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Enzymatic digestion in stomachless fishes: how a simple gut accommodates both herbivory and carnivory

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Abstract The lack of a stomach is not uncommon amongst teleost fishes, yet our understanding of this reductive specialisation is lacking. The absence of a stomach does not restrict trophic preference, resulting in fishes with very similar alimentary morphology capable of digesting differing diets. We examined the digestive biochemistry of four beloniform fishes: two herbivorous halfbeaks (Hemiramphidae) and two carnivorous needlefish (Belonidae) to determine how these fishes digest their respective diets with their simple, short gut. We found that although the halfbeaks showed significantly greater α-amylase activity than that of the needlefish ($P < 0.01$), trypsin, lipase, aminopeptidase and maltase activity were not substantially different between the two families. We also found that habitat (freshwater vs. marine) appears to play a significant role in digestive capability, as the two freshwater taxa and the two marine taxa were significantly different (ANOSIM; dietary Gobal $R = 0.544$, $P = 0.001$, habitat Global $R = 0.437$, $P = 0.001$), despite their phyletic and dietary similarities. Our findings offer partial support for the adaptive modulation hypothesis, support the Plug-Flow Reactor model of digestion in herbivorous halfbeaks and also support the compartmental model of digestion but suggest that another model is required to describe stomachless carnivorous needlefish.

Introduction

Nearly all vertebrates have an alimentary tract that comprises the same basic components, the oesophagus, stomach, intestine and rectum, which serve to process, digest, absorb and egest food with the help of ancillary organs such as the liver, pancreas and gallbladder (Stevens and Hume 1995). While this general gut plan is relatively conservative among vertebrates, stomach morphology shows astonishing diversity (Smith et al. 2000). Typically, the vertebrate stomach is either a saccular or muscular organ separated by sphincters from the oesophagus and the intestine, and is characterised by specialised cells (oxynticopeptic cells in lower vertebrates, and parietal and chief cells in higher vertebrates) that produce hydrochloric acid and the protease pepsin. The stomach's primary functions are to store food, mechanically process it and begin digestion through protein denaturation and hydrolysis (Stevens and Hume 1995; Smith et al. 2000).

In a number of disparate teleost fish lineages, including some of the most speciose families of freshwater and marine fishes such as the Cyprinidae, Labridae and Gobiidae, the stomach has been secondarily lost (Barton 2007). Understanding how the loss of the stomach affects digestion in these fishes is difficult, as digestion in fishes is

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less well-studied in comparison to terrestrial vertebrates (Choat and Clements 1998; Clements et al. 2009), and because stomachless fishes are defined more by what is absent (a stomach) rather than by any insight into form or function of the alimentary canal. This loss does not appear to impose dietary constraints, as stomachless fishes cover the entire trophic spectrum, including herbivory, omnivory, detritivory and carnivory (Logothetis et al. 2001; Crossman et al. 2005; Horn et al. 2006; German 2009; German et al. 2010). Herbivory in stomachless fishes has been described as relying on a robust pharyngeal jaw apparatus to mechanically process food and rapid gut passage rates through a short gut in order to maximise gut throughput (Horn and Messer 1992). However, some stomachless herbivorous fishes have long food retention times (Clements and Rees 1998), a pharyngeal jaw apparatus that cannot effectively triturate algae (Clements and Bellwood 1988; Clements 1991) and a reliance on microbial fermentation to digest an algal diet (Clements 1991; Montfort et al. 2002; Skea et al. 2005).

Comparatively little physiological work has been done on stomachless carnivores. In a study comparing several species of stomachless minnows (Cyprinidae), German (2009) found notable differences in the digestive enzyme activity levels and localisation patterns between a carnivorous minnow and four species of closely related herbivorous species. In a study of three species of stomachless silversides (Atherinopsidae) with different diets (herbivory, omnivory and carnivory), Horn et al. (2006) compared gut length, gut surface area and enzyme activity level. Not only did they find significant differences between each of the dietary groups, but also between conspecific herbivorous and carnivorous populations. Despite the relative lack of study, these findings point to a difference in how stomachless herbivores and carnivores digest their respective diets.

A common theme to the studies by Horn et al. (2006) and German (2009) is that of the herbivores investigated all have relatively long guts (at least longer than their body length), whereas the carnivores have guts that are generally shorter than their body length (Horn et al. 2006; German et al. 2010). In contrast to this, fishes in the order Beloniformes all have similar, short (approximately 50% of their body length; Tibbetts 1991; Manjakasy et al. 2009) and stomachless digestive tracts (Verigina 1991), yet they have diets ranging from herbivory, to planktivory and carnivory (Fig. 1). All beloniforms also appear to have a large, well-developed pharyngeal jaw apparatus. There may be functional differences, however, in how herbivorous and carnivorous beloniforms digest their food. For instance, the pharyngeal jaws of the halfbeak (Hemiramphidae) act as a grinding mill (Tibbetts and Carseldine 2003) and ingesta throughput is rapid and continuous through a large bore
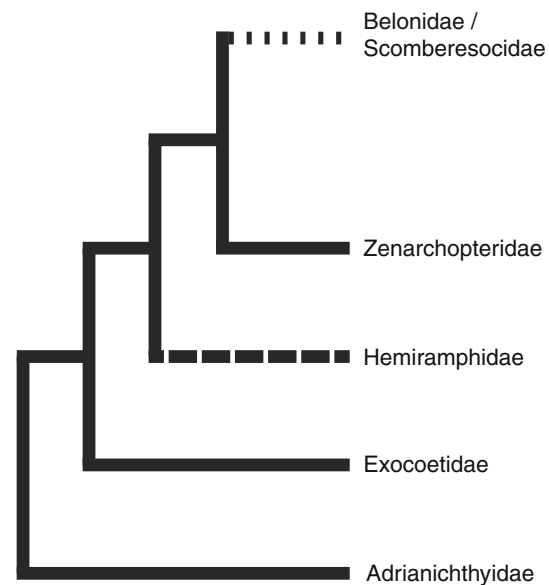

