Investigations into Early Larval Feeding Practices for the Tiger Barb

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Abstract
The Tiger Barb *Puntigrus tetrazona* is one of the highest trade volume freshwater species in the ornamental fish industry. Culture of larval Tiger Barb is largely dependent on live feeds at first feeding and throughout early life stages, leading to increased cost relative to the use of commercially produced microparticulate diets (MDs). Potential for the successful culture of Tiger Barb by using MDs from first feeding was evaluated here, with a focus on the physiological characteristics that limit digestive capacity in larval cyprinids, as well as the hypothetical benefit of including feed attractants in formulated larval feeds. Comparable growth and survival were achieved for the first 14 d of feeding with one of three MDs when compared to feeding with brine shrimp *Artemia* spp. Histological preparation revealed evidence for a fully functional pharyngeal jaw structure, including pharyngeal teeth and a pharyngeal pad, from 6 d posthatch, which coincided with first feeding. The masticatory function of these structures likely facilitated the breakdown and subsequent utilization of the relatively complex macronutrients that are characteristic of MDs. Inclusion of top-coated potential attractants (tryptophan, taurine, trimethylglycine betaine, or a mix of the three) with the most successful MD from the original trial failed to induce an increased feeding response, as evidenced by the observation of similar feeding incidence, total larval protein content, and trypsin enzyme activity relative to a negative control MD without added attractants. The results of this research suggest that the successful culture of larval Tiger Barb is possible with the use of commercially available MDs, potentially leading to cost savings and increased resilience of producers in the ornamental aquaculture industry.

The ornamental fish industry accounts for an estimated US$15–30 × 10^9 globally, and propagation of freshwater ornamental fishes is one of the largest contributing sectors to the aquaculture economy in the USA (Chapman et al. 1997; Evers et al. 2019). The Tiger Barb *Puntigrus tetrazona* (Cyprinidae) is domestically produced in high volume for the ornamental trade (Chapman et al. 1997; Tamaru and Ako 2000). The intensive larval rearing of Tiger Barb from first feeding is almost entirely accomplished using live feeds, with brine shrimp *Artemia franciscana* nauplii being chiefly employed for this purpose (Tamaru et al. 1997; Lim et al. 2003). *Artemia* spp. are commercially collected from the wild in the cyst stage from brine lakes and primarily from the Great Salt Lake in Utah (Bengtson et al.

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1991; Lavens and Sorgeloos 2000). Although dormant *Artemia* cysts are easily stored and cultured for use relative to other live feeds, their collection is dependent on environmentally induced cyst blooms, which can result in market volatility and inflated costs (Cahu and Zamponino Infante 2001; Hamre et al. 2013). These negative aspects of *Artemia* use as a live feed, coupled with biosecurity concerns inherent to introducing wild organisms into the rearing environment, have prompted numerous studies evaluating alternative first feeds (Lazo et al. 2000; Wang et al. 2005; Sautter et al. 2007; Patterson et al. 2016; Lipscomb et al. 2020a).

Microparticulate diets (MDs) have been most commonly investigated as alternatives to *Artemia* and other live feed organisms for first-feeding larval fishes. Microparticulate diets are not only more convenient than *Artemia* and require less labor; they are also subject to less market volatility, can be formulated according to a species’ nutritional requirements, and are more biosecure (Kolkovski 2001; Hamre et al. 2013). Although these attributes are compelling, the underdevelopment of the digestive system in altricial/agastic larval fish has led to widespread failure in introducing MDs from first feeding (Cahu and Zamponino Infante 2001; Faulk and Holt 2009; Thompson et al. 2019; Lipscomb et al. 2020b). Additionally, the early introduction of MDs has been reported to increase heterogeneity of growth, potentially leading to increased rates of cannibalism in predatory fish species (Kestemont et al. 2003).

In the case of cyprinids and other agastic species, the larvae not only lack a stomach and associated enzymatic activity at first feeding; this organ never develops and is absent in adult specimens (Castro et al. 2013). Despite this anatomical similarity among larvae of both cyprinids and altricial species, cyprinids are able to successfully feed on MDs throughout their life and often from first feeding (Dabrowski and Poczyczyński 1988). Although many cyprinids appear to be physiologically capable of digesting and assimilating nutrients in MDs, a potential hurdle still exists in food particle recognition and palatability (Rønnestad et al. 2013). Relative to live feeds, MDs can be largely devoid of feeding stimuli or attractants that are detected by the olfactory rosette and that elicit an endocrine-regulated orexigenic response in the fish (Volkoff et al. 2009). Feed attractants are generally small, water-soluble molecules, such as amino acids, nucleotides, and nucleosides, that continually emanate from live prey (Volkoff et al. 2009; Hoskins and Volkoff 2012). As additives to manufactured diets, compounds like tryptophan, taurine, and trimethylglycine betaine, among others, have been shown to elicit transcription-level endocrine responses, increase feed intake, modulate feeding-related behaviors, and increase growth and/or survival in Striped Bass *Morone saxatilis*, genetically improved farmed tilapia *Oreochromis* sp., Rainbow Trout *Oncorhynchus mykiss*, Lake Trout *Salvelinus namaycush*, and Lake Whitefish *Coregonus clupeaformis*, as well as cyprinids, including Goldfish *Carassius auratus* and Crucian Carp *Carassius carassius* (Papatriphon and Soares 2000; Hara 2006; Salze and Davis 2015; Olsén and Lundh 2016; Zou et al. 2017).

