°C , and 100 μl of each supernatant was transferred to a new microcentrifuge tube. In addition, a negative control with 100 μl of reagent grade deionized water, as well as a positive control with 100 μl of 50 $\mu\text{g/ml}$ pepsin solution were aliquoted into microcentrifuge tubes. With tubes in a water bath at 37°C , 500 μl of hemoglobin substrate (60 mM HCl, 2% w/v equine hemoglobin) was added to each sample. After 20 min, 1 ml of 5% trichloroacetic acid was added to the tubes to quench the reaction. For each sample, a blank was created by adding 1 ml of 5% trichloroacetic acid prior to the addition of the hemoglobin substrate. Samples and blanks were centrifuged for 10 min at 1700 G and 20 μl of each supernatant was pipetted into a 96-well plate in triplicate, in addition to positive and negative controls. The concentration of tyrosine liberated from hydrolysis of the substrate was measured as absorbance at 280 nm using a BioTek spectrophotometer (Synergy HTX Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA). Pepsin activity was expressed as μmol of tyrosine liberated per minute.

Samples of 10 fish for both trypsin and bile-salt dependent lipase were homogenized in a buffer solution (20 mM Tris–HCl, 1 mM EDTA, 1 mM CaCl_2 , 7.5 pH) in 1.5 ml microcentrifuge tubes and centrifuged for 10 min at 1700 G at 4 °C. To measure trypsin activity, 20 μl of each sample supernatant, as well as 15 $\mu\text{g}/\text{ml}$ porcine trypsin solution (positive control) and homogenization buffer (negative control) were pipetted in triplicate into a 96-well plate resting on an aluminum cooling plate in ice. Trypsin substrate solution (100 μL , 1 mM N_α -benzoyl-DL-arginine *p*-nitroanilide dissolved in dimethyl sulfoxide) was quickly added to each well using a multi-channel pipette. At 30 °C, liberation of *p*-nitroaniline from hydrolysis of the substrate was measured as absorbance at 410 nm every 45 s for 30 min. Trypsin activity was expressed as μmol of *p*-nitroaniline liberated per minute (U).

For bile-salt dependent lipase, 10 μl of each sample supernatant, 7.5 $\mu\text{g}/\text{ml}$ lipase enzyme standard solution (positive control) and homogenization buffer (negative control), were pipetted in triplicate into a 96-well plate resting on an aluminum cooling plate in ice. Quickly, 200 μl of lipase substrate solution (0.35 mM 4-nitrophenyl *n*-Caproate (4-NPC) dissolved in 70 μl ethanol, 0.5 M Tris–HCl, 6 mM sodium taurochlorate, 1 M NaCl, pH=7.4) was added to each well. At 30°C, hydrolyzation of 4-NPC was measured at 400 nm absorbance and units were expressed as 1 μM 4-NPC hydrolyzed per minute (U).

Statistical analysis

Water quality parameters among treatments were compared using one-way ANOVA following verification of assumptions of normality using a Shapiro-Wilks test and homogeneity of variance using a Bartlett's test. Growth rate recorded during the GI characterization study was determined using linear regression. Proportional survival data for each trial was evaluated using the *glm* function (family = binomial) in R to perform a logistic regression on the Bernoulli distributed survival data (Crawley 2005). Effect of treatment on survival was evaluated using a likelihood ratio test, followed by a post hoc pairwise Tukey HSD test on the log odds of survival if a significant effect of treatment was detected. All survival data are reported as mean percent survival \pm standard error (SE) of the proportion. Standard lengths and digestive

enzyme activities resulting from different treatment groups were compared using one-way ANOVA following confirmation of assumptions of normality and homogeneity of variance. If a significant effect of treatment on standard length or enzyme activity was detected, a post hoc pairwise Tukey HSD test was used to compare differences between individual treatments. Values for SL and digestive enzyme activity (U) are reported as mean \pm standard error of the mean. If individual tanks had zero surviving larvae at the conclusion of the trial, these tanks were omitted from analysis of SL and an unbalanced design was employed. A priori significance levels were set at $\alpha=0.05$ and all analyses were completed in Program R (RStudio, V. 0.99.903 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA).

Results

Nutritional trial

Survival varied significantly among treatments following 10 days of initial feeding with either *Artemia*, MD1, MD2, or MD3 ($\chi^2_4=1,187.300$, $P<0.001$, Fig. 2a). Feeding behavior in each treatment group was confirmed visually. Survival of *P. innesi* larvae fed *Artemia* nauplii from first feeding (4 dph) until 14

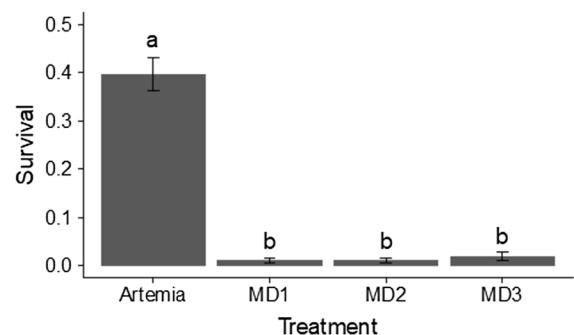


Fig. 2 Proportional survival \pm standard error following 10 days of feeding with either newly hatched *Artemia* nauplii or one of three commercial microparticulate diets: MD1 (Larval AP-100, < 100 μm , Ziegler Bros Inc., Gardners, PA, 50% protein, 12% fat), MD2 (Golden Pearls, 50–100 μm , Brine Shrimp Direct, Ogden, UT, USA, 60% protein, 8% fat), and MD3 (Otohime A1, 75–150 μm , Marubeni Nisshin Feed Co. LTD, Tokyo, Japan, 53% protein, 8% fat). Different letters above columns denote statistically significant differences ($P \leq 0.050$)

dph ($39.8 \pm 3.3\%$; 4.71 ± 0.09 mm) was significantly higher than the survival resulting from MDs (mean: $1.4 \pm 0.6\%$, $P < 0.001$).

