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Why can't young fish eat plants? Neither digestive enzymes nor gut development preclude herbivory in the young of a stomachless marine herbivorous fish

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ABSTRACT

Most young fishes lack the ability to function as herbivores, which has been attributed to two aspects of the digestive system: elevated nitrogen demand and a critical gut capacity. We compared the digestive morphology and biochemistry of two size classes of the marine herbivore *Hyporhamphus regularis ardelio*, pre-ontogenetic trophic shift (pre-OTS, <100 mm) and post-ontogenetic trophic shift (post-OTS, >100 mm), to determine what limits the onset of herbivory and how their digestive processes fit with current models of digestion. Two gut-somatic indices comparing gut length to body length (relative gut length) and body mass (Zihler's Index) demonstrated a significant decrease (RGL 0.59 → 0.49, $P<0.01$; ZI 3.24 → 2.44, $P<0.01$) in gut length relative to body size. There was little difference in enzyme activity between the two classes, with juveniles showing similar levels of carbohydrase and lipase and less protease compared with adults, indicating that juveniles did not preferentially target nitrogen and were as capable of digesting an herbivorous diet. These findings suggest that herbivory in this fish is not limited by the function of the post-oesophageal digestive tract, but rather the ability of the pharyngeal mill to mechanically process plants. Our findings offer partial support for the current model of stomachless digestion, indicating that further refinement may be necessary.

1. Introduction

Ontogenetic trophic shifts are changes in diet that occur during an animal's life history, and are common amongst vertebrates that are herbivores as adults (White, 1985). Such shifts present an opportunity to investigate the developmental and physiological requirements of herbivory, as they represent the point at which herbivory becomes a feasible trophic specialisation. Marine fishes offer a challenging case study for understanding the potential limiting factors of herbivory, as substantially less is known about herbivorous fishes than about terrestrial herbivores (Choat and Clements, 1998; Clements et al., 2009) and comparative studies of digestive physiology on either side of a carnivore-to-herbivore trophic shift have been limited (Moran and Clements, 2002; Elliott and Bellwood, 2003; Drewe et al., 2004; German et al., 2004).

The apparent inability of juvenile fishes to thrive on an herbivorous diet has been attributed to their need to meet an elevated nitrogen (N) demand and the lack of some critical development of the alimentary system and accessory organs. In order to maintain a rapid growth rate, juveniles may have elevated N requirements, forcing them to consume a protein-rich, animal diet until this high N demand subsides (White, 1985). Alternatively, alimentary systems of juveniles may lack the ability to adequately process plant matter, as trophic shifts tend to coincide with gut lengthening (Montgomery, 1977; Stoner and Livingston, 1984; Kramer and Bryant, 1995a; Drewe et al., 2004; German and Horn, 2006). Herbivores tend to have longer, more complex guts than carnivores (Al-Hussaini, 1947; Kramer and Bryant, 1995b; Elliott and Bellwood, 2003), which may enhance digestive efficiency through increased mucosal surface area (Frierson and Foltz, 1992; Horn et al., 2006; German, 2009a; German et al., 2010) or by achieving a necessary gut volume and processing capability to assimilate sufficient energy to meet metabolic needs (Benavides et al., 1994).

The halfbeaks (Hemiramphidae) are a predominantly herbivorous family of fishes (Randall, 1967; Carr and Adams, 1973; Robertson and Klumpp, 1983; Carseldine and Tibbetts, 2005) with ontogenetic trophic shifts from carnivory to herbivory that have been well characterised at species-specific points in development (Robertson

Abbreviations: AMH, adaptive modulation hypothesis; CV, coefficient of variation; K_m, Michaelis–Menten constant; N, Dietary nitrogen; OTS, ontogenetic trophic shift; PFR, plug flow reactor; post-OTS, pre-ontogenetic trophic shift = <100 mm SL; pre-OTS, post-ontogenetic trophic shift = >100 mm SL, RGL, relative gut length $\frac{gut\ length}{standard\ length}$; SL, standard length, measured from the tip of the upper jaw to the caudal flexure; TSGA, total standardised gut activity; U, enzymatic activity units (1 μmol product liberated per minute); ZI, Zihler's Index $\frac{gut\ length}{10 \times \sqrt[3]{body\ mass}}$.

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Fig. 1. Pre-OTS (65 mm SL) and post-OTS (187 mm SL) *Hyporhamphus regularis ardelio*, below and above respectively, with excised intestines. Intestines are positioned approximately according to where they lie in the visceral cavity. Gut region divisions for digestive enzyme analyses are marked with arrowheads. These sections are referred throughout as posterior, mid and distal segments.