Previous studies investigating feeding attractants have focused not only on quantifying growth parameters and survival, but also on feeding behavior, feed intake, feed conversion ratio, protein efficiency ratio, and nutrient assimilation in juvenile fish (Shankar et al. 2008; Zou et al. 2017). The techniques employed to collect data related to these parameters are logistically challenging when considering larval fish as the subjects, simply due to the minute size of many first-feeding fishes (Conceição et al. 2010). Postprandial detection of isotopic tracer inclusion has been successfully used to quantify intake and assimilation of food particles in larval fishes; however, such methodologies can be expensive, time consuming, and hazardous when radioactive isotopes are involved (Conceição et al. 2007). In the current study, we endeavored to quantify feed intake using two approaches: (1) comparing total protein content using a Bradford assay, and (2) evaluating tryptic enzyme activity of fish fed various MDs with various feed attractants included. Because individual variability associated with feeding behavior is minimized in first-feeding larvae, we theorized that any observed differences in total larval protein content at these early life stages would reflect the protein intake obtained from different food ingestion levels among diets. Trypsin activity is positively modulated by feed ingestion and has been previously used as an indicator of nutritional quality of feeds as well as a proxy for feed intake (Ueberschär 1995; Tillner et al. 2014).

In this study, we first evaluated survival and growth resulting from commercially available MDs as the sole diet of first-feeding Tiger Barb compared to a diet of *Artemia* nauplii through the first 14 d of feeding. Next, we characterized the histological development of the digestive tract, the histochemical distribution of mucins produced in the goblet cells of the digestive mucosa throughout the larval period, and the ontology of two critical pancreatic enzymes: trypsin (enzyme number 3.4.21.4; IUBMB 1992) and bile salt-dependent lipase (3.1.1.13). Finally, we evaluated feed intake, total protein content, trypsin activity, survival, and growth resulting from the inclusion of potential feed attractants, including taurine, tryptophan, betaine, a mix of the three, or a control. The results of these investigations will improve techniques for the intensive aquaculture of Tiger Barb and will also add to the collective understanding of larval cyprinid digestive physiology.

**METHODS**

**Larval rearing conditions.**—Tiger Barb larvae were obtained either from a local ornamental aquaculture
operation (5D Tropical, Inc., Plant City, Florida) or from inhouse spawning at the University of Florida’s Tropical Aquaculture Laboratory (TAL), Ruskin. Larvae from offsite were transferred to the TAL at 3 d posthatch (dph), hand counted, and stocked into experimental tanks. For inhouse production, Tiger Barb broodfish were conditioned on a diet of bloodworms (Bio-Pure Frozen Bloodworms; Hikari Sales USA, Inc., Hayward, California) and extruded pellets (AquaMax 500; Purina Animal Nutrition LLC, Gray Summit, Missouri) that were administered to satiation twice daily. Pairs of broodfish (1 male, 1 female) were stocked into fifty 10-L spawning tanks filled with a static mixture of degassed well water and reverse-osmosis-filtered water (hardness = 102.6 mg CaCO$_3$/L) and were allowed to volitionally spawn on large bottle brushes. After spawning, broodfish were removed and embryos were hatched in the spawning tanks, after which larvae (3 dph) were counted into experimental tanks. Dissolved oxygen, pH, temperature, total ammonia nitrogen, and nitrite-N were monitored weekly for each experimental tank in each trial. Dissolved oxygen and temperature were measured using an optical dissolved oxygen meter (Pro-Comm II; YSI, Yellow Springs, Ohio). Total ammonia nitrogen, nitrite, and pH were measured using colorimetric assays according to the manufacturer’s protocols (Hach Company, Loveland, Colorado).