Digestive ontogeny characterization study

Larval *P. innesi* growth from 3 to 29 dph was linear ($R^2=0.887$) and NL or SL increased at a rate of 0.154 mm/day (Fig. 3). Mean NL at 3 dph was 3.26 ± 0.03 mm and increased to a mean SL of 7.25 ± 0.26 mm at 29 dph. Yolk was present at 3 dph (3.26 ± 0.03 mm) and was nearly completely absorbed by 5 dph (3.61 ± 0.04 mm). The GI tract persisted as a straight, uniform tube divided into esophagus, intestine (including stomach anlage), and rectum from 3 dph (3.26 ± 0.03 mm) until 20 dph (5.24 ± 0.20 mm), when differentiation of the stomach was observed (Fig. 4). The buccopharyngeal cavity and esophagus were characterized by simple squamous epithelial cells and lacked extensive mucous cells prior to 9 dph (3.98 ± 0.07 mm). At 9 dph (3.98 ± 0.07 mm), PAS positive mucous cells began to proliferate and flame cells were apparent in the esophagus, a pattern that persisted throughout the observed developmental period. Intestinal rugae proliferated and deepened at 7 dph (3.50 ± 0.06 mm) in the stomach anlage region. Intestinal epithelial cells were columnar in shape, becoming cuboidal in the stomach region following differentiation. The muscularous layer of the esophageal region began to thicken and was more pronounced following 14 dph (4.71 ± 0.10 mm).

While there was a distinct lack of teeth in the mouth region throughout the observed developmental stages, pharyngeal tooth buds appeared in the pharynx at 17 dph (5.56 ± 0.12 mm) and erupted pharyngeal teeth appeared at 20 dph (5.24 ± 0.20 mm). Intestinal epithelial cells exhibited extensive supranuclear vacuoles beginning at 20 dph (5.24 ± 0.20 mm), which proliferated distally throughout the GI. Mucous cells proliferated in the rectal region of the GI beginning at 14 dph. At 20 dph (5.24 ± 0.20 mm), gastric glands were present in the stomach and a pyloric valve separated the stomach and the anterior intestine (Figs. 4 and 5). The GI exhibited a fold initiating between the pyloric region of the stomach and the anterior intestine (Fig. 4c). By 29 dph (7.25 ± 0.26 mm), further folding along the entirety of the GI occurred, as well as proliferation of gastric glands in the lamina propria and the appearance of sections of 6 distinct pyloric caeca (Figs. 4d and 5d). The muscularous layer thickened in conjunction with stomach differentiation at 20 dph. Histochemical preparations with PAS stain revealed a distinct absence of neutral mucopolysaccharides in the stomach anlage prior to gastric gland formation (Fig. 6a). Following the appearance of gastric glands and concurrent stomach differentiation, the apical surface of the stomach epithelium as well as much of the stomach lumen were strongly PAS positive, indicating the presence of neutral mucopolysaccharides in these regions (Fig. 6c, d).

While trypsin activity was detectable from 3 dph (3.26 ± 0.03 mm), activity on a per fish basis

Fig. 3 Growth curve for *P. innesi* fed exclusively newly hatched *Artemia* nauplii from 3 to 29 days post hatch (dph) from larvae in digestive ontogeny characterization trial. Values are either notochord length (mm, < 20 dph) or standard length (mm, ≥ 20 dph) \pm standard error