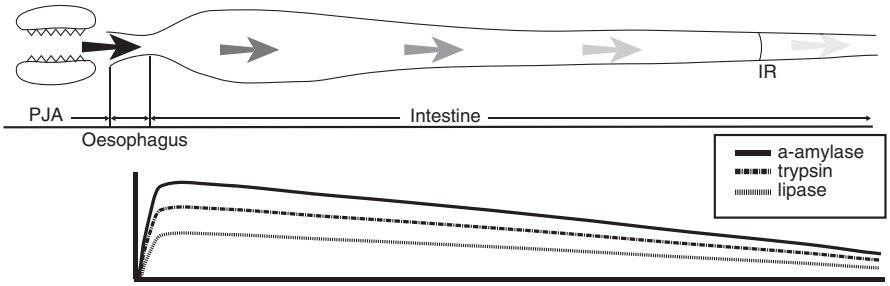

Fig. 2. Schematic diagram of digestive function predicted by Plug Flow Reactor theory. Ingesta enter the intestine directly from the oesophagus, after mechanical disruption by the pharyngeal jaw apparatus (PJA), if present, or oral jaws. Arrow shading corresponds to nutrient concentration and reaction gradient (i.e., digestive enzyme activities), with darker arrows indicating greater levels of both. Ingesta are immediately exposed to pancreatic digestive enzymes, which persist throughout the entirety of the intestine. The PFR is characterised by a high throughput rate and relatively steep gradients of nutrient concentrations and digestive enzyme activities.

and Klumpp, 1983; Tibbetts and Carseldine, 2005). However, little is known about changes in digestive tract morphology that might be associated with the hemiramphid trophic shift. Juveniles have seldom been studied and the bulk of our knowledge about their digestion stems from work on the morphology of adult halfbeaks, which have a digestive tract comprising a short, wide diameter intestine that extends as a straight tube from oesophagus to anus, lacking convolutions, a stomach, and gastric diverticulae (Fig. 1; Klumpp and Nichols, 1983; Robertson and Klumpp, 1983; Tibbetts, 1991; Tibbetts and Carseldine, 2003). Herbivory in these fishes appears to be dependent upon a well developed pharyngeal mill, which mechanically processes food (Tibbetts and Carseldine, 2003). In what appears to be the only ontogenetic trophic study of halfbeaks, the development of the pharyngeal mill, particularly the number, size and coverage area of pharyngeal teeth, has been found to correlate with the onset of herbivory and this development may play a critical role in facilitating the onset of herbivory by conferring the ability to adequately rupture plant cell walls (Tibbetts et al., 2007).

To date, the only model of digestive tract function that fits stomachless fishes is the theoretical plug-flow reactor (PFR) model, proposed by Penry and Jumars (1987) and applied to herbivorous fishes by Horn and Messer (1992). In a PFR gut (Fig. 2), a robust pharynx, if present, mechanically processes food (*sensu* Horn and Messer, 1992) and a relatively short intestine serves as the sole site of chemical reaction. This model is characterised by high intake and throughput rate, which allows the fish to access sufficient nutrients from their relatively poor quality diet. Based solely upon observations of alimentary tract morphology including the milling function of the pharyngeal jaws (Tibbetts, 1991; Tibbetts and Carseldine, 2003) and measurements of a gut passage rate of approximately 4 hours (Klumpp and Nichols, 1983), halfbeaks appear to fit the PFR model, as described by Horn and Messer (1992).

Table 1
Hypothesized patterns of GI tract characteristics in *Hyporhamphus regularis ardelio*.

Characteristic	Function	Hypothesized pattern based on PFR model[a]	Prediction based on AMH[b]
Gut length	Gut capacity	N/A	Larger in post-OTS fish
Enzyme activities			
Amylase	Hydrolyzes starch	Decreasing activity in distal intestine	Elevated in post-OTS fish
Trypsin	Hydrolyzes protein	Decreasing activity in distal intestine	Elevated in pre-OTS fish
Lipase	Hydrolyzes lipids	Decreasing activity in distal intestine	Elevated in pre-OTS fish

[a] Pattern of enzymatic activity based on a plug flow reactor (PFR) model, as described by Horn and Messer (1992).
[b] According to the adaptive modulation hypothesis (AMH; Karasov and Martinez del Rio, 2007) an animal's diet leads to the *a priori* expectation of elevated digestive enzyme activities against those compounds in high concentration in the animal's diet. The carnivorous diet of pre-OTS fish contains approximately 50% protein, 4% carbohydrate, and 9% lipid (Dall et al., 1991), whereas the herbivorous diet of post-OTS fish contains approximately 9% protein (Birch, 1975), 40% carbohydrate (Montgomery and Targett, 1992), and 4% lipid (Nichols et al., 1982). Thus, we expected amylase activity to be elevated in post-OTS fish, whereas trypsin and lipase activities were expected to be elevated in pre-OTS fish.