Water quality parameters did not vary among treatment groups in any of the trials and were within the acceptable range for larval Tiger Barb. Water quality parameters and associated statistics are as follows: flow (0.165 ± 0.002 L/min; $F_0 = 0.750$, $P = 0.564$), pH (7.814 ± 0.004; $F_0 = 0.220$, $P = 0.925$), dissolved oxygen (7.172 ± 0.020 mg/mL; $F_0 = 0.202$, $P = 0.935$), temperature (25.73 ± 0.1°C; $F_0 = 0.031$, $P = 0.998$), total ammonia nitrogen (0.20 ± 0.00 mg/mL), and nitrite-N (0 ± 0 mg/mL). Values are mean ± SE.

**Dietetics trial.**—Fifty 3-dph larvae obtained from a local producer were stocked into each of sixteen 3-L, rectangular, plastic experimental tanks fitted with 200-μm screened baffles and maintained on flow-through well water that was degassed and aged. Specifically, well water was degassed through three packed columns and stored in a 3,785-L holding tank prior to being pumped into the experimental tanks. Beginning at 6 dph (first feeding), larvae in each tank were offered newly hatched, first to second instar stage *Artemia* nauplii or one of three MDs: MD1 (Golden Pearls, 50–100 μm; 60% protein, 8% fat; Brine Shrimp Direct, Ogden, Utah), MD2 (Otohime A1, 75–150 μm; 53% protein, 8% fat; Marubeni Nissin Feed Co. Ltd., Tokyo, Japan), and MD3 (Larval AP-100, <100 μm; 50% protein, 12% fat; Ziegler Bros, Inc., Gardens, Pennsylvania). Larvae were fed twice daily (per industry standard in Florida commercial aquaculture operations; Eric Cassiano, Aquaculture Extension Faculty, Institute of Food and Agricultural Sciences, University of Florida, personal communication) to beyond satiation, as confirmed by the presence of feed particles remaining in the tanks 15 min after feeding (~0.5 *Artemia* nauplii/mL; ~20 mg MD/tank per day). After 14 d of feeding (i.e., at 20 dph), all surviving larvae from each tank were harvested and enumerated. Ten larvae from each tank were photographed using a trinocular dissecting microscope outfitted with a digital camera and were measured using ImageJ version 1.50i (National Institutes of Health, Bethesda, Maryland).

**Characterization of digestive system ontogeny.**—For characterization of digestive system ontogeny, one-thousand 1-dph Tiger Barb larvae originating from approximately 30 brood pairs housed at the TAL were stocked into each of three 10-L tanks maintained on flow-through well water that was degassed and aged. Beginning at 6 dph, larvae were exclusively fed *Artemia* nauplii to beyond satiation twice daily (~0.5 *Artemia* nauplii/mL). Twenty larvae were aggregated from all three tanks and sampled at 2, 4, 6, 8, 11, 13, 15, 17, and 20 dph. Samples from 6 to 20 dph were taken prior to initial daily feedings. On each sampling day, all larvae were photographed under a trinocular dissecting microscope and measured for notochord length (from 2 to 6 dph) and SL (from 8 to 20 dph) as previously described. Ten larvae were placed in a 1.5-mL microcentrifuge tube containing Trump’s fixative for histological and histochemical processing, and 10 larvae were gently dried on a Kimwipe, transferred to a 1.5-mL microcentrifuge tube, and stored at ~80°C for trypsin and bile salt-dependent lipase assays.

Histological and histochemical samples were embedded in paraffin, longitudinally sectioned, and mounted on glass slides. Two slides were produced for each sampling day: one was stained with hematoxylin and eosin, and the other was stained with alcian blue (AB) and periodic acid-Schiff (PAS). Slides were viewed and photographed under a trinocular compound light microscope with a digital camera attachment.