Fig. 1 Simplified phylogeny of the Beloniformes, including familial dietary preference. *Solid lines* indicate planktivory, *dashed lines* indicated herbivory and *dotted lines* indicate macrocarnivory. Phylogeny based on Lovejoy et al. (2004)

tube with little to no axial mixing (Klumpp and Nichols 1983; Robertson and Klumpp 1983). Conversely, needlefish (Belonidae) have been found to use the pharyngeal jaws to grasp and manipulate prey into a "head first" position rather than for mastication, after which the prey item is moved directly into the posterior portion of the intestine where it is retained until it is digested and absorbed (Manjakasy et al. 2009). What is clearly missing is an understanding of how the biochemistry of digestion varies among herbivorous and carnivorous beloniforms.

In the present study, we compared digestive enzyme activities among herbivorous halfbeaks (*Hyporhamphus regularis ardelio* and *Arrhamphus sclerolepis krefftii*) and carnivorous needlefish (*Tylosurus gavialoides* and *Strongylura krefftii*) (Figs. 1, 2). We chose to employ a biochemical approach because the mechanical components of digestion in these fishes have been well described (Tibbetts and Carseldine 2003; Manjakasy et al. 2009) and based on this knowledge, endogenous digestive enzymes are expected to provide the bulk of biochemical digestive function (Skea et al. 2007). Digestive enzyme assays have been shown to be a useful measure of digestive capabilities, such as understanding rates of reaction and diet composition in a number of fishes with a range of dietary habits (Zambonino Infante and Cahu 2001; German et al. 2004; Skea et al. 2005, 2007; German 2009; German and Bittong 2009; German et al. 2010). The use of closely related taxa allowed for better phylogenetic control (German et al. 2010) than many previous comparisons of digestive enzyme activities in herbivorous and carnivorous

Fig. 2 The four beloniform taxa used in this study, from *top* to *bottom*: The herbivorous halfbeaks: *Hyporhamphus regularis ardelio* (HRA) and *Arrhamphus sclerolepis krefftii* (ASK); the carnivorous needlefish: *Tylosurus gavialoides* (TG) and *Strongylura krefftii* (SK). *Scale bar* in bottom right represents 1 cm

fishes, including stomachless fishes (Hofer and Schiemer 1981; Chakrabarti et al. 1995; Hidalgo et al. 1999). Based on the adaptive modulation hypothesis (AMH; Karasov and Hume 1997; Karasov and Martinez del Rio 2007) and the previous findings of the pharyngeal and intestinal morphology of these taxa, we hypothesise that, despite a similar pharyngeal and alimentary morphology, biochemical digestion in these fishes occurs in a substantially different manner with a bias towards carbohydrate digestion in halfbeaks and protein digestion in needlefish and that localisation of these enzymes will correlate with how food is processed in the gut (Table 1).

Methods

Study specimens

Two species of halfbeak (Hemiramphidae), *Hyporhamphus regularis ardelio* (HRA) and *Arrhamphus sclerolepis krefftii* (ASK), and two species of needlefish (Belonidae), *Tylosurus gavialoides* (TG) and *Strongylura krefftii* (SK), were used in this study. To avoid confounding results, we

selected collection sites that had both a halfbeak and a needlefish species in residence. The marine taxa, *H. r. ardelio* and *T. gavialoides*, were caught via seine net (50 m long, 8 mm mesh) and angling, respectively, from shores of Dunwich, North Stradbroke Island, Queensland (27°30′21′′S 153°24′29′′ E) between the hours of 1000 and 1200. The taxa from freshwater, *A. s. krefftii* and *S. krefftii*, were caught via seine net from Lake Awoonga, Gladstone, Queensland (24°05′43′′ S 151°16′15′′ E) between the hours of 0900 and 1100. All fish were collected in December, when the sun rises prior to 0500, thus mid-morning and later capture times ensure that halfbeaks have fed sufficiently to fill their guts (Tibbetts and Carseldine 2005). However, due to the sporadic and highly episodic nature of feeding opportunities for macrocarnivores, it was not possible to ensure that needlefish had fed.

Immediately following capture, fishes were sacrificed via a blow to the head and subsequent severing of the spinal cord, frozen and transported on dry ice (–40°C) to the laboratory at the University of Queensland in Brisbane, where the intestinal tissue was prepared as described below.