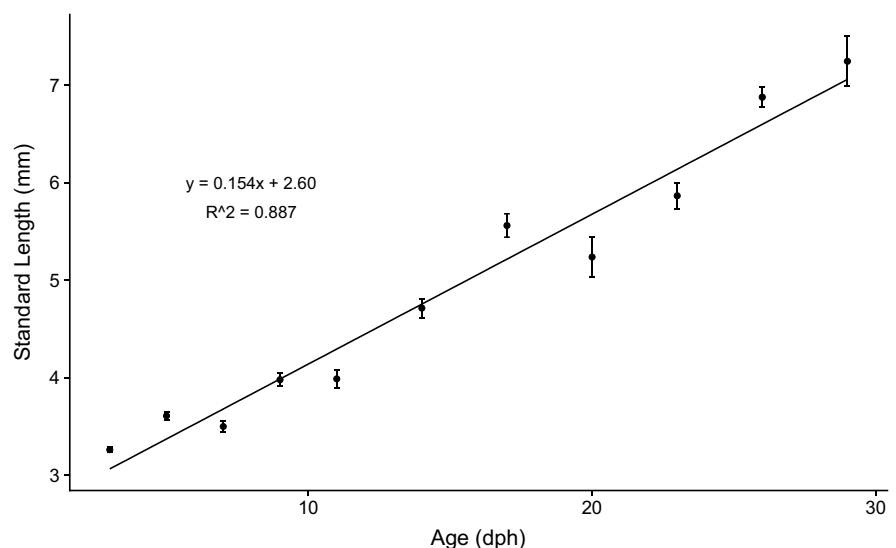
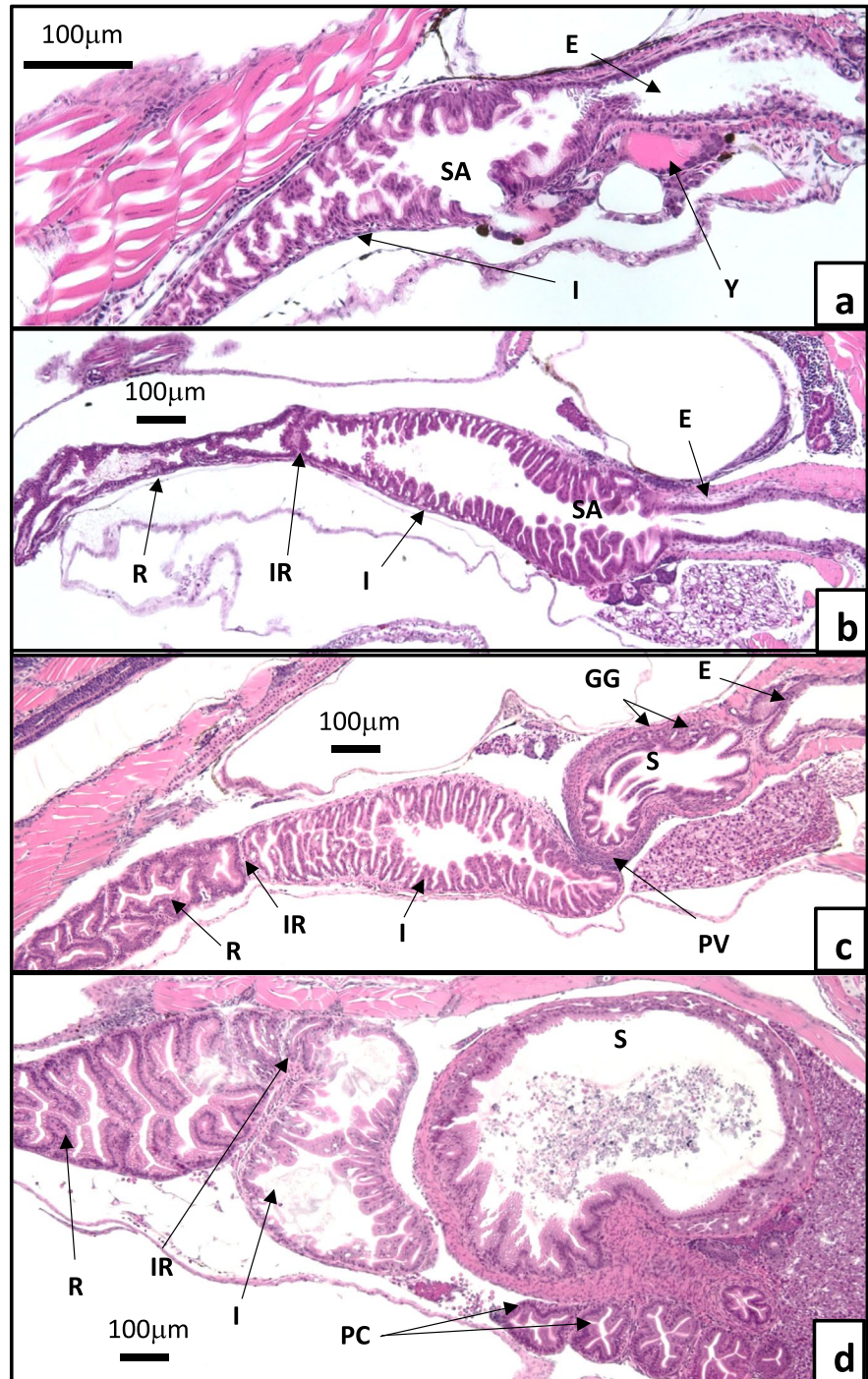


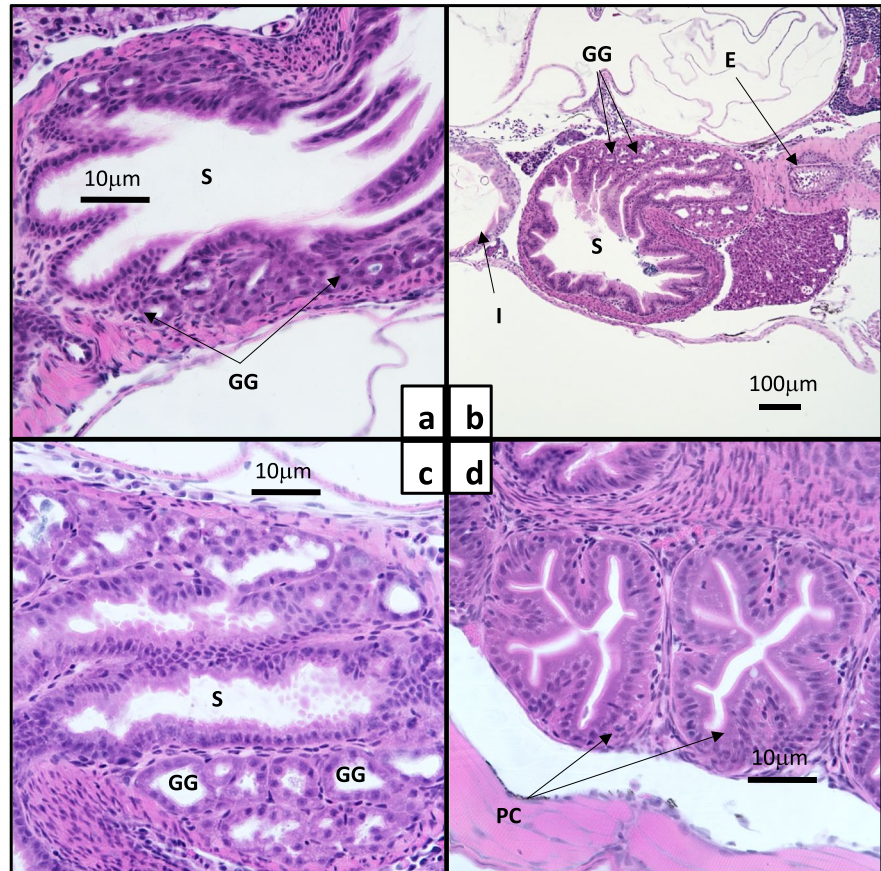
Fig. 4 Photomicrographs of histological preparations (H & E stain) of *P. innesi* gastrointestinal tract at **a**) 5 days post hatch (dph, 200x), **b**) 14 dph (100x), **c**) 20 dph (100x), and **d**) 29 dph (100x) from larvae in digestive ontogeny characterization trial. Esophagus (E), stomach anlage (SA), stomach (S), gastric glands (GG), pyloric valve (PV), pyloric caeca (PC), intestine (I), ileo-rectal valve (IR), rectum (R), yolk (Y)