**Feed attractants trial.**—Experimental feeds were created prior to conducting the trial by top-coating MD3 (the best-performing MD from the dietetics trial) with each of four potential attractants at an inclusion rate of 0.5% by weight: taurine (Chemical Abstracts Service [CAS] Number 107-35-7; Sigma-Aldrich, St. Louis, Missouri), L-tryptophan (CAS Number 73-22-3; Sigma-Aldrich), betaine (CAS Number 107-43-7; Sigma-Aldrich, St. Louis, Missouri), and MD3 (Larval AP-100, <100 μm; 50% protein, 12% fat; Ziegler Bros, Inc., Gardens, Pennsylvania). Larvae were fed twice daily (per industry standard in Florida commercial aquaculture operations; Eric Cassiano, Aquaculture Extension Faculty, Institute of Food and Agricultural Sciences, University of Florida, personal communication) to beyond satiation, as confirmed by the presence of feed particles remaining in the tanks 15 min after feeding (~0.5 *Artemia* nauplii/mL; ~20 mg MD/tank per day). After 14 d of feeding (i.e., at 20 dph), all surviving larvae from each tank were harvested and enumerated. Ten larvae from each tank were photographed using a trinocular dissecting microscope outfitted with a digital camera and were measured using ImageJ version 1.50i (National Institutes of Health, Bethesda, Maryland).
and aged. Beginning at 6 dph, larvae were fed either MD3 top-coated with taurine, tryptophan, betaine, a mix, or the control at a rate of 40 mg twice daily, an amount that was demonstrated in previous experiments to be in excess of satiation. Following the morning feeding at 6, 8, and 10 dph, 10 larvae were sampled 15 min after feed was offered. Sampled larvae were photographed under a trinocular dissecting scope, and feeding incidence was enumerated by visual inspection of translucent larvae. Larvae with ingesta present in the gastrointestinal tract were counted as fed, while those without ingesta were counted as unfed. Larvae were placed into 1.5-mL microcentrifuge tubes and stored at −80°C prior to being assayed for trypsin activity and protein content. After 14 d of feeding (i.e., at 20 dph), the remaining larvae were enumerated, and survival was assessed by dividing the remaining larvae by the original stocking number minus the number of larvae sampled throughout the trial. Ten larvae were photographed and measured for final SL according to previously described methods.

Biochemical assays.—Digestive enzyme activity assays were performed according to Faulk et al. (2007). On the morning of each assay, samples were thawed in an aluminum cooling chamber nested in ice. 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 CaCl₂, pH 7.5) in 1.5-mL microcentrifuge tubes and centrifuged for 10 min at 1,700 g and 4°C. To measure trypsin activity, 20 µL of each sample supernatant, as well as 15-µg/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 Nα-benzoyl-DL-arginine p-nitroanilide dissolved in dimethyl sulfoxide) was quickly added to each well using a multichannel 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 micromoles of p-nitroaniline liberated per minute (U), standardized on a per-fish basis.

For bile salt-dependent lipase, 10 µL of each sample supernatant, as well as 7.5-µg/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 of ethanol, 0.5-M Tris-HCl, 6-mM sodium taurocholate, 1-M NaCl, pH 7.4) were added to each well. At 30°C, hydrolyzation of 4-NPC was measured at 400-nm absorbance and units were expressed as micromoles of 4-NPC hydrolyzed per minute (U). Protein concentration of the supernatant from each homogenate of 10 larvae collected during the feeding stage of the feed attractants trial was evaluated using the Bradford assay (Pierce Coomassie Plus [Bradford] Assay Kit; Thermo Fisher Scientific, Inc., Waltham, Massachusetts). All spectrophotometric biochemical assays were conducted using a BioTek spectrophotometer (Synergy HTX Multi-Mode Microplate Reader; BioTek Instruments, Inc., Winooski, Vermont).

Statistical analysis.—Growth rate observed during characterization of digestive system ontogeny was obtained through linear regression. Survival of treatment groups following the dietetics and feed attractants trials was modeled using a logistic regression on the Bernoulli-distributed data of living and dead larvae using the glm function in program R (family = binomial; Crawley 2005). Following model convergence, a likelihood ratio test was used to evaluate the effect of treatment on survival. Finally, if the likelihood ratio test revealed a significant effect, a post hoc pairwise Tukey honestly significant difference (HSD) test was used to compare the log odds for survival of each treatment group.

Differences in final SL for both the dietetics trial and the feed attractants trial were evaluated using one-way ANOVA after confirming the assumptions of normality of residuals and homogeneous variance using a Shapiro–Wilk test and Bartlett’s test, respectively. The coefficient of variation (CV) of SL was calculated for each tank in the dietetics trial by using the following equation:

\[
CV = \frac{SD\ of\ SL}{Mean\ of\ SL} \times 100.
\]

Variation in CV among treatment groups was evaluated using one-way ANOVA. The Bernoulli-distributed data of feeding and nonfeeding larvae were modeled using the glm function in program R, with age and feed attractant type included as independent variables. Following model convergence, a likelihood ratio test was used to evaluate the effect of treatment and age on feeding incidence. Finally, if the likelihood ratio test revealed a significant effect, a post hoc pairwise Tukey HSD test was used to compare the log odds for feeding at each age or resulting from each treatment.

Protein and trypsin activity were evaluated using a two-way ANOVA, with age and feed attractant types as independent variables. Post hoc pairwise comparisons following a significant result were conducted using a Tukey HSD test. A priori significance levels were set at \( \alpha = 0.05 \), and all analyses were completed in program R (RStudio version 0.99.903; RStudio, Inc., Boston). All values presented in the text and in the figures are expressed as mean ± SE.