Upon dissection and clearing of the gut of its contents, it was observed that the guts both species of halfbeak were full of plant material, in agreement with the findings of Tibbetts and Carseldine (2005), which showed HRA fed nearly exclusively on the seagrass *Zostera muelleri* and ASK fed predominantly on filamentous algae. Nearly all needlefish specimens had guts with ingested contents, although they did not show the same degree of gut fullness as the halfbeaks. Seven of the nine TG guts were found to contain ingesta, which was dominated by relatively large fish along with some crustaceans, in agreement with Manjakasy et al. (2009). Nine of the ten SK guts were found to contain ingesta. Although both fish and freshwater prawns were found in SK guts as well, fish made up a greater proportion of contents in the SK gut than the TG gut. The prey items ingested by SK were also smaller,

Table 1 Hypothesised patterns of enzymatic digestion in the herbivorous halfbeaks *Hyporhamphus regularis ardelio* (HRA) and *Arrhamphus sclerolepis krefftii* (ASK) and the carnivorous needlefish *Tylosurus gavialoides* (TG) and *Strongylura krefftii* (SK)

Family (species)	Diet	Site of digestion and absorption[a]	Carbohydrase activity (Amylase + Maltase)[b]	Protease activity (Trypsin + Aminopeptidase)[b]	Lipase activity[b]
Hemiramphidae (HRA + ASK)	Herbivorous	Along the entirety of the gut in a distally decreasing gradient	High; distally decreasing	High; distally decreasing	Low; uniform distribution
Belonidae (TG + SK)	Carnivorous	High in posterior intestine, intermediate in mid intestine, low in anterior intestine and rectum	Low; uniform distribution	Locally high in the posterior intestine	Locally high in the posterior intestine

[a] Site of digestion and absorption hypothesis in Hemiramphidae is based on the Plug-Flow Reactor model (Horn and Messer 1992) that predicts a nutrient and reaction gradient in stomachless herbivores. In Belonidae, the hypothesis is based on morphological observations of food distribution in the gut

[b] Enzyme activity hypotheses are based on the adaptive modulation hypothesis (Karasov and Hume 1997; Karasov and Martinez del Rio 2007), which predicts that the activity levels of digestive enzymes will be positively correlated with the concentration of the corresponding substrate in the diet

relative to the size of the fish, than those ingested by the more robust TG.

Tissue preparation

To quantify gut enzyme function, the guts from ten HRA (SL range 154.0–181.0 mm, average $L_s = 161.5 \pm 2.69$ mm; standard length is measured from the tip of the upper, i.e. not elongated, jaw to the caudal flexure in halfbeaks), ten ASK (SL range 143.0–174.0 mm, average $L_s = 154.6 \pm 3.03$ mm), ten SK (SL range 246.0–284.0 mm, average SL = 262.8 ± 3.79 mm; measured from the tip of the upper jaw and thus including jaw length in needlefish) and nine TG (SL range 307.0–568.0 mm, average $L_s = 406.33 \pm 26.57$ mm) were excised, cut longitudinally and cleared of contents. The guts were divided into four sections (proximal, middle, distal and rectal) of approximately equal length to evaluate the difference in enzyme activity along the gut. Gut sections were homogenised individually with a Tissue-Tearor homogeniser (Biospec Products, Bartlesville, OK, USA) in 19 volumes (v/w) of ice cold 50 mM Tris–HCl, pH 7.4 (Amresco 0234, Solon, Ohio) for all fishes except ASK, which was homogenised in 29 volumes (v/w) of buffer. Homogenate-buffer solution was centrifuged at $5,500 \times g$ 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.

Assay conditions

Assays were run in duplicate at $25° \pm 1°C$ to reflect the average summer water temperature in Moreton Bay (Queensland Environmental Protection Agency Coastal Services Unit 2005) and Lake Awoonga (pers. comm. Gladstone Area Water Board). Assays were run in Greiner-Bio One 96 well microplates (Interpath Services, West Heidelberg, Vic.) and absorbance was read with a FLUOstar OPTIMA microplate reader (BMG Labtech, Mornington, Vic.). All pH values for enzyme assay solutions were at $25 \pm 1°C$ and all reagents were purchased from Sigma-Aldrich (Sydney, NSW, Australia) unless specified otherwise. Every reaction was run against homogenate and substrate blanks for each assay and all assays were run at saturating substrate concentrations as determined with preliminary optimisations (German et al. 2004). Incubation times were also optimised prior to assays to ensure incubation time period was within linear range.

Enzyme assays

Enzyme assays are described in detail by Day et al. (2010) and are based on assays described by German et al. (2004). Briefly, α-amylase (EC 3.2.1.1) activity was assayed using

a modified Somogyi–Nelson method with a substrate of 3% starch in a 0.8 M sodium citrate (pH 7) buffer, of which 50–75 µl was added to 5–10 µl homogenate and incubated at 25°C for 30–60 min. The reaction was stopped by adding 1 M NaOH and Somogyi–Nelson reagent A. The tube was immersed in boiling water for 10 min to accelerate the copper–glucose complex. Somogyi–Nelson reagent B was then added and allowed to develop for 5 min. The solution was then diluted in water and centrifuged at $8,500 \times g$ 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 87.5–95 µl 2.00 mM Nα-benzoyl-L-arginine-p-nitroanilide in 100 mM Tris–HCl (pH 8.0) was added to 5–12.5 µl homogenate and read continuously for 20 min at 410 nm. 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 with 20 µl 10 mM p-nitrophenyl myristate substrate in 95% ethanol and a buffer solution containing 57.5–65 µl 7.0 mM sodium cholate bile salt solution and 250 mM Tris–HCl buffer (pH 9) and 2.5 µl 10 mM 2-methoxyethanol over a 15 min incubation at 25°C. Absorbance was measured continuously at 405 nm for 20 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.

Maltase (EC 3.2.1.20) activity was assayed using 20 µl homogenate and 10 µl maltose substrate (56 mM maltose in 0.1 M maleate buffer, pH 6.0), which were incubated at 25°C for 45–60 min. Following incubation, 300 µl glucose oxidase/peroxidase (Sigma G-3660) and o-dianisidine (Sigma D-2679) mixture was added and incubated for a further 30 min at 37°C. The sample was then read at 450 nm and maltase activity was determined using a glucose standard curve and maltase activity was expressed in U (1 µmol glucose liberated per minute) per gram wet weight of gut tissue.