In this study, we used the marine halfbeak *Hyporhamphus regularis ardelio* as a model in which to investigate the physiological and morphological changes of the digestive tract that occur at the onset of herbivory. First, we investigated whether the digestive biochemistry of pre-ontogenetic trophic shift (OTS) and post-OTS fish was predicated on diet. Using digestive enzyme assays, we measured the activity levels of amylase, trypsin and lipase, which hydrolyse starches, proteins and lipids, respectively (Table 1), all of which are synthesised in the pancreas and secreted into the gut lumen via the hepatopancreatic duct. Based on the adaptive modulation hypothesis (AMH; Karasov and Hume, 1997; Karasov and Martinez del Rio, 2007) we expected digestive enzyme activities to be matched to dietary biochemistry and reflect the relative importance of ingested nutrients. Second, we investigated whether relative gut length and Zihler's index–two morphometric indices of gut size – correlated with the OTS. Finally, we analysed the patterns of enzyme activity along the gut to determine whether the digestive tracts of pre- or post-OTS halfbeaks function as PFRs, as described by Horn and Messer (1992; Table 1). Overall, the results of this study were used to characterize the digestive strategy of the stomachless herbivore, *H. regularis ardelio*, to assess whether they fit into current models and theories of digestive physiology.

2. Materials and methods

2.1. Fish collection

Hyporhamphus regularis ardelio were collected in June 2006 in the vicinity of the Moreton Bay Research Station (MBRS), Dunwich, North Stradbroke Island (27°30'S, 153°24'E), Queensland, Australia. Juveniles were collected with dip nets on partially submerged sand flats and adults were collected with a seine net (50 m long, 8 mm mesh) over seagrass beds. Fish were transported back to MBRS, where they were killed with a sharp blow to the head, frozen in a −80 °C freezer, and subsequently transported to the University of Queensland, Brisbane on dry ice and processed upon arrival. Fish were captured and killed according to approved animal ethics protocol CMS/332/06/URG of the University of Queensland Animal Ethics Committee. Upon dissection, gut contents were examined to confirm that they matched the dietary description of Tibbetts and Carseldine (2005), in which terrestrial insects and aquatic crustaceans dominated the diet of pre-OTS fish and seagrass (*Zostera muelleri*) dominated the diet of post-OTS fish.

2.2. Gut-somatic indices

Morphometric measurements were obtained from formalin fixed specimens to reduce the influence of tissue displacement during dissection. Specimens were blotted dry, weighed (±0.01 g wet weight), and measured from the tip of the upper jaw to the caudal flexure (standard length, ±0.1 mm). In the same manner, the length and mass of the gut, from the termination of the oesophagus to the anus, were determined. Zihler's index (ZI; Zihler, 1982) and relative gut length (RGL; Al-Hussaini, 1947) were compared as a function of size class.

2.3. Assays of digestive enzyme activity

To quantify ontogenetic changes in gut enzyme function, the post-oesophageal alimentary tracts were excised from seven pre-OTS (54.0–72.0 mm S_L) and ten post-OTS (134.0–185.5 mm S_L) *H. r. ardelio* (Tibbetts and Carseldine, 2005). The guts were divided into three approximately equal sections to evaluate the amount of enzyme activity in different regions of the gut: the anterior intestine, including the hepatopancreas and the gall bladder (proximal intestine); the posterior intestine delimited posteriorly by the ileorectal valve (mid intestine) and the rectum (distal intestine; Fig. 1), extending from the ileorectal valve to the anus. Liver and gall bladder tissues were included in the proximal section due to the diffuse nature of the pancreas (Tibbetts, 1991) and the resultant uncertainty of where pancreatic tissue may be located. Gut sections containing their contents were homogenised individually with a Tissue-Tearor homogeniser (Biospec Products, Bartlesville, OK, USA) in 30 volumes (v/w) of ice cold 50 mM Tris–HCl, pH 7.4 (Amresco 0234, Solon, Ohio). Homogenate-buffer solution was centrifuged at 5500 *g* for 5 min at 4 °C and the supernatant was separated into 500 µl aliquots and stored at −80 °C until used in enzyme assays.

Assays were run in duplicate at 25 °C in Greiner-Bio One 96 well microplates (#655151, Interpath Services, West Heidleberg, Vic., Australia) and absorbance was read with a FLUOstar OPTIMA microplate reader (BMG Labtech, Mornington, Vic., Australia). All pH values for enzyme assay solutions were taken at room temperature (23–26 °C) and all reagents were purchased from Sigma-Aldrich (Sydney, NSW, Australia) unless specified otherwise. Every reaction was run against homogenate and substrate blanks (Skea et al., 2005), and all assays were run at saturating substrate concentrations as determined with preliminary optimisations (German et al., 2004). End-point assays (α-amylase and trypsin) were also time optimised to ensure incubation period fell within linear range.