RESULTS

Dietetics Trial

There was a significant effect of treatment on Tiger Barb survival after 14 d of feeding (\( \chi^2 = 11.901, P = \)
Post hoc pairwise comparison revealed that *Artemia* and MD3 treatments performed similarly in terms of survival (*Artemia*: 43.5 ± 7.0%; MD3: 33.5 ± 6.6%; *P* = 0.170). Feeding *Artemia* resulted in significantly higher survival than feeding both MD1 and MD2 (MD1: 28.0 ± 6.2%, *P* = 0.007; MD2: 31.0 ± 6.3%, *P* = 0.049). There were no significant pairwise differences detected between any of the MD treatments with respect to survival (*P* ≥ 0.633; Figure 1a). Growth was not significantly affected by treatment (F3 = 1.275, *P* = 0.327), and mean final SL across all treatments was 5.38 ± 0.04 mm (Figure 1b). The CV of SL likewise did not vary among treatments (mean CV = 5.810 ± 0.667%; F3 = 0.967, *P* = 0.440).

**Characterization of Digestive System Ontogeny**

Fish body length increased at a rate of 0.11 mm/d from 4.25 ± 0.06 mm (notochord length) at 2 dph to 6.34 ± 0.04 mm (SL) at 20 dph (Figure 2). Trypsin activity was detectable from 2 dph (84.8 U/larva) and increased daily until a peak at 11 dph (513.2 U/larva), followed by a slight decrease at 13 dph (417.0 U/larva), an increase until another peak at 17 dph (780.2 U/larva), and finally another decrease at 20 dph (489.7 U/larva; Figure 3a). Lipase was likewise detectable from 2 dph (1,009.5 U/larva) but remained relatively low until 17 dph, when a marked increase occurred to 11,115.5 U/larva, followed by a decrease at 20 dph to 9,045.6 U/larva (Figure 3b).

Yolk was present at 4 dph and was nearly entirely depleted by 6 dph, when exogenous nutrition was initiated (Figure 4). The digestive tract at 4 dph consisted of a mouth, pharynx, and esophagus and a straight, uniform intestine situated immediately dorsal to the yolk. Developing pharyngeal teeth were present beginning at 4 dph and were emergent and functional by 6 dph (Figure 5a, c). The dorsal muscular layer of the pharynx was greatly enlarged relative to the opposing side from 2 dph and was populated by thick bundles of striated muscle, as evidenced at 4 dph (Figure 5a). A keratinized pharyngeal pad appeared at 6 dph on the epithelial surface at this location in the pharynx (Figure 5c). The pharyngeal pad progressively thickened throughout development, and pharyngeal teeth proliferated in the opposing epithelial layer (Figure 5a, c, d: Figure 6a, c, e, f). Beginning at 17 dph, the intestine exhibited elongation and folding (Figure 4d), which progressed further at 20 dph.

Both AB- and PAS-positive goblet cells were present in the pharynx and esophagus beginning at 4 dph, while only AB-positive goblet cells were present in the intestine, which exhibited a very narrow lumen at this stage (Figure 6a, b). The intestinal lumen expanded greatly and proliferation of intestinal villi occurred following the onset of exogenous nutrition at 6 dph (Figure 6d). In addition, AB- and PAS-positive goblet cells proliferated in the pharynx...
and esophagus at 6 dph (Figure 6c). Goblet cells continued to proliferate and hypertrophy at 13 dph and again at 20 dph, when copious AB-positive mucins were observable in the lumen of the pharynx and esophagus (Figure 6e, f). Periodic acid–Schiff-positive neutral mucopolysaccharides were distinctly absent from the epithelial surface and lumen in the entirety of the intestine.

Feed Attractants Trial

Inclusion of potential feed attractants did not have an effect on feeding incidence, protein content, or trypsin activity after a 15-min feeding period at 6, 8, and 10 dph (Figures 7, 8, and 9, respectively). Although feeding incidence was similar among attractant treatments and the control ($\chi^2 = 4.963, P = 0.291$), feeding incidence was significantly different among age-groups ($\chi^2 = 96.232, P < 0.001$). Feeding incidence was lowest at 6 dph (29.5 ± 12.3%; $P < 0.001$), increased to 65.0 ± 14.4% at 8 dph ($P < 0.023$), and was highest at 10 dph (77.0 ± 11.3%; $P < 0.023$; Figure 7).