Aminopeptidase (3.4.11.2) activity was measured using 80–90 µl of 2.04 mM L-alanine-p-nitroanilide HCl dissolved in 200 mM NaH_2PO_4/Na_2HPO_4 (pH 7.0), to which 10–20 µl gut homogenate was added. Absorbance was read continuously at 410 nm for 30 min and activity was determined using a p-nitroaniline standard curve. Activity was expressed in U (1 µmol p-nitroaniline liberated per minute) per gram wet weight of gut tissue.

Total standardised gut activity (TSGA) was calculated for each enzyme by multiplying the activity for each section by the weight of the gut tissue and summing the results

for each enzyme per species in order to quantify the digestive capacity of the entire gut for each enzyme.

Statistical analysis

One-way ANOVAs followed by a Tukey's HSD multiple comparison test with a family error rate set at $P \leq 0.05$ were used to perform intraspecific analyses of digestive enzyme activities, compared between each of the four segments of the intestine, and interspecific total standardised gut activity, made between each enzyme for all four species using TSGA, using R v2.10.1 (R Foundation for Statistical Computing, Austria).

TSGA data were also used to compare enzyme activity using multidimensional scaling (MDS), with each of the five enzymes as a dimension, and two-way crossed analysis of similarity (ANOSIM), using diet (herbivore or carnivore) and habitat (freshwater or marine) as factors, on Euclidean distances on 999 permutations using untransformed, unstandardised data. Similarity percentage (SIMPER) analysis was performed on untransformed, unstandardised data to determine the contribution of each of the five enzymes to the differences observed between taxa. All multivariate analyses were performed using PRIMER 5.24 (PRIMER-E Ltd., United Kingdom).

Results

Enzyme activities

Both halfbeaks showed a pattern of distally declining α-amylase activity (Fig. 3), though this trend was not significant. The needlefish, however, showed a significant difference in activity among regions of the gut, with activity in the mid-intestine segment significantly greater than in the rectum in TG ($F_{3,31} = 3.31$; $P = 0.03$) and activity in the proximal section significantly higher than the three other more distal sections in SK ($F_{3,36} = 5.18$; $P < 0.01$). Though no significant α-amylase expression pattern was found between gut sections in the halfbeaks, they showed much higher levels of relative α-amylase activity than the two needlefish. This pattern was borne out with TSGA analysis (Fig. 4), which demonstrated that both halfbeaks showed significantly greater total α-amylase activity than the two needlefish, with HRA showing the greatest amount overall ($F_{3,151} = 36.03$; $P < 0.01$).

HRA demonstrated relatively low maltase activity (Fig. 3), with significantly more in the proximal intestine
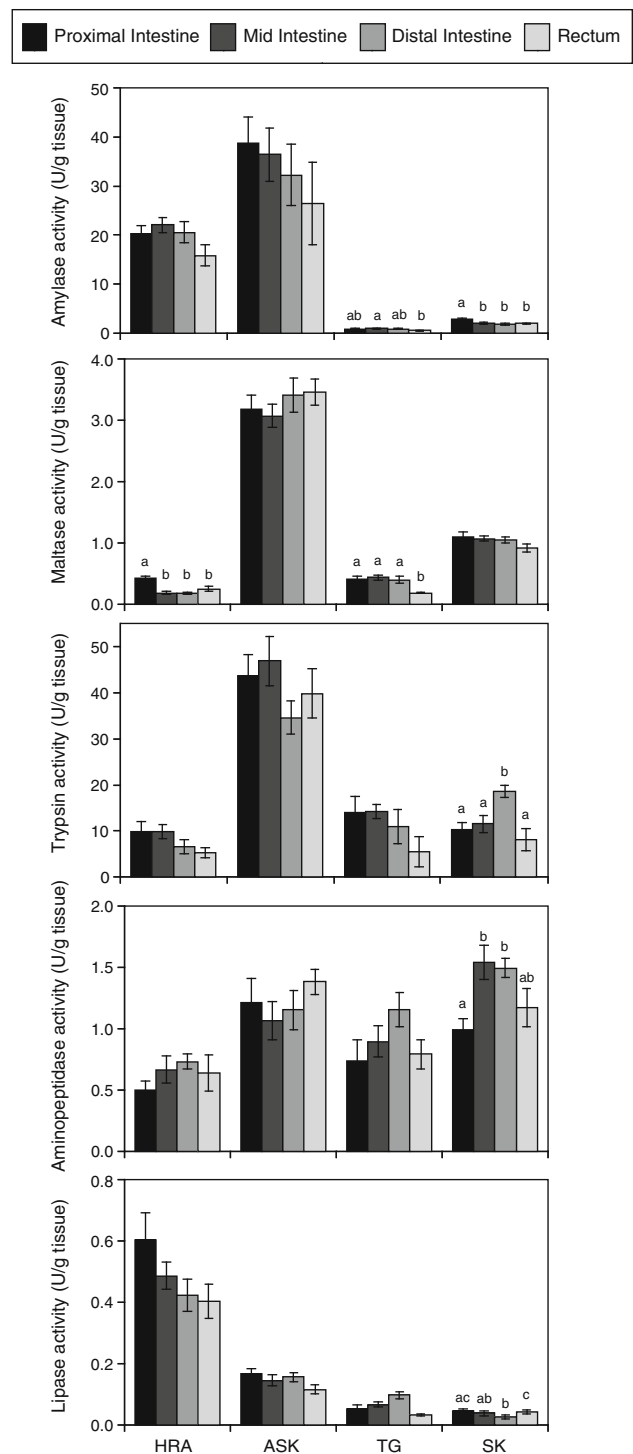