remained relatively low (δ 144.95 U/fish) until 29 dph (7.25 ± 0.26 mm), when a 6.42-fold increase to 931.06 U/fish occurred (Fig. 7a). Lipase exhibited a similar pattern, with enzyme activities of $\leq 12,655.71$ U/fish occurring between 3 (3.26 ± 0.03 mm) and 20

dph (5.24 ± 0.20 mm), followed by sharp increases at 23 (5.87 ± 0.13 mm), 26 (6.88 ± 0.32 mm), and 29 dph (7.25 ± 0.26 mm) to 45,672.15 U/fish (Fig. 7b). Pepsin activity was undetectable until 11 dph (3.99 ± 0.09 mm), when very slight activity (0.342

Fig. 5 Photomicrographs of histological preparations (H & E stain) of *P. innesi* gastrointestinal tract at **a**) 20 dph (400x), **b**) 29 dph (100x), **c**) 29 dph (400x), and **d**) 29 dph (400x) from larvae in digestive ontogeny characterization trial. Esophagus (E), stomach (S), gastric glands (GG), pyloric caeca (PC), Intestine (I)



U/fish) was detected. Pepsin activity was low or not detected (1.58 U/fish) until 29 dph (7.25 ± 0.26 mm), when activity increased 3.78 times relative to the previous peak (5.97 U/fish, Fig. 7c).