To ensure that all enzyme assays were run under saturating conditions, the Michaelis–Menten constants (K_m) were determined for each reaction using nonlinear regression with GraphPad Prism 5 (GraphPad Software, La Jolla, California). Homogenate from the proximal gut portion of each adult fish in order to include hepatopancreatic tissue. The K_m for α-amylase was determined with 7 starch concentrations from 0.06% to 2.07%, for lipase with 8 p-nitrophenyl myristate concentrations ranging from 0.077 to 3.60 mM and for trypsin with 7 N_α-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) concentrations ranging from 0.04 to 1.30 mM.

The activity of α-amylase (EC 3.2.1.1) was assayed using the modified Somogyi–Nelson method described by German et al. (2004). Substrate was prepared by adding 2% starch to 0.8 M sodium citrate (pH 7) buffer and boiling the solution for 5 min. After cooling the starch solution, 87.5 µl starch and 12.5 µl homogenate were added to a microfuge tube and incubated at room temperature for 30 min. The reaction was stopped by adding 20 µl 1 M NaOH and 200 µl Somogyi–Nelson reagent A. The tube was immersed in boiling water for 10 min to accelerate the copper–glucose complex. 200 µl Somogyi–Nelson reagent B was then added and allowed to develop for 5 min. The solution was then diluted in water and centrifuged at 8500 *g* for 5 min. The supernatant was read at 650 nm to determine glucose content and a glucose standard curve was used to determine α-amylase activity. Activity was expressed in U (1 µmol glucose liberated per minute) per gram wet weight of gut tissue.

Trypsin (E.C. 3.4.21.4) activity was determined using a modified version of the German et al. (2004) assay following Preiser et al. (1975). However, while German and colleagues used enterokinase to activate pancreatic trypsin, we evaluated endogenously activated trypsin that was present in the gut tissue and digesta, so enterokinase activation was not necessary. In a microfuge tube, 70 µl homogenate was added to 80 µl 2.00 mM BAPNA substrate and 70 µl 100 mM tris–HCl (pH 8) buffer, and left to incubate for 45-min at room temperature. The reaction was stopped by adding 200 µl of 0.2 N HCl. 50 µl of the Bratton–Marshall reagents, 0.1% sodium nitrite, 0.5% ammonium sulfamate, 0.05% N-(1-naphthyl)ethylenediamine HCl in 95% ethanol, were then added (in listed order) at 3-min intervals. After 5-min room temperature incubation, 100 µl was plated and absorbance was read at 550 nm and activity was determined with a p-nitroaniline standard curve constructed using Bratton–Marshall reagents. Trypsin activity was expressed in U (1 µmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Non-specific bile-activated lipase (EC 3.1.1.–) activity was assayed following the modified Iijima et al. (1998) method adapted from German et al. (2004). 71.5 µl 7.0 mM sodium cholate bile salt solution in 250 mM Tris–HCl buffer (pH 9) and 2.5 µl 10 mM 2-methoxyethanol were added to 5 µl homogenate to activate lipase in the homogenate over a 15 min incubation at room temperature. Once activated by the bile salt solution, 21 µl 10 mM p-nitrophenyl myristate substrate, dissolved in 95% ethanol, was added and absorbance measured continuously at 405 nm for 15 min. Lipase activity was determined with a p-nitrophenol standard curve and results were expressed in U (1 µmol p-nitrophenol liberated per minute) per gram wet weight of gut tissue.

All enzymatic activity data were compared using linear regression to protein content of the gut homogenate samples assayed using the Bradford reaction (Bradford, 1976). No correlation between enzyme activity and protein content was observed, so activities were more appropriately expressed as U per gram wet weight of gut tissue rather than U per mg protein, although we acknowledge the potential for variations in tissue water content may introduce some degree of variability to the results.

To compare the total capacity for enzymatic digestion, the total standardised gut activity (TSGA; µmol substrate liberated per min) was calculated, which allowed activity to be expressed in terms of the entire gut rather than per gram of gut tissue. TSGA was determined by multiplying activity expressed in U per gram wet weight by the weight of the gut sample used to get total activity per section and then summing the activity from the three gut regions for each enzyme.

2.4. Statistical analysis

To examine whether the digestive tract of *H. regularis ardelio* functions as a PFR, digestive enzyme activities were compared between the three gut sections within pre-OTS and post-OTS fish for each enzyme using one-way ANOVAs followed by a Tukey's HSD multiple-comparison test with a family error rate set at $P \leq 0.05$. To test the AMH, TSGA was compared between the two size classes (pre-OTS versus post-OTS) using Welch two-sample *t*-tests, with the significance level set at $\alpha = 0.05$. To test the gut capacity hypothesis, RGL and ZI were compared between pre- and post-OTS fish using a Wilcoxon Sign Rank test with the significance level set at $\alpha = 0.05$. All statistical analyses were done using R v2.10.1 (R Foundation for Statistical Computing, Austria).