Fig. 3 Activity levels of five digestive enzymes by gut segment in each species. Enzyme activity values are expressed in U (1 µmol substrate liberated per minute per gram wet weight of gut tissue) with *bars* indicating mean ± SE. *Bars* marked with differing *letters* are significantly different than others within the same species, as determined by one-way ANOVA followed by Tukey's HSD with a family error rate of $P = 0.05$ for intraspecific comparisons
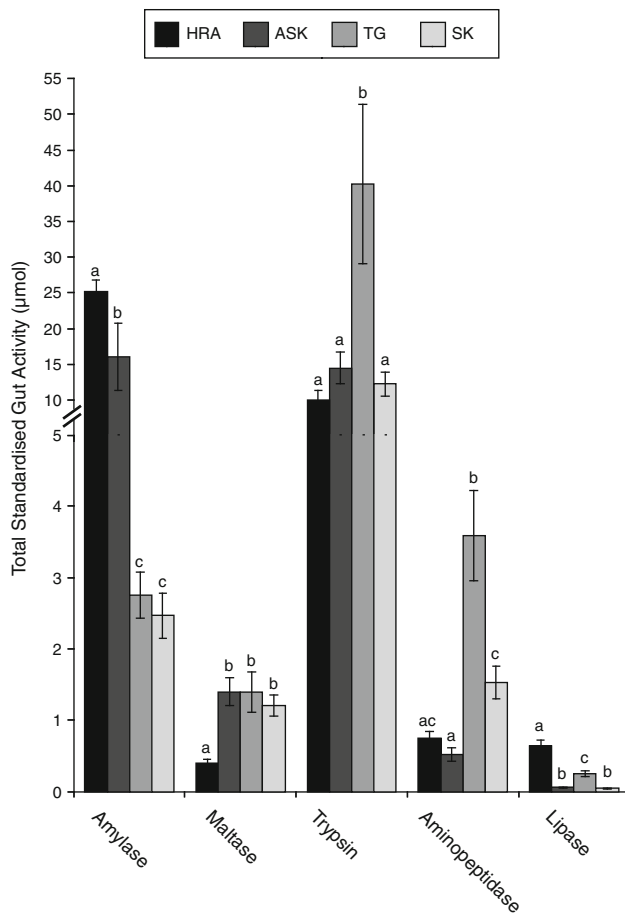
Fig. 4 Total standardised gut activity of the five digestive enzymes assayed in the entire intestine. Values are mean ± SE, expressed as μmol substrate liberated per minute. *Bars* marked with differing *letters* are significantly different than others within the same enzyme, as determined by one-way ANOVA followed by Tukey's HSD with a family error rate of $P = 0.05$ for intraclass comparisons. A break in the scale is indicated by the *parallel diagonal lines* on the y-axis

than in the three more distal sections ($F_{3,36} = 12.475$; $P < 0.01$). TG showed significantly less activity in the rectum than in the three more proximal sections ($F_{3,31} = 7.05$; $P < 0.01$). These two marine species showed less relative activity than the freshwater species ASK and SK, and when TSGA levels were compared (Fig. 4), HRA maltase activity was significantly lower than that of the other three fishes ($F_{3,151} = 14.89$; $P < 0.01$).

Trypsin activity (Fig. 3) in the halfbeaks again showed an overall trend towards a distally decreasing gradient, though it was more variable in ASK and again was not significant. Surprisingly, ASK demonstrated the highest relative levels of trypsin expression, higher than that of either of the carnivorous needlefish. Amongst the needlefish, there was a clear pattern of decreased activity in the rectum of both fishes, and SK showed a significant localisation, as the distal intestine demonstrated higher levels of activity than proximal, middle or rectal intestinal segments

($F_{3,36} = 6.23$; $P < 0.01$). When standardised for gut tissue weight (Fig. 4), levels for trypsin showed that TG had significantly more trypsin activity than the three other taxa ($F_{3,150} = 10.90$; $P < 0.01$) and that the two halfbeaks had activity levels on par with that of the other needlefish, SK.

Aminopeptidase activity differed little among the four taxa (Fig. 3), though HRA showed somewhat less activity than the other three fishes. SK was the only species to show a significant localisation pattern, as activity was significantly greater in the two middle sections than in the proximal section ($F_{3,36} = 4.81$; $P < 0.01$). This trend was also seen in HRA and TG, though it was not significant. In terms of TSGA (Fig. 4), TG showed significantly greater activity than the other three taxa and SK was significantly greater than ASK ($F_{3,151} = 34.37$; $P < 0.01$). Both halfbeaks showed similar levels of activity.

The two halfbeaks demonstrated the greatest relative lipase activities (Fig. 3), with HRA demonstrating substantially greater levels that the other three taxa and ASK showing somewhat more than the needlefish. The distally decreasing enzyme activity pattern in the halfbeaks continued with lipase activity. TG demonstrated a localisation pattern of lipase, with activity in the distal intestine higher than any other section and activity in the rectal segment lower than any other section ($F_{3,31} = 10.29$; $P < 0.01$). No other species demonstrated a significant pattern. When converted to TSGA (Fig. 4), HRA demonstrated the greatest lipase activity and TG lipase activity was greater than that of SK and ASK, which were both similar ($F_{3,151} = 34.38$; $P < 0.01$).