Total protein content was likewise similar among attractants and the control ($F_4 = 1.043, P = 0.394$) but varied significantly by age ($F_2 = 12.143, P < 0.001$). Protein content was significantly lower at 6 dph (26.7 ± 1.4 μg/fish) than at 8 dph (32.0 ± 2.2 μg/fish; $P < 0.001$) or 10 dph (30.4 ± 1.62 μg/fish; $P = 0.004$) but did not vary significantly between 8 and 10 dph ($P = 0.328$; Figure 8). Trypsin activity on a per-fish basis was also similar among attractant additives and the control ($F_4 = 0.258, P = 0.903$) but varied significantly by age ($F_2 = 54.885, P < 0.001$). Trypsin activity increased between 6 dph (100.7 ± 20.0 U/larva) and 8 dph (176.1 ± 35.6 U/larva; $P < 0.001$) and again between 8 and 10 dph (238.3 ± 53.6 U/larva; $P < 0.001$; Figure 9).

Survival after 14 d of feeding with top-coated feeds was significantly different among attractant treatments and the control ($\chi^2 = 29.521, P < 0.001$). Feeding with MD3 top-coated with taurine resulted in lower survival (18.1 ± 2.5%) than feeding with the betaine treatment (24.4 ± 3.3%; $P = 0.036$) or a mix of all three attractants (29.0 ± 3.4%; $P < 0.001$) but was similar to survival resulting from the tryptophan treatment (19.1 ± 2.9%; $P = 0.989$) and the control (23.4 ± 3.1%; $P = 0.115$). Survival after feeding with the control was similar to that of all feed attractant treatments ($P \geq 0.1145$), while feeding MD3 top-coated with a mix of all three attractants resulted in the highest numerical survival, which was statistically greater than that of the taurine and tryptophan treatments ($P < 0.001$; Figure 10a). Growth was similar among all attractant treatments after 14 d of feeding and resulted in a mean SL of 5.51 ± 0.06 mm ($F_4 = 0.360, P = 0.837$; Figure 10b).

**Figure 3.** Ontogeny of (a) trypsin activity and (b) bile salt-dependent lipase activity in Tiger Barb that were fed Artemia nauplii from 2 to 20 d posthatch (dph) during trials for characterizing digestive system ontogeny. Values are from pooled samples of 10 fish standardized to activity (U) per fish, so error bars are not present.

**Discussion**

Trypsin activity and bile salt-dependent lipase activity were detectable well before exogenous feeding in Tiger Barb, as has been the case in many other species, and this is likely related to nutrient processing of yolk reserves (Zambonino Infante and Cahu 2001). Similar to previous studies, activities for both pancreatic enzymes increased rapidly shortly after the onset of exogenous feeding, with peaks for each occurring at 17 dph. Proteins and lipids are the two most important groups of macronutrients for larval fish growth and development and are therefore critical components when considering larval diets (Rønnestad et al. 2003). Lipids—specifically in the phospholipid and triacylglycerol forms—are likely not hydrolyzed prior to entering the intestinal lumen, as is the case in mammals (Rønnestad et al. 2013). Alternatively, these macronutrients enter the intestine, where pancreatic lipases together with emulsifiers present in bile (chiefly bile salt-dependent lipase) begin lipid hydrolysis (Rønnestad et al. 2013).
Protein components, or amino acids, are not only the building blocks for tissue production, but also can be efficiently catabolized in larval fish (Rønnestad et al. 2007; Holt 2011). In the absence of a stomach and associated pepsin, such as in altricial larvae or throughout the life span of agastric cyprinids, trypsin and trypsin-like serine

FIGURE 4. Photomicrographs of histological sections of Tiger Barb stained with hematoxylin and eosin at (a) 4 d posthatch (dph; 10x), (b) 6 dph (40x), (c) 15 dph (40x), and (d) 17 dph (40x). Labeled structures are the pharynx (P), esophagus (E), intestine (I), and yolk (Y).

FIGURE 5. Photomicrographs of histological sections of Tiger Barb stained with hematoxylin and eosin at (a), (b) 4 d posthatch (dph; 40x); (c) 6 dph (40x); and (d) 15 dph (40x). Labeled structures are the pharynx (P), pharyngeal tooth (PT), pharyngeal pad (PP), striated muscle (StM), olfactory rosette (OR), and olfactory bulb (OB).
proteases originating from the pancreas contribute most to protein hydrolysis (Rønnestad et al. 2013). In altricial larval species, the introduction of MDs prior to the onset of acid protease activity in lieu of live feed items has been largely unsuccessful, primarily due to the insufficient hydrolysis of large, water-insoluble proteins found in MDs by trypsin alone (Rønnestad et al. 2003; Santamaría et al. 2004; Faulk et al. 2007; Faulk and Holt 2009; Lipscomb et al. 2020b). This is in contrast to the case of larval cyprinids, which never develop a stomach but have been successfully reared on manufactured diets from first feeding (Dabrowski and Poczyczyński 1988; Abi-Ayad and Kestemont 1994; Kolkovski 2001). This was also observed in the current study, where both survival and growth were similar between larvae fed MD3 and Artemia from first feeding through 20 dph. Although MD1 and MD2 resulted in poor survival relative to Artemia, simple differences in physical characteristics of the MDs (i.e., observed differences in buoyancy) may have led to this result as opposed to any inadequacies related to digestibility. As was noted by Lipscomb et al. (2020a), as well as by Cahu and Zambonino Infante (2001), species-specific feeding behaviors lend themselves to certain diets. In this case, MD3 was far more negatively buoyant than MD1 and MD2, which may have served to benefit Tiger Barb larvae.