3. Results

3.1. Gut-somatic indices

RGL significantly decreased in *H. r. ardelio* from a pre-OTS ($n = 17$) mean of 0.59 ± 0.01 to a post-OTS ($n = 10$) mean of 0.49 ± 0.01 ($W = 164$, $d.f. = 24$; $P < 0.01$; Fig. 3a). Similarly, ZI significantly decreased from a pre-OTS mean of 3.24 ± 0.06 to post-OTS mean of 2.44 ± 0.05 ($W = 170$, $d.f. = 24$; $P < 0.01$; Fig. 3b).

3.2. Digestive enzyme activity

The Michaelis–Menten constants (K_m) of α-amylase, trypsin and lipase are given in Table 2. The mean activity values in each gut portion of each enzyme in pre- and post-OTS *H. r. ardelio* are given in and shown by section in Fig. 4.

Both pre- and post-OTS fishes displayed similar patterns of α-amylase enzyme activity when expressed in terms of activity per g tissue (Fig. 4A). Within post-OTS fish, α-amylase activity was significantly lower in the proximal portion of the gut than in either of the two more distal sections ($F_{2,27} = 3.99$; $P = 0.03$). Similarly, activity in the proximal section in pre-OTS fish was lower than in the two more distal sections, though the difference was not significant ($F_{2,18} = 3.11$; $P = 0.069$). When activity along the entire gut was converted to TSGA, post-OTS fish showed significantly greater activity than pre-OTS fish ($t = -8.74$, $d.f. = 9.14$; $P < 0.01$).

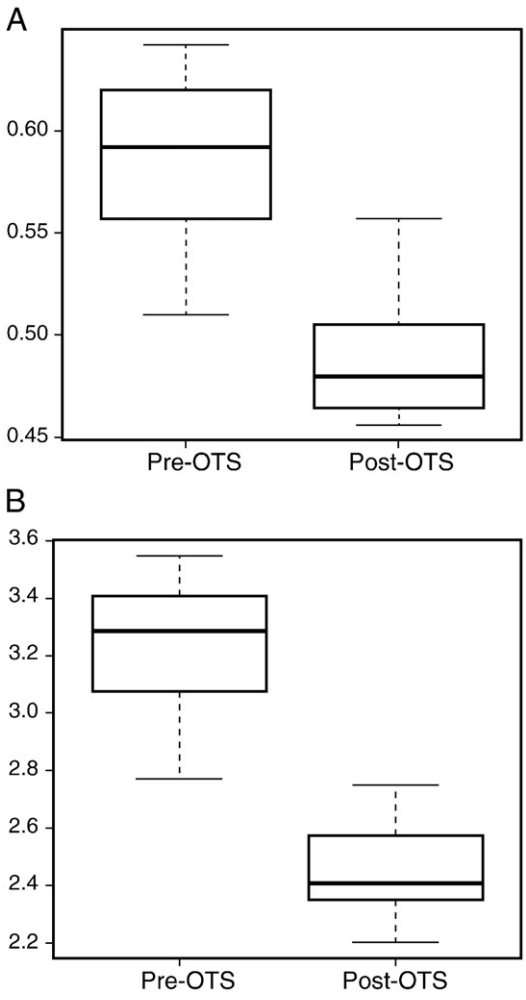

Fig. 3. Morphometric analyses of gut length in relation to fish length and mass. A) Relative gut length and B) Zihler Index in *Hyporhamphus regularis ardelio*. Mean RGL in pre-OTS fish was 0.59 ± 0.01 ($n = 17$) and significantly decreased to 0.49 ± 0.01 ($n = 10$) in post-OTS fish as determined by a Wilcoxon Sign Rank test with $\alpha = 0.05$ ($W = 164$, $d.f. = 24$; $P < 0.01$). Mean ZI in pre-OTS fish was 3.24 ± 0.06 ($n = 17$) which significantly decreased to a mean of 2.44 ± 0.05 ($n = 10$) in post-OTS fish as determined by a *t*-test with $\alpha = 0.05$ ($W = 170$, $d.f. = 24$; $P < 0.01$).