Multivariate analyses

The MDS plot resulted in strong congeneric halfbeak and needlefish clusters according to enzyme expression by diet, as well as a freshwater cluster with the two marine taxa on the peripheries when habitat was considered (Fig. 5). Both of these clusters were found to be significantly different (ANOSIM; dietary Gobal $R = 0.544$, $P = 0.001$, habitat Global $R = 0.437$, $P = 0.001$), indicating that enzyme activity was similar between confamilial taxa and that the two families were significantly different. The similarity within the halfbeaks was driven primarily by α-amylase, whereas the similarity in the needlefish was driven primarily by trypsin (Fig. 5).

SIMPER analysis supported the findings of the MDS plot, demonstrating that diet played the greatest role in similarity, with an average similarity of approximately 72% between the herbivorous halfbeaks and 63% between the needlefish (see supplemental Table S1 in online version of this article). Between the two herbivorous halfbeaks, HRA and ASK, α-amylase and trypsin activities accounted for nearly all similarity. The same enzymes resulted in the vast majority of similarity in the carnivorous needlefish,
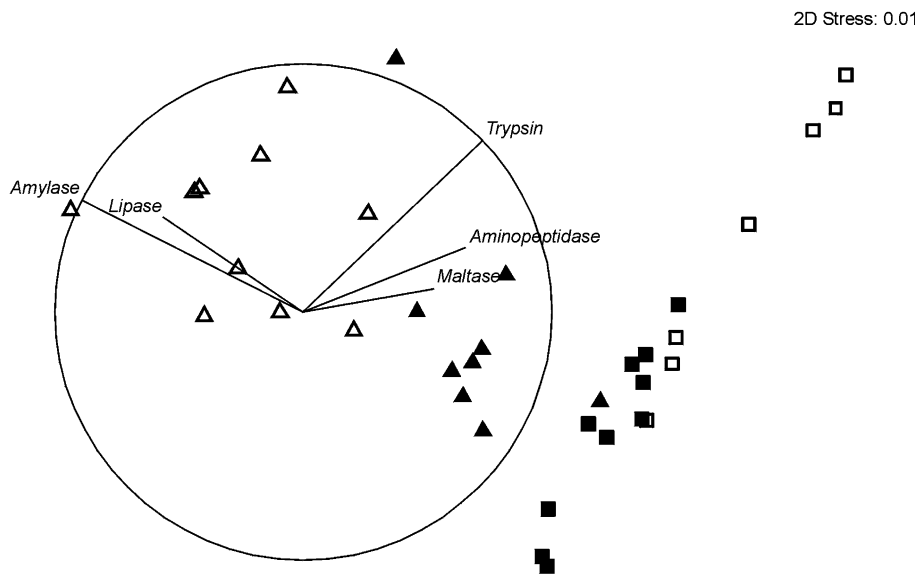
Fig. 5 Multidimensional scaling (MDS) plot of the total activity (TSGA) of each of five digestive enzymes as a function of species and diet (*squares* denote carnivorous needlefish, *triangles* denote herbivorous halfbeak) and habitat (*filled shape* denotes freshwater, *open shape* denotes marine). The distance between any two points reflect their relative similarity, with closer points showing greater similarity. HRA (*open triangle*) and ASK (*filled triangle*) cluster together, as do TG (*open square*) and SK (*filled square*), demonstrating a confamilial grouping of similar enzyme activities. These groupings are supported by ANOSIM analysis (Gobal $R = 0.544$, $P = 0.001$). Additionally, the freshwater taxa ASK and SK clustered in the middle, with the two marine taxa on the peripheries, indicating a significant role of habitat in enzyme activity (Global $R = 0.437$, $P = 0.001$). Each of the assayed enzymes are shown on the plot along with a vector depicting its directional influence, which shows what role each enzyme played in determining the distance between data points. The stress level of the MDS plot (0.01) relates to how well the plot fits all of the factors into two dimensional spaces, with values less than 0.1 considered to fit well

TG and SK, as well, though trypsin had a substantially greater impact than α-amylase (Table S1). Not surprisingly, α-amylase and trypsin contributed the most dissimilarity between the two groups. Though the remaining three enzymes contributed only a marginal amount of similarity within the groupings, the brush border enzymes did follow a priori predictions, with maltase contributing slightly more similarity to the herbivores and aminopeptidase contributing slightly more to the carnivores.

When clustered according to habitat, freshwater taxa were found to have about 65% similarity and marine taxa about 55% similarity. Similarity in both clusters was again driven primarily by trypsin and secondarily by α-amylase, and dissimilarity between the two clusters was divided evenly between α-amylase and trypsin (Table S1). Lipase and brush border enzymes had little contribution towards similarity, though aminopeptidase contributed slightly more than maltase or lipase in the marine fishes, whereas maltase was slightly more influential in the freshwater fishes.