Abolhasani et al. (2014) observed total mortality of 8.33–9.16% over a 14-d period when larval Tiger Barb were fed either enriched or unenriched Artemia nauplii, which represents far greater survival than was observed in the current research. However, while initial age was not indicated in the Abolhasani et al. (2014) study, initial TL was listed as 6.15–6.19 mm, which is larger than the final lengths of larvae in any of the treatment groups in our dietetics trial (5.38 ± 0.04 mm) and our feed attractants trial (5.51 ± 0.06 mm). Differences between survival in the current research and survival observed by Abolhasani et al. (2014) are potentially attributable to the difference in age and developmental stage of the larvae at initial stocking. Additionally, the feeding frequency employed in the Abolhasani et al. (2014) study could partially account for
the higher observed survival, as those authors offered *Artemia* four times per day relative to the practice of feeding twice per day in the current study. However, the feeding frequency administered herein is more reflective of industry standards in Florida, where larval Tiger Barb are fed twice daily for the initial 2–3 weeks of rearing (Casiano, personal communication). Further research investigating the optimal feeding frequency and rations for Tiger Barb larvae is merited.

Differences in survival between larvae that were fed *MD3* in the dietetics trial (33.5 ± 6.6%) and the feed attractants trial (23.4 ± 3.1%) may have resulted from the repeated stress of sampling fish from each tank at 6, 8, and 10 dph, which occurred in the feed attractants trial but not in the dietetics trial. Additionally, observed differences may have resulted from the genetic disparity between the brood sources of the two trials.

Previous authors have attributed the early digestive competence observed in cyprinids to the presence of a pharyngeal jaw structure that includes pharyngeal teeth, an opposing keratinized pharyngeal pad, and thick muscular fibers that effectively preprocess complex macronutrients through mastication (Hofer 1991; Sibbing et al. 1998). Using electron microscopy, Huysseune et al. (1998) observed invagination of germ cells destined to be the dental organ in the pharyngeal region of *Zebrafish Danio rerio* embryos as early as 2 d postfertilization (0 dph), and they observed an erupted, functional tooth at 6 d postfertilization (4 dph). The development of pharyngeal teeth in Tiger Barb is slightly delayed relative to *Zebrafish*, wherein the deposition of dentine matrix and an enamel cap are apparent in the dental organ at 4 dph and a functional, erupted pharyngeal tooth is first detected at 6 dph.

The keratinization of the thickened pharyngeal pad that is situated on the dorsal epithelial surface directly opposing the pharyngeal teeth was first apparent in Tiger Barb at 6 dph, which coincides with eruption of the first pharyngeal teeth as well as exogenous feeding. The presence of both of these structures signals the completion of the pharyngeal masticatory organ in Tiger Barb, which greatly aids
digestive function and thereby prey diversity in cypriniform fishes (Sibbing 1988; Sibbing et al. 1998). It is possible that the presence and functionality of the pharyngeal jaw structure in Tiger Barb at first feeding facilitate the successful introduction of MDs at this stage.

Goblet cells producing both acidic, AB-positive mucins and neutral, PAS-positive mucins were present in epithelial cells in the mucosa surrounding the pharyngeal jaw structure beginning at 4 dph, and those producing acidic mucins greatly proliferated through 20 dph. These mucins serve to lubricate ingestion of food particles, aid in immune functions, and—specifically in larval fishes—facilitate efficient osmoregulation (Holt 2011). Numerous goblet cells with varying shapes and mucin types were also present throughout the intestine, which agrees with previous descriptions of goblet cells in adult Tiger Barb (Leknes 2014). Leknes (2014) theorized that the observed prevalence and diversity of goblet cells facilitate digestion of a diet rich in cellulose.