Trypsin activity expression differed markedly between pre-OTS and post-OTS fishes, as the former displayed a distally increasing pattern of activity whereas the latter displayed a distally decreasing pattern (Fig. 4B). The decreasing trend in pre-OTS fish was not found to be significant. Additionally, trypsin activity showed the greatest variation in activity in pre-OTS fishes (coefficient of variation $CV = 0.86$). In post-OTS fish, activity in the proximal section was found to be significantly greater than in the two more distal sections ($F_{2,27} = 6.29$; $P < 0.01$). When activity was converted to TSGA, post-OTS fish displayed significantly greater trypsin activity ($t = -8.09$, $d.f. = 9.023$, $P < 0.01$).

Table 2
Michaelis–Menten constants (K_m) for α-amylase, trypsin and lipase in post-OTS *Hyporhamphus regularis ardelio*.

	α-amylase (% starch)	Trypsin (mM BAPNA)	Lipase (mM p-NPM)
K_m	0.72 ± 0.39	0.23 ± 0.08	0.56 ± 0.24

Note: Values (mean ± SEM; $n = 10$) determined by nonlinear regression. BAPNA = Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride; p-NPM = *p*-nitrophenyl myristate.

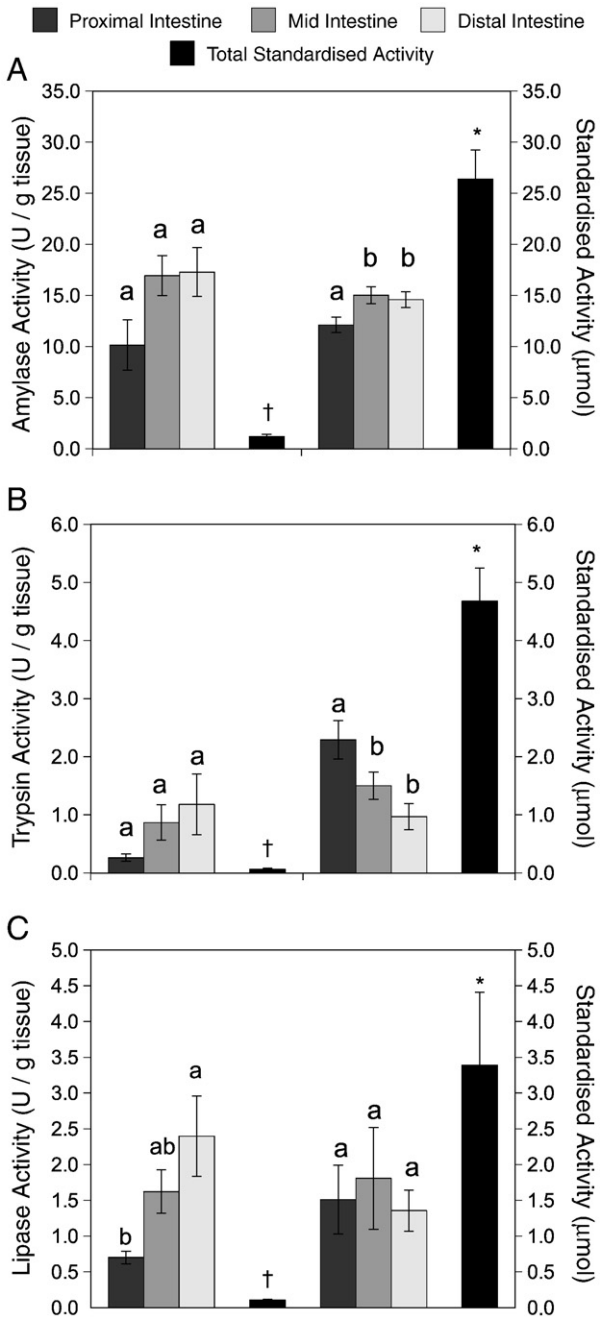

Fig. 4. Activity levels of A) α-amylase, B) trypsin and C) lipase in three segments of the gut in pre-ontogenetic trophic shift (54.0–72.0 mm SL measured from the tip of the upper jaw to the caudal flexure) and postontogenetic shift (134.0–185.5 mm SL) *Hyporhamphus regularis ardelio*. Regional enzyme values are mean ± SE, expressed as U (1 µmol substrate liberated per minute per gram wet weight of gut tissue) with scale shown on the left *y*-axis. Total standardised activity values are mean ± SE, expressed as µmol substrate liberated per minute with scale shown on the right *y*-axis. Bars marked with differing letters are significantly different than others within the same age class, as determined by one-way ANOVA followed by Tukey's HSD with a family error rate of $P = 0.05$ for intraclass comparisons. Total Standardised Activity values marked with differing symbols (* or †) are significantly different between age classes (α-amylase: $t = -8.74$, $d.f. = 9.14$; $P < 0.01$; trypsin: $t = -8.09$, $d.f. = 9.023$, $P < 0.01$; lipase: $t = -3.23$, $d.f. = 9.00$; $P = 0.01$) as determined by Welch *t*-tests with $\alpha = 0.05$.