Discussion

Despite having superficially similar alimentary tracts, biochemical digestion in the stomachless belonid halfbeaks and needlefishes differs substantially and reflects their dietary specialisations. The digestive enzyme activity profile of the two carnivorous needlefish species partially followed our predictions, with an emphasis on protein digestion, as evidenced by the dominant role of trypsin and high levels of aminopeptidase. However, lipase activity was not as high as predicted for these carnivores, indicating that lipids are either not as preferentially targeted as expected or are common enough to allow for sufficient assimilation despite low lipase activity. Amongst the two halfbeaks, α-amylase activity was high, and as predicted, greatly contributed to the significant similarity of digestive enzyme patterns of these herbivorous taxa. However, the activity of the other digestive enzymes did not support our hypotheses. Both trypsin and lipase activities in the halfbeaks were either on par with or greater than those of the needlefish. In fact, the freshwater halfbeak *Arrhamphus sclerolepis krefftii* featured the greatest relative levels of trypsin activity and the marine halfbeak *Hyporhamphus regularis ardelio* displayed the greatest relative levels of lipase activity. Furthermore, the herbivorous *H. r. ardelio* was found to have the lowest activity of the brush border enzyme maltase, both in terms of relative activity and when standardised for gut mass, which stands in contrast to our predicted activity patterns for this enzyme. Maltase functions to hydrolyse the end products of amylolytic digestion of starches into glucose, making this low level particularly

surprising, especially in comparison to other stomachless herbivorous fishes (Horn et al. 2006; German 2009).

The patterns of localisation of digestive enzyme activities along the gut were largely as expected for all four fishes. The halfbeaks displayed an overall distally decreasing pattern of digestive enzyme activity and the needlefish tended to display the greatest enzymatic activity in the middle segments of the intestine, with a notable drop-off in activity in the rectum. Compared to a previous study of digestive enzymes in a halfbeak (Day et al. 2010), the pattern displayed by both halfbeak species in this study follow the Plug-Flow Reactor model of digestion in stomachless herbivores (Horn and Messer 1992), which predicts distally decreasing enzyme activities along with high rates of digestion. However, the distal decrease is not as prominent as might be expected, suggesting that half-beaks are capable of digestion, and presumably absorption, all the way through the gut, including the rectum. Indeed, unaided visual inspection of freshly dissected halfbeak intestines have revealed a highly transient hypervascularised zone, which has been alternatively found both anterior and posterior to the ileo-rectal valve (R.D. & I.T. personal observation), which may serve as a means of maximising nutrient assimilation in spite of such a short gut and rapid passage rate (approximately 4.4 h; Klumpp and Nichols 1983).

The difference in digestive enzyme patterns observed between halfbeaks and needlefishes in this study characterise the dietary needs that result from their respective diets. Buddington et al. (1987) determined that herbivores and carnivores require absorption of similar levels of protein in their diet, and that herbivores acquire sufficient protein from their low protein diet through ingesting greater quantities of food. Our results support this hypothesis and demonstrate how biochemical digestive strategies must differ in order to achieve a similar nitrogen requirement. The halfbeak employs an aggressive, "shotgun" approach to nutrient assimilation, targeting a wide range of nutrients available from its narrow dietary range of plant material that has been rendered by the pharyngeal jaw apparatus into a high surface area:volume bolus. This shotgun approach is characterised by producing relatively large amounts of multiple enzymes to take advantage of a large amount of food constantly flowing through the gut and using the entirety of the gut for absorption. The end result is a surfeit of available energy and the limiting nutrient, protein, is extracted from their plant diet to meet their nitrogen budget. In fact, it is possible that other nutrients may play important roles in halfbeak digestion that were not investigated in this study. Sugars such as sucrose are the photosynthates in seagrasses and are important storage saccharides in marine plants (Kuiper-Linley et al. 2007), and as such, are a potential target of

marine herbivores that consume seagrass. Hence, sucrase activities would be relevant to measure in future studies of seagrass-eating fishes. Similarly, while the possibility that the incidental ingestion and digestion of epiphytic material on seagrass may contribute to meeting nitrogen demands of halfbeaks cannot be ignored, isotopic evidence suggests that any role played by epiphytic material is exceedingly small (Carseldine and Tibbetts 2005). Needlefish, on the other hand, were more specific in their "rifle" approach to enzyme production, with activity levels modulated to specific nutrient levels. This approach was characterised by high trypsin activity, directed at the abundant protein available from its range of large, low surface area:volume prey items, along with low levels of α-amylase and lipase activity that serve to scavenge glycogen, lipids and any plant matter ingested by prey in order to maximise energy, which may serve as a limiting factor in the carnivorous diet (Carbone et al. 2007). Enzymatic digestion was more prominent in the mid-gut of the needlefish, which was composed of the mid- and posterior intestinal segments, and is believed to occur at a slower rate than the constant throughput of the halfbeaks, although throughput rates of needlefish remain to be investigated.

The piscivorous diet of the needlefish is more protein rich than the marine seagrass and freshwater algae diet of the halfbeak. Based on the predictions of the compartmental model of digestion (Raubenheimer and Simpson 1998; Clements and Raubenheimer 2006), needlefish, as consumers of the more nutrient rich diet, should tend towards maximisation of nutrient extraction and assimilation, resulting in longer retention times at the cost of re-feeding. This approach is evidenced in the rifle strategy of enzyme production found in the needlefish. Halfbeaks, on the other hand, should be expected to approach their respective diets with the aim of maximising nutrient gain, which means sacrificing carbohydrates while preferentially targeting proteins, as evidenced by their constant maintenance of a full gut (90–100% full throughout daylight hours; Klumpp and Nichols 1983) and a particularly rapid gut throughput rate (4.4 h; Klumpp and Nichols 1983). The surprisingly low activity level of maltase in the marine halfbeak HRA also offers support for this strategy, as the relatively high levels of α-amylase suggest that there is no shortage of maltase substrate, yet low activity suggests that energy requirements (e.g. glucose assimilation) are met with relatively low investment in maltase production.