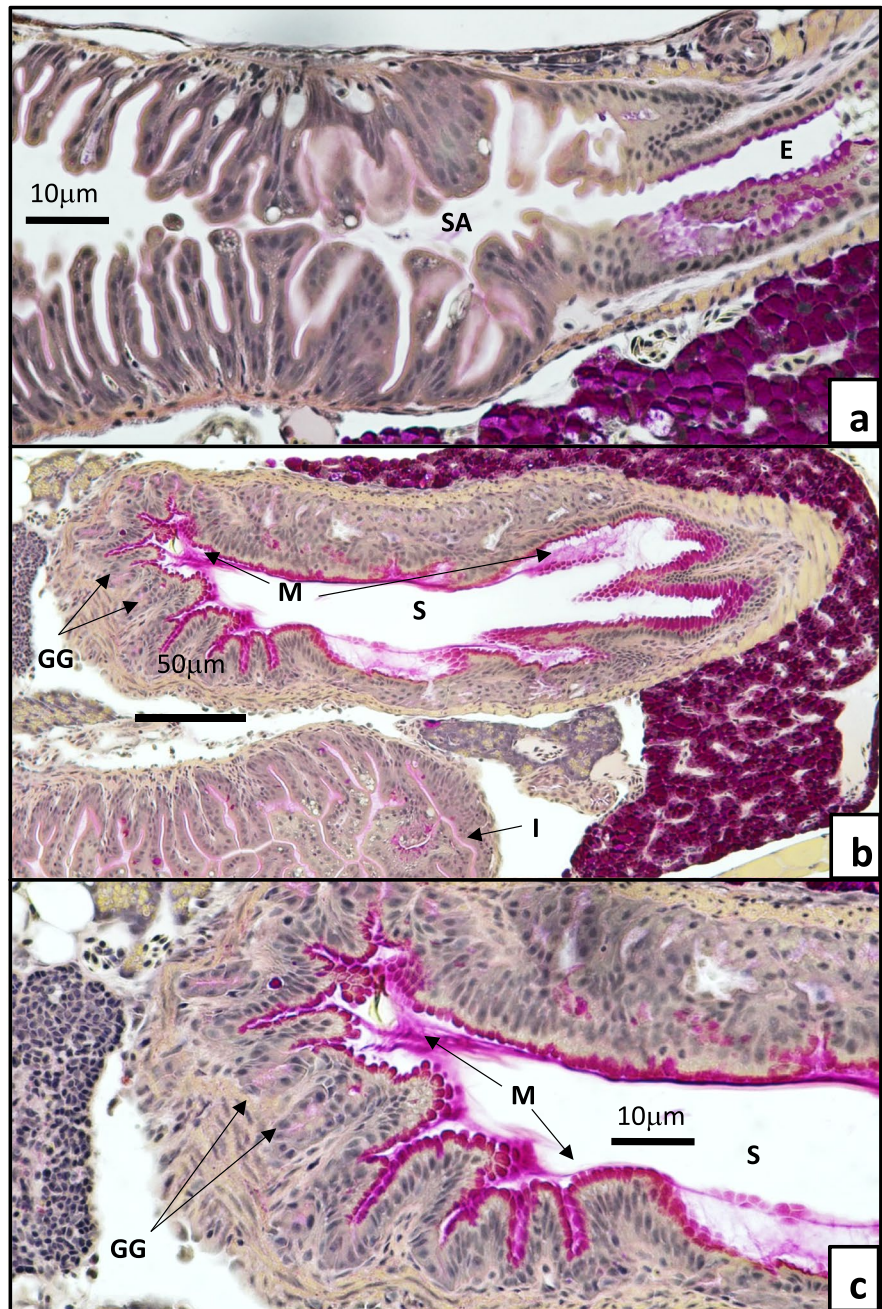
Weaning study

Survival at 32 dph (7.38 ± 0.08 mm) varied significantly among weaning treatments ($X^2_5=712.64$, $P<0.001$), with exclusively feeding MD1 from first feeding resulting in the lowest survival ($0.75 \pm 0.33\%$). Fish fed exclusively *Artemia* and those receiving weaning treatments W1 and W2 resulted in similar survival ($22.20 \pm 1.62\%$, $21.50 \pm 1.72\%$, and $22.20 \pm 1.85\%$, respectively, $P \geq 0.988$). Treatment W3 resulted in higher survival ($16.20 \pm 1.30\%$) than MD1, but lower survival than the other treatments ($P<0.001$, Fig. 8a). Because survival was exceedingly low for fish fed

exclusively MD1, SL and digestive enzyme activities for this treatment group were not included in those analyses. Growth among the remaining treatments was similar ($F_3=1.830$, $P=0.142$), with an overall final mean standard length of 7.38 ± 0.08 mm (Fig. 8b).

Trypsin activity varied significantly among treatments ($F_3=3.755$, $P=0.049$), however, post hoc pairwise comparison did not reveal significant relationships between individual treatments, due to the conservative nature of Tukey HSD for protection against type I error ($P \geq 0.066$, Fig. 9a). Mean trypsin activity at 32 dph (7.38 ± 0.08 mm) for all treatments was $1,281.91 \pm 124.91$ U/fish. Activities for both lipase and pepsin did not vary significantly among treatments ($F_3=0.520$, $P=0.678$, and $X^2_3=1.752$, $P=0.625$, respectively). Mean lipase activity for all treatments at 32 dph (7.38 ± 0.08 mm) was $28,316.35 \pm 2,698.40$ U/fish and mean pepsin activity was 18.87 ± 1.23 U/fish (Fig. 9b, c).

Fig. 6 Photomicrographs of histochemical preparations of *P. innesi* gastrointestinal tract stained with periodic acid Schiff (PAS) at **a**) 14 dph (400x), **b**) 23 dph (200x), and **c**) 23 dph (400x) from larvae in digestive ontogeny characterization trial. Esophagus (E), PAS+ neutral mucopolysaccharides (M), stomach anlage (SA), stomach (S), gastric glands (GG)

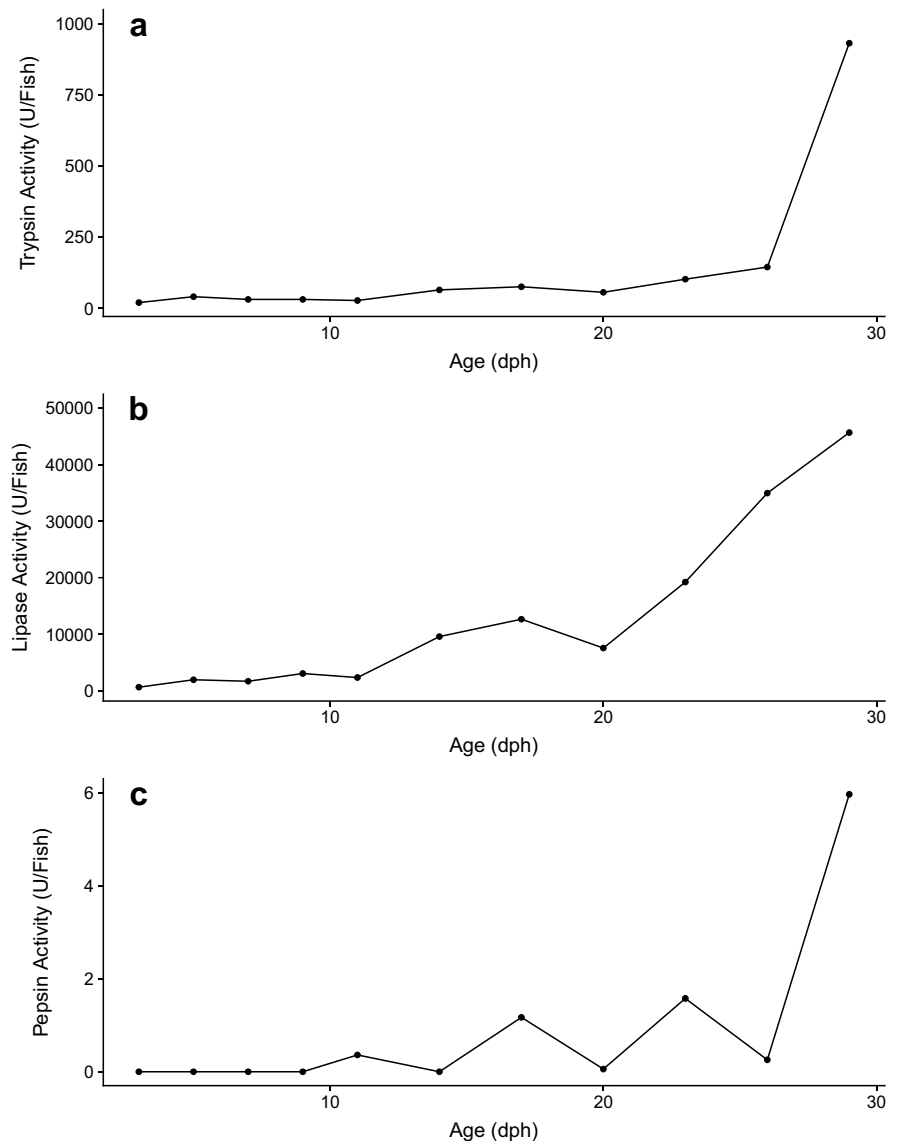


Discussion

Evaluation of the larval nutritional trial confirmed that, at least for the three MDs evaluated, *P. innesi* was incapable of thriving on an MD from first feeding, particularly when compared to larvae fed *Artemia* (Fig. 2). This pattern was confirmed in the weaning trial, where larvae fed exclusively MD3 from first