Similar to trypsin, lipase activity was found to display a distally increasing pattern in pre-OTS fish (Fig. 4C), with significantly greater activity in the distal section than in the proximal section ($F_{2,18} = 5.1287$; $P = 0.01$). No significant differences were found in post-OTS fish as activity was relatively uniform along the gut. Of the three enzymes assayed, lipase showed the greatest variability among post-OTS samples (CV = 0.97). TSGA was significantly greater in post-OTS fishes than in pre-OTS fishes ($t = -3.23$, $d.f. = 9.00$; $P = 0.01$).

4. Discussion

4.1. Gut-somatic indices and analysis

Our findings agree with previous studies that suggest the alimentary system of the halfbeak *Hyporhamphus regularis ardelio* is atypical amongst herbivorous fishes, as it lacks a stomach and the intestine is short and straight, with a relative gut length of approximately 50% of the body length (Tibbetts and Carseldine, 2003). This short, simple gut appears to be common throughout the Beloniformes (Manjakasy et al., 2009). The RGL measurements are amongst the lowest observed in fishes, and are substantially less than the 2–21 range commonly found in herbivores (Al-Hussaini, 1947; Kapoor et al., 1975; Kramer and Bryant, 1995b).

As halfbeaks have a long and narrow, sagittiform body, this study also employed Zihler's Index (Zihler, 1982) to evaluate whether body mass may more accurately describe the relationship between the length of the gut and the size of the fish. Again, the gut to body mass ratio of *H. r. ardelio* is exceedingly low, with a ZI less than 3, falling far below the range attributed to herbivores of 11–95 (Kramer and Bryant, 1995b). As hemiramphids were previously known to have remarkably short guts, these findings are not particularly surprising. It was unexpected, however, to find that gut length relative to body size, according to both indices, decreased significantly in post-OTS fish, indicating that carnivorous, pre-OTS fish have a relatively longer gut than herbivorous adults. For both indices, pre-OTS halfbeaks tended toward the low end of the scale, fitting into a range which is associated with a planktivorous diet (Horn, 1989), which is appropriate for the diet of pre-OTS fish. Following the ontogenetic shift, however, the decrease in halfbeak relative gut length departs from the predictions of these gut-somatic indices, suggesting that the gut grows at a slower rate than the body in larger fish. Thus, gut length does not play a substantial role in facilitating herbivory in these fish. We cannot completely discount the role of gut development, however, as the gut-somatic indices used do not account for any increase in the volume of the gut, which has been suggested to play an important role in terms of the energetics of diet (Benavides et al., 1994; Bouchard and Bjorndal, 2005).

4.2. Digestive enzyme activities

The K_m values, which describe the affinity with which an enzyme reacts with its substrate, were similar for *H. r. ardelio* to those of other fishes measured with identical methodology (German et al., 2004; German et al., 2010). However, halfbeak α-amylase K_m was higher than several other fishes, including several cyprinid herbivores, with only *Carassius carassius* showing higher amylase K_m (Kuz'mina et al., 1996). Halfbeak trypsin K_m was on the low end of the scale in a range of values reported and cited by Castillo-Yáñez (2005).

Digestive enzyme activities of pre-OTS *H. r. ardelio* did not follow the Plug Flow Reactor model and the AMH was only partially supported. All three enzymes showed distally increasing activity gradients, suggesting that the gut operates in a manner that departs from Horn and Messer's (1992) description of PFR function, perhaps due to their carnivorous diet. This apparent breakdown of PFR function has previously been described in herbivores during periods of starvation (Anderson, 1991) and, as predicted by the AMH (Karasov and Hume, 1997), in response to decreased food intake after a switch to a higher quality diet (Rosenthal and Paffenhöfer, 1972; Klumpp and Nichols, 1983; German, 2009b). In both cases, this modulation, along with increased retention time (Fris and Horn, 1993; Horn et al., 1995; German, 2009a; German et al., 2010), has been suggested as a mechanism that maximises nutrient absorption. As such, it appears that the diet of pre-OTS halfbeaks precludes the currently understood functions of the PFR model.

Despite this apparent protein maximisation strategy in pre-OTS halfbeaks, the increase in α-amylase activity distally along the gut corresponds with the pattern found in a range of herbivores (Anderson, 1991; Tengjaroenkul et al., 2000; Logothetis et al., 2001; Papoutsoglou and Lyndon, 2006). Furthermore, in contrast to AMH predictions, pre-OTS halfbeaks have mass-specific α-amylase activity levels on par with post-OTS halfbeaks, suggesting that pre-OTS *H. r. ardelio* are as capable of enzymatic hydrolysis of starches as herbivorous adults. While the possibility that pre-OTS hemiramphids are gaining nutrition from an unidentified source of starches cannot be ignored, this activity level may indicate that pre-OTS halfbeaks are carnivorous not because they lack the biochemical capability to digest plant material, but rather they lack the mechanical wherewithal. The development of the pharyngeal mill has been shown to play a significant role in the onset of the trophic shift in two species of herbivorous halfbeak (Tibbetts et al., 2007), and the results of the present study support the hypothesis that pharyngeal development is more likely to be the primary factor in facilitating the shift to herbivory, as its development allows for the proper triturating treatment of plant cell walls, freeing the nutritive cellular contents.