The herbivorous shotgun versus carnivorous rifle approach has been demonstrated in several previous studies through comparisons of digestive enzyme activities. Although Hofer and Schiemer (1981) found relatively high protease activities in several carnivorous cyprinid taxa, intermediate levels in omnivorous species and low levels in herbivorous species, when protease activity was calculated

for an entire day, the herbivorous species were found to produce as much as six times more than carnivores. Chakrabarti et al. (1995) and Hidalgo et al. (1999) found that stomachless herbivorous and omnivorous cyprinids had α-amylase levels greater than, and protease levels similar to or greater than, that of several unrelated carnivorous species. This lead Hidalgo et al. (1999) to determine that although protease activities provide no real insight into the dietary habits of study species, α-amylase activity resulted in two distinct physiological groups: carnivores with stomachs and omnivores without. Furthermore, Horn et al. (2006) observed that herbivorous *Atherinops affinis* had significantly higher levels of α-amylase activity and similar levels of trypsin, aminopeptidase and lipase than that of related carnivorous species. In a study with similar enzyme assay methodology as ours, German (2009) found similar implementation of shotgun versus rifle strategies in herbivorous and carnivorous minnows, with comparatively high levels of α-amylase in the herbivores and trypsin levels that showed less variation with diet. Maltase, aminopeptidase and lipase activities were generally similar between all five species (with some exceptions), which further agrees with our findings. The common thread here is that α-amylase activity tends to be elevated in herbivores, whereas protease activities are usually similar among herbivores and carnivores (German et al. 2010), much as we observed in this investigation. Perhaps, cases of herbivores with particularly high protease levels indicate some inefficiency in protein digestion. Low levels of protein digestibility have been reported in herbivores feeding on seagrasses (Bjorndal 1980; Montgomery and Targett 1992), suggesting potential difficulties in either hydrolysis or absorption. Whether elevated protease levels may help to overcome these difficulties remains to be demonstrated.

An unexpected finding of this study was the strong signal in digestive enzyme activity profiles that was attributable to habitat; whether the fish lives in fresh or salt water. Despite the different diets and digestive strategies employed, the two freshwater fish, *A. s. krefftii* and *S. krefftii* and the two marine fish, *H. r. ardelio* and *T. gavialoides*, were found to be significantly similar. A number of studies have found differences in growth rate and nutrient assimilation capabilities related to the salinity of the water in which a fish is reared, and some evidence suggests that this difference may result from the effect of salinity on digestive enzyme activity (Moutou et al. 2004; Harpaz et al. 2005; Tsuzuki et al. 2007). Although our study cannot be directly compared to these other accounts, as we are comparing different species of fishes from freshwater and marine habitats rather than two conspecific populations reared in the differing habitats, these findings suggest that salinity plays a role in enzymatic digestion,

which may account for our unexpected habitat groupings. Harpaz et al. (2005) found enhanced brush border enzyme activity in fishes given dietary supplements of salt, which they attribute to the beneficial effect readily available salt ions, specifically Na^+ in this study, have on the Na^+/K^+ ATPase pumps that move glucose and amino acids across the cell membrane, thus allowing for a reduced reaction end product and enhanced reaction kinematics. Marine fishes should experience a similar effect through seawater taken in when feeding and drinking, potentially resulting in the differences we found. These results suggest that the effect salinity has on digestive physiology requires further study, as it may have implications for understanding the invasion of the oceans by freshwater fishes, freshwaters invasions by marine fishes, digestive pressures resulting from diadromy, as well as for the aquaculture industry, particularly in the case of euryhaline fishes.

In conclusion, this study provides support for digestion according to the AMH in the needlefishes, but departure from the predicted digestive enzyme pattern within the halfbeaks. We found low levels maltase activities in the herbivorous halfbeak *H. r. ardelio* and low lipase activities in both needlefish species, and high activities of lipase and trypsin in *H. r. ardelio* and *A. s. krefftii*, respectively. These findings suggest digestion in these fishes may be understood better through the predictions of the compartmental model of digestion (Raubenheimer and Simpson 1998; Clements and Raubenheimer 2006). Additionally, we verified the previous suggestion (Day et al. 2010) that the gut of herbivorous halfbeak operates according to the predictions of the PFR digestive model (Horn and Messer 1992) and support the proposal offered by Manjakasy et al. (2009) that the intestine of the needlefish may operate as an agastric stomach analogue. Furthermore, these differing methods of digestion result in herbivorous halfbeaks and the carnivorous needlefish targeting similar nutrients in a very different manner, with an unspecific "shotgun" nutrient targeting strategy in halfbeaks and targeted "rifle" strategy along with nutrient scavenging in needlefish. Finally, we found that while the role diet plays in digestive enzyme expression cannot be understated, habitat and salinity may also significantly affect enzymatic activity.

Digestive physiology in fishes is still a developing field with a number of gaps is knowledge (Clements et al. 2009), and as such, it is not surprising that our knowledge of digestion in stomachless fishes is wanting. In order to understand this prominent divergence from the otherwise ubiquitous vertebrate body plan, future investigations in a number of directions are required. In terms of understanding why the stomach was lost, research into the energetics of digestion to assess whether there is an energetic benefit to agastric digestion is necessary. Similarly, investigation into whether the lack of a stomach offers any

adaptive advantage besides energy savings may also allow for further understanding of this reduction. Finally, expanding the extant knowledge of the physical and biochemical digestive processes in a variety of stomachless fishes will enable the refinement of current models of digestion and may lead to additional ways of understanding digestion in these fishes.

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