feeding survived very poorly ($0.75 \pm 0.33\%$) relative to all other treatments (Fig. 8a). When taken with the evidence for an extended, agastric larval phase revealed through the physiological characterization of developmental ontogeny (Figs. 4, 5, 6 and 7), it appears that limited digestive capacity due to low digestive enzyme activity and the absence of acidic digestive function likely contributed to this result.

Fig. 7 Development of digestive enzyme activities [trypsin (**a**), lipase (**b**), pepsin (**c**)] of *P. innesi* fed exclusively newly hatched *Artemia* nauplii between 3 and 29 days post hatch (dph) from larvae in digestive ontogeny characterization trial. Errors bars are absent as each data point represents enzyme activity of a pooled sample of ten larvae standardized to enzyme activity per fish

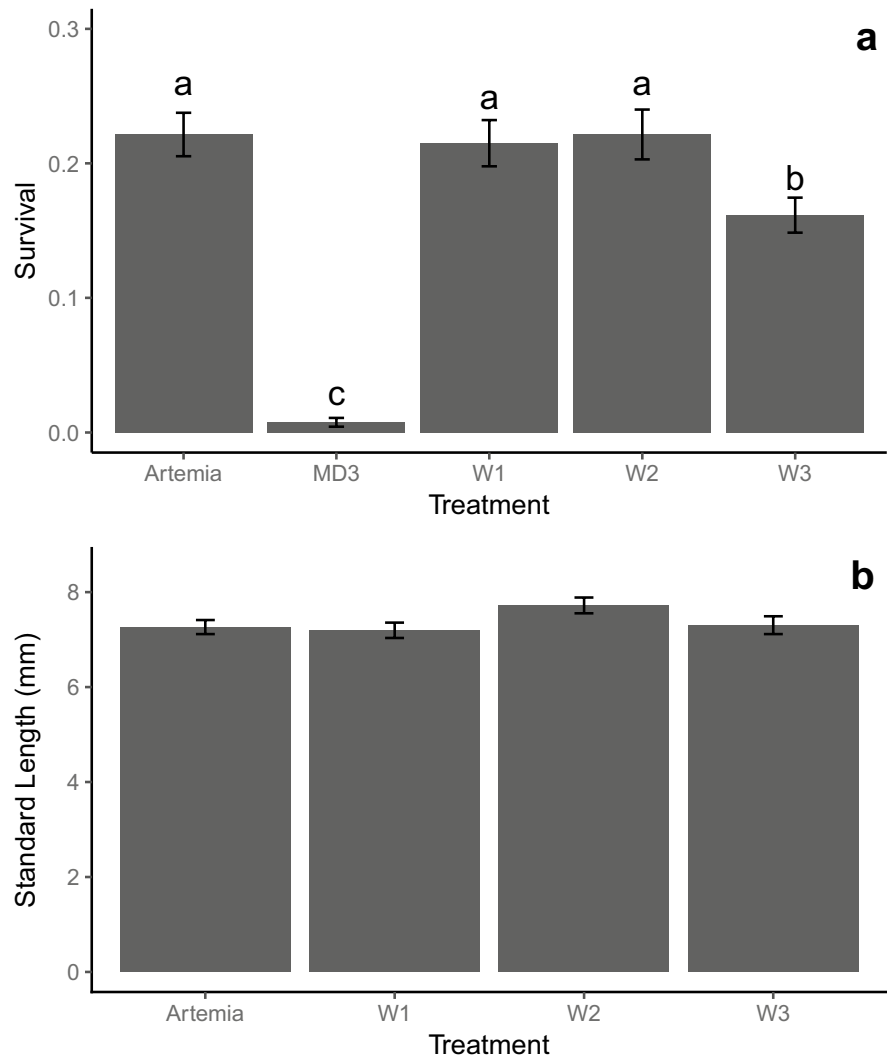


However, inherent physical characteristics of each type of diet, including palatability, color, sinking rate, shape of the pellet, etc., may have also contributed to the limited observed success. Further investigations, including nutritional trials evaluating ration and feeding frequency for each diet, are required to optimize feeding protocols for this species.

During early development of larval teleosts there exist three modes of digestive system ontogeny: agastric, precocial, and altricial (Ronnestad et al. 2013). In agastric fishes (i.e. Cyprinidae, Gobiidae), there is a complete reliance on alkaline, pancreatic proteases for hydrolysis of proteins over the entirety of the

fishes life, and digestive capacity increases as a function of gut length as the larvae develops, as well as the development of other structures like pharyngeal teeth (Dabrowski 1984; Dabrowski and Poczyczyński 1988; Hur et al. 2016). In precocial fishes (i.e. Salmonidae, Bagridae), larvae develop a fully functioning stomach with concomitant acid protease activity prior to the onset of exogenous feeding, vastly increasing their digestive capacity relative to agastric fishes and allowing for introduction of MDs from first feeding (Dabrowski 1984; Yang et al. 2010). Finally, altricial fishes (i.e. Sparidae, Paralichthyidae, Characidae) exhibit an agastric larval phase, where protease