After the trophic shift, halfbeak digestion follows AMH predictions, with relatively high levels of α-amylase and similar levels of trypsin and lipase. Whether it adheres to the PFR model is not clear, as little difference in digestive enzyme activity was found along the gut. While α-amylase activity increased slightly in the distal intestine, trypsin activity decreased significantly and lipase activity did not change. Distally decreasing enzyme and reaction gradients are common in teleosts (Fish, 1960; Cockson and Bourn, 1973; Hofer and Schiemer, 1981; Bitterlich, 1985; Chakrabarti et al., 1995; Tengjaroenkul et al., 2000; Logothetis et al., 2001; German, 2009a; German and Bittong, 2009) and reflect the role of the proximal portion of the gut as the primary site of reaction and absorption (Leger et al., 1977; Stroband and Van Der Veen, 1981; Honkanen et al., 1985; German, 2009b; German et al., 2010). However, some species have shown enzyme activity patterns along the gut that range from a relatively uniform distribution (Smoot and Findlay, 2000) to distally increasing (Logothetis et al., 2001). It has been suggested that fishes with unusually short guts may absorb nutrients throughout the length of the gut (Ferraris and Ahearn, 1984), which may explain the relatively uniform distribution of enzyme activity along the gut of *H. regularis ardelio*, particularly in regard to α-amylase and lipase. Further investigations into digestive function, including histological examination of the gut and measurements of nutrient transport rates, must be undertaken to clarify this issue.

Trypsin activity in post-OTS halfbeaks was found to significantly decrease in the distal intestine, which may indicate that digestive function is geared towards a maximisation of protein assimilation in exchange for decreased carbohydrate assimilation efficiency, which may be an advantageous trade-off for an herbivore. That a post-OTS, herbivorous hemiramphid has the enzymatic capability for the efficient digestion of protein is not surprising, as they are able to subsist almost entirely on animal material in highly modified environments, such as urban marine canal developments (Waltham and Connolly, 2006), and herbivorous halfbeaks that live in temperate regions have be found to undergo seasonal and diel shifts to carnivory in response to low seagrass abundance or high zooplankton abundance (Coetzee, 1981; Klumpp and Nichols, 1983; Robertson and Klumpp, 1983). Herbivorous fish have also been shown to preferentially select plant and algal material that is either comparatively high in protein (Neighbors and Horn, 1991) or particularly easy to assimilate (Pillans et al., 2004). Zosteraceae seagrasses dominate the diet of *H. r. ardelio* (Tibbetts and Carseldine, 2005), possibly due to their relatively high levels of nitrogen (Birch, 1975) and protein (Klumpp and Nichols, 1983). Furthermore, elevated activity levels of proteases and lipases have been reported in herbivorous fish and are understood to allow herbivores to maximise the assimilation of any available proteins and lipids in their diet, which are in great demand and can be considered limiting nutrients. Thus protein must be utilised efficiently (Horn, 1989; Gerking, 1994; Hidalgo et al., 1999; Tengjaroenkul et al., 2000; German et al., 2004; De Almeida et al., 2006; German et al., 2010).

4.3. Conclusion

Although we are unable to support or refute PFR function in *Hyporhamphus regularis ardelio*, we have shown that neither digestive enzyme activities nor the length of the gut are the best predictors of the onset of herbivory in this stomachless fish, lending further support to the hypothesis that halfbeak herbivory is limited primarily by the development of the pharyngeal mill (Tibbetts et al., 2007). Halfbeak digestion fails to be adequately described by any of the existing models of herbivory in fishes, including digestive-somatic indices and chemical reactor modelling, indicating that our understanding of herbivory is still developing (Choat and Clements, 1998; Clements et al., 2009). Furthermore, the digestive physiology of ontogenetic trophic shifts requires a great deal more work to understand how the interplay of digestive physiology and morphology govern the onset of herbivory in fishes. Research into the rate of food intake and gut transit time, along with what is already known about dietary preference and dietary capability, may help clarify any changing nutritional demands that correspond with ontogenetic trophic shifts. Evaluating brush-border enzymes and nutrient transport rates will provide insight into how these nutritional demands are met, allowing for a full characterisation of the digestive strategy in the halfbeak.

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