Although digestion, assimilation, and subsequent survival and growth are possible after first feeding with MDs in Tiger Barb, increased survival and growth may be possible through the incorporation of feed attractants. Feed attractants have been investigated extensively for use in aquaculture. In general, most studies have evaluated the efficacy of using either free amino acids or nucleotides to increase feeding incidence of diets containing high levels of plant-based ingredients (Dias et al. 1997; Kubitza et al. 1997; Papatriphon and Soares 2000). Amino acids like betaine, taurine, tryptophan, alanine, arginine, and glycine, as well as nucleotides like inosine, can increase feeding incidence while enhancing growth and survival when compared to diets devoid of them (Kubitza et al. 1997; Papatriphon and Soares 2000; Zou et al. 2017). Attractants are first detected at the chemosensory olfactory rosette situated in the olfactory pit of the nares, where innervated sensory cells transmit signals to the olfactory bulb in the brain, eliciting a stimulus (Barth et al. 1996). The ontogeny of the olfactory rosette, arising from the olfactory placodes, occurs very early on in embryonic development in Zebrafish, with innervation and connection to the brain occurring at 1 d postfertilization (Barth et al. 1996). In Tiger Barb, an olfactory rosette was apparent at 4 dph, indicating the potential for functional olfactory detection.

Stimuli resulting from the detection of appetiteregulating compounds act on the endocrine appetite control system, with effective feed attractants inducing increases in orexigenic agents, such as neuropeptide Y and ghrelin, while simultaneously suppressing the expression of anorexigenic agents, such as leptin and cholecystokinin (CCK; Volkoff et al. 2009; Hoskins and Volkoff 2012; Zou et al. 2017). The onset of gene expression for specific olfactory receptor nerves occurs asynchronously in larval Zebrafish, possibly demonstrating elevated prey acuity at these early stages (Barth et al. 1996). Although the inclusion of taurine, tryptophan, betaine, or a mixture of the three in an MD did not elicit differential responses with regard to feed intake, total protein content, or trypsin activity in Tiger Barb, all of these parameters increased with age. This may be due to an increase in the diversity of specific olfactory receptor neurons, effectively decreasing olfactory specificity and increasing prey recognition. In concert with other developmental milestones that impact feeding success (i.e., development of finnage, increased locomotor capacity, and behavioral habituation), this may contribute to observed differences in temporal feeding success. Evaluation of gene expression for the specific olfactory nerves related to the attractants used in this study could confirm this assertion. Alternatively,
differential feeding success among individuals may have resulted in attrition of non-feeding individuals, which subsequently would have positively shifted the observed parameters.

Trypsin activity in fish is controlled by a positive feedback loop, wherein ingestion of protein-rich food items elicits an increase in trypsin production by the pancreas and subsequent enzyme activity in the intestinal lumen. Previous researchers have theorized that the mechanism of this feedback loop is reliant on the hormone CCK, which acts on the pancreas to induce trypsinogen production (Zambonino Infante and Cahu 2007; Tillner et al. 2014). In this scenario, trypsin in the gut lumen hydrolyzes CCK in the absence of appropriate substrate, thereby limiting overproduction of the zymogen. When proteins and shorter polypeptides are present, trypsin preferentially binds and hydrolyzes them, allowing CCK to induce more trypsinogen production and release by the pancreas (Zambonino Infante and Cahu 2007). Indeed, trypsin activity has been shown to be acutely modulated by ingested feed items at a differential rate, possibly related to the variable binding affinity of trypsin for various substrates (Zambonino Infante et al. 2008; Tillner et al. 2014). Trypsin activity increased in a similar fashion to feeding incidence in the feed attractants experiment across all treatments, which may be explained by the aforementioned positive feedback scenario. Differences among attractant treatments were not observed with regard to protein content. Although protein content increased between 6 and 8 dph, this could have been due to growth and tissue deposition in the larvae as opposed to differential feed intake between the two ages.

The results of this study indicate that introducing commercially manufactured MDs at first feeding for Tiger Barb results in adequate survival and growth relative to feeding with Artemia. This is likely due to the early development of the pharyngeal jaw and associated masticating elements that can effectively pre-process large, insoluble macronutrients prior to entering the intestine and encountering pancreatic enzymes. Trypsin and bile salt-dependent lipase were detectable prior to first feeding and increased until peaks at 17 dph, indicating increases in enzymatic production as well as digestive competency in Tiger Barb over this time period. Although the potential feed attractants evaluated did not result in significantly different feeding incidence, protein content, or trypsin activity, these parameters were variable with age, indicating either an increasing affinity for MDs between 6 and 10 dph or an increased capture efficiency as the larvae developed. Taken together, these results illuminate the digestive ontogeny of Tiger Barb and how it relates to the early production stages of this ornamental cyprinid. Experimental results show that introduction of an MD at first feeding in Tiger Barb larval culture can be successful, which provides an opportunity to refine current feeding practices for the benefit of production efficiency and reducing the reliance on live feed organisms.

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REFERENCES