Fig. 8 Proportional survival \pm standard error (**a**) and standard length \pm standard error (mm, **b**) at 32 dph following feeding with either exclusively newly hatched *Artemia* nauplii, exclusively MD3, or one of three weaning regimes: W1 = cofeeding beginning at 12 dph followed by exclusively MD3 at 17 dph; W2 = cofeeding beginning at 17 dph followed by exclusively MD3 at 22 dph; W3 = cofeeding beginning at 22 dph followed by exclusively MD3 at 27 dph. Different letters above columns denote statistically significant differences ($P \leq 0.050$)



activity is due to alkaline proteases at first feeding until which time the stomach differentiates and gastric digestion begins (Zambonino Infante and Cahu 2007; Ronnestad et al 2013; Yúfera et al. 2018; Nolasco-Soria et al. 2020). In altricial fishes, the successful introduction of MDs in an aquaculture setting largely hinges on the differentiation of a functional stomach, as established by previous authors (Faulk and Holt 2009; Ronnestad et al. 2013; Thompson et al. 2019). While histological evaluation of the GI in *P. innesi* is absent from the literature, *P. axelrodi* gastrointestinal physiology has been described in adult samples, which indicate gastric digestive function (Gómez-Ramírez et al. 2011).

Stomach differentiation in *P. innesi* appeared to occur by 20 dph (5.24 ± 0.20 mm), evidenced by appearance of a pyloric valve, gastric glands, and PAS positive neutral mucopolysaccharide production (Figs. 4, 5 and 6). Pepsin activity increased sharply at 29 dph, indicating a delay between histologically evident organ differentiation and functional gastric digestion, which has been observed in meagre *Argyrosomus regius* (Solovyev et al. 2016). Also, this is similar timing to the ontogeny of the stomach of another freshwater altricial neo-tropical fish, *Hemisorubim platyrhynchos*, in which the first gastric glands were observed at 15 dph and the fully differentiated stomach develop at 20 dph (Faccioli et al.

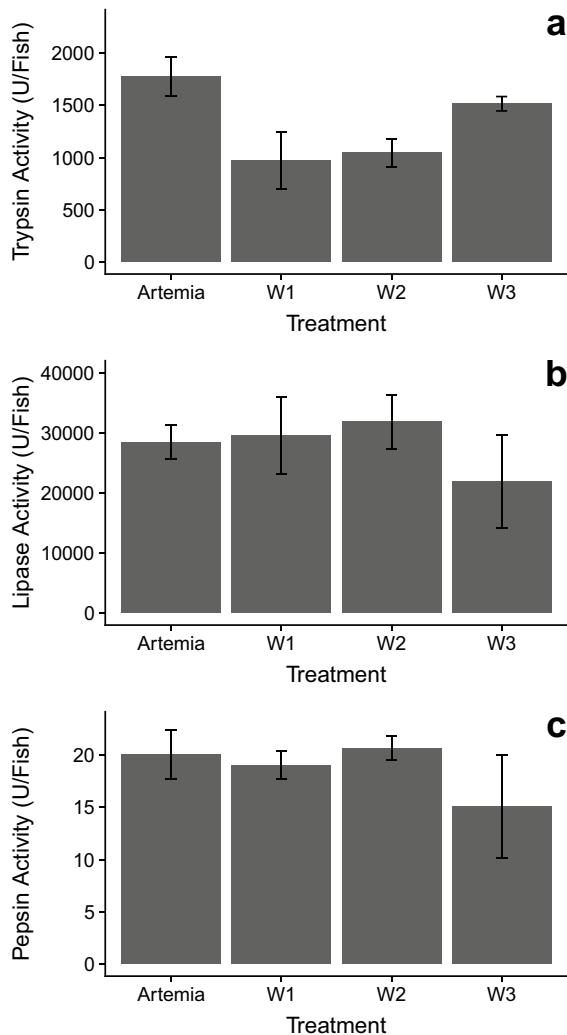


Fig. 9 Digestive enzyme activities [trypsin (a), lipase (b), pepsin (c)] of *P. innesi* at 32 dph following feeding with either exclusively newly hatched *Artemia* nauplii or one of three weaning regimes: W1=cofeeding beginning at 13 dph followed by exclusively MD3 at 18 dph; W2=cofeeding beginning at 18 dph followed by exclusively MD3 at 23 dph; W3=cofeeding beginning at 23 dph followed by exclusively MD3 at 28 dph

2016). The digestive ontogeny of the Pacu is more accelerated with gastric gland formation occurring at 10–13 dph and functional stomach differentiation occurring from 10–16 dph (Portella et al. 2014). Slight peaks at 11 (3.99 ± 0.09 mm) and 17 dph (5.56 ± 0.12 mm) (Fig. 7c) may be the result of accelerated development of a few fish that were present in the pooled sample of ten fish for the pepsin assays,

introducing variation in the sample result. Alternatively, previous authors who observed a similar phenomenon in leopard grouper *Mycteroperca rosacea* ascribed these early peaks in acid protease activity to other acid enzymes in other regions of the body of the larvae (Martínez-Lagos et al. 2014). Thompson et al. (2019) performed micro-dissections of pigfish *Orthopristis chrysoptera* larvae, effectively eliminating the potential for this type of false positive, which could have clarified the results in this study. However, given that feeding began at 4 dph and yolk absorption was nearly complete by 5 dph (3.61 ± 0.04 mm) (Fig. 4a), and that there was a complete lack of pepsin activity until 11 dph (3.99 ± 0.09 mm), a period of agastric digestion is apparent.

The presence of PAS positive neutral mucopolysaccharides beginning at 20 dph (5.24 ± 0.20 mm) provides further evidence for the initiation of acidic digestive function beginning at this age. Neutral mucins containing PAS positive mucopolysaccharides have been theorized to be produced by epithelial cells located in the mucosa of the stomach to protect this layer from the activity of the concurrently produced hydrochloric acid and pepsin by the oxynticopeptic cells of the gastric glands (Scocco et al. 1996; Pradhan et al. 2013; Ghosh and Chakrabarti 2015). The mucins in question effectively create a boundary between the acidic environment of the stomach lumen and the active pepsin, protecting the apical surface of the stomach epithelium from autolysis. The production of these compounds signals a functional shift, where acid and pepsin are actively being produced (Scocco et al. 1996).

Despite some species of small bodied, altricial, freshwater fish being capable of digesting MDs at first feeding (Lipscomb et al. 2020a), other studies have implicated a similar pattern of agastric larval digestive function as causative in low survival following first feeding with MDs. Hamlin et al. (2000) described the larval digestive system ontogeny in haddock *Melanogrammus aeglefinus*, which begin exogenous feeding at 2 dph, but do not exhibit stomach differentiation and gastric gland formation until 33 dph. The authors of this study indicated that weaning *M. aeglefinus* from live feeds to an MD should be conducted following this transition (Hamlin et al. 2000). Likewise, a study that described digestive enzyme ontogeny and evaluated weaning from live feeds to an MD in larval *O. chrysoptera* showed that pepsin

activity remained low until 30 dph and weaning at 32 dph yielded significantly higher survival than earlier weaning times (Thompson et al. 2019). Additionally, we showed a similar pattern of development in *G. ternetzi*, a characid relative of *P. innesi* (Lipscomb et al. 2020b). In the case of *G. ternetzi*, pepsin activity, gastric gland formation, stomach differentiation and neutral mucopolysaccharide production all occurred at 22 dph, although weaning with cofeeding initiated at 13 dph resulted in similar survival to later weaning times (Lipscomb et al. 2020b).

While *P. innesi* exhibit histological and histochemical stomach differentiation at 20 dph (5.24 ± 0.20 mm), cofeeding beginning at 13 dph resulted in similar survival and growth to fish fed exclusively *Artemia* from first feeding through 32 dph, as well as cofeeding initiated at 17 dph. Given that cofeeding ceased and exclusive feeding with MD3 occurred at 17 dph for weaning treatment W1, it appears that weaning to an MD prior to completion of stomach formation is possible for *P. innesi* (Fig. 8). This may be explained by the increased activity of lipase and a slight increase in pepsin that were concurrent with this weaning period (Fig. 7).

Alternatively, authors have suggested that initiating larval nutrition with live feeds may infer digestive benefits due to an increase the production of endogenous enzymes in larval fish (Hjelmeland et al. 1988). Hjelmeland et al. (1988) observed that ingestion of either copepods or *Artemia* in larval herring *Clupea harengus* elicited higher levels of trypsin in the intestine than inert particles, although the inert particles did elicit some response. This interaction between live prey and larval digestive function could help explain the observed results of successful weaning prior to complete stomach differentiation in *P. innesi*. Curiously, larvae weaned beginning at 23 dph (treatment W3; 5.87 ± 0.13 mm) exhibited reduced survival relative to those weaned at 13 and 18 dph (treatments W1 and W2, respectively Fig. 8a). While further research is required to elucidate the cause of this result, one possible explanation is that the prolonged live feed phase in W3 resulted in difficulty in transitioning to an inert particle, potentially due to habituation to *Artemia*.

Timing of weaning in *P. innesi* did not significantly impact pepsin or lipase activities, and although a significant effect on trypsin activity was detected ($F_3=3.755$, $P=0.049$), pairwise comparisons did

not reveal differences between individual treatments (Fig. 9). Given that samples were taken after a period of overnight fasting, positive feedback interactions between ingested feed items and enzyme activity were likely limited. This means that observed digestive enzyme activities can be interpreted as basal zymogen production, and not acutely modulated by ingesta. This observation agrees with the previous assertion by Zambonino Infante, and Cahu (2001) that diet may modulate peaks in enzyme activities following ingestion, but timing of increases and decreases in activity is largely genetically determined.

The results of this study show that live feed dependence of *P. innesi* at first feeding is likely due to an altricial pattern of GI development, where the stomach and associated functions are absent during the initiation of exogenous feeding, and do not appear until between 11 (3.99 ± 0.09 mm) and 20 dph (5.24 ± 0.20 mm). Weaning prior to the differentiation of the stomach organ and appearance of gastric glands, pyloric valve, and PAS positive neutral mucins is possible, with weaning beginning at 13 dph inferring similar survival to exclusively feeding *Artemia*. This result is consistent with previous work with another characid, *G. ternetzi*, which potentially reveals a pattern of digestive function common to small-bodied characids, although further research into more species is warranted (Lipscomb et al. 2020b). In conclusion, commercial production of *P. innesi* can be accomplished with minimal live feed inputs if the early weaning protocols established herein are implemented. This potentially increases the resilience of this aquaculture endeavor in periods of *Artemia* shortage, as well as provides an overall economic advantage for the critical larval culture stage in this ornamental fish.

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and reviewed/edited the manuscript. MD conceptualized the research, assisted in conducting the experiments, and reviewed/edited the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Code availability The statistical analyses and figures herein were created in Program R (RStudio, V. 0.99.903 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). The custom code is available upon reasonable request.

Declarations

Ethics approval All research herein was conducted under approval by and in compliance with standards developed by the Institutional Animal Care and Use Committee of the University of Florida (IACUC study #202011169).

Consent to participate Not applicable.

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