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RESEARCH ARTICLE

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Structure and function of the stomachless digestive system in three related species of New World silverside fishes (Atherinopsidae) representing herbivory, omnivory, and carnivory

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Abstract We explored possible diet-related specializations in the digestive tract of stomachless fishes by summarizing the diets, verifying the absence of a stomach, and comparing gut lengths, microvilli surface areas, and activities of five digestive enzymes in four taxa of silversides from southern California coastal waters. For the comparisons, we examined these gut features in *Atherinops affinis* from both estuarine and kelp-forest habitats, and *Atherinopsis californiensis* and *Leuresthes tenuis* from open coastal habitats. *A. affinis* was found to be primarily herbivorous in estuaries and carnivorous in kelp forests, whereas *As. californiensis* was shown to be somewhat omnivorous but mainly carnivorous, and *L. tenuis* strictly carnivorous. Estuarine *A. affinis* exhibited the longest gut, largest microvilli surface area, and highest amylase and maltase activities, all arguably reflecting responses to an algal diet. In contrast, kelp-forest *A. affinis* displayed the highest trypsin activity and generally similar microvilli surface areas and aminopeptidase, amylase, and maltase activities to the two other carnivorous taxa. All four taxa showed similar lipase activities that compared closely with published values for other fishes. Taken together, our results reveal striking differences in gut structure and function among the four taxa, but especially between the estuarine and kelp-forest populations of *A. affinis*. Further studies are required to assess the roles of genetic variation and phenotypic plasticity in explaining the differences in these herbivorous and carnivorous taxa.

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Introduction

Any consistent differences in the structure and function of the digestive system of herbivorous fishes compared to carnivorous species have been difficult to reveal except perhaps in those herbivores with elaborate digestive tracts containing microbial symbionts specialized for fermentation of algal polysaccharides (see Rimmer and Wiebe 1987; Horn and Messer 1992; Clements and Choat 1995; Mountfort et al. 2002). For those seaweed-eating species with relatively simple guts, the distinction between these herbivores and their carnivorous relatives seems to be subtle or non-existent. Even the classic feature of longer guts in herbivorous taxa, while holding true in many comparisons, can disappear in teleost fishes with short guts regardless of diet as in the Hemiramphidae (Tibbetts 1991).

The differences that may exist in the digestive systems of herbivorous and carnivorous fishes have been slow to be recognized because of the paucity of studies in which phylogenetically related species representing these two types of consumers are compared. Recent works (Chan et al. 2004; German et al. 2004; German and Horn 2006) have helped to discern differences in related species of herbivorous and carnivorous prickleback (Stichaeidae). Even though the pricklebacks are characterized by relatively short and simple guts, their digestive tracts contain the standard teleost components of stomach, pyloric caeca, and intestine (German et al. 2004). The guts of certain other teleost fishes consist only of a short, tubular intestine whether the species are carnivores, herbivores, or even detritivores. Fishes in this category include the

Labridae (incorporating Odacidae and Scaridae), Blenniidae, Hemiramphidae, and Atherinopsidae (Clements and Bellwood 1988; Tibbetts 1991; Horn and Ojeda 1999; Logothetis et al. 2001).

Members of the New World silversides (Atherinopsidae) appear to have short, simple guts even though only one species, *Atherinops affinis*, has been studied in this regard. Logothetis et al. (2001) showed that this silverside assimilates energy and nutrients from macroalgae as efficiently as other marine herbivorous fishes despite lacking a stomach and any obvious physical mechanism for breaking down algal cells. In addition to *A. affinis*, members of the Atherinopsini clade within the Atherinopsidae include *Atherinopsis californiensis*, *Leuresthes tenuis*, *L. sardina*, *Colpichthys regis*, and *C. hubbsi* (White 1985; Crabtree 1987, 1989; Dyer 1997). *A. affinis*, *As. californiensis*, and *L. tenuis* occur in the northeastern Pacific, mainly along the coasts of California and Baja California (Miller and Lea 1972; Eschmeyer et al. 1983). *A. affinis* also occurs in the upper Gulf of California, and *L. sardina* and the two species of *Colpichthys* are confined to this upper gulf region (Crabtree 1987, 1989). *As. californiensis* and *L. tenuis* are common and well-recognized fishes in California waters (Miller and Lea 1972; Eschmeyer et al. 1983), but virtually nothing is known of the feeding ecology and digestive physiology of these two species, especially *L. tenuis*, beyond anecdotal information (MHH, EAL, JWC, personal observation). The same general lack of information on feeding and digestion also is apparent for *C. regis* and *C. hubbsi* (Crabtree 1989). The limited observations on all four of these species indicate that they have short, simple digestive tracts, and mainly carnivorous diets.

Atherinopsids are ideal taxa for a comparative study in which the primary focus is on discerning any existing differences in gut structure and function among taxa with simple digestive tracts but different diets. With these points in mind, we chose to investigate feeding and digestion in three species of atherinopsids—*A. affinis*, *As. californiensis*, and *L. tenuis*—all from populations in southern California coastal waters, including an estuarine and a kelp-forest population of *A. affinis*. The study of gut structure and function in *A. affinis* (Logothetis et al. 2001) mentioned above was conducted only on an estuarine population. This species, however, occurs in other types of coastal habitats and appears to be a phenotypically plastic species, showing differences in diet (Smith 2002) and body shape (O'Reilly and Horn 2004) between populations from mainland estuarine and island kelp-forest habitats.

The possibility exists that the variation among populations of *A. affinis* has either a genetic or phenotypic basis. Hubbs (1918) recognized several subspecies within the species, but Crabtree (1986) in a morphological and allozyme study of *A. affinis* populations concluded that members of the species represent a broadly distributed "monophyletic assemblage of morphologically differentiated forms". Given the obvious variability of the species but the lack of currently recognized subspecies or other genetically differentiated populations, we referred to the estuarine and kelp-forest *A. affinis* in our study as "populations". Thus, our investigation included three species and two populations of one of the species, and, for clarity and word economy, we refer to them collectively as "the four taxa" throughout the rest of the paper.

Our study had five components: (1) A dietary summary for the four taxa presented as the proportions of animal, algal, and detrital material using our heretofore unpublished data for three of the four taxa and that from Smith (2002) for kelp-forest *A. affinis*. (2) Measurements of gut pH and assays for pepsin activity in *As. californiensis* and *L. tenuis* to confirm (or not) that these two species lack a stomach. We expected stomachlessness in both species based on previous work (Logothetis et al. 2001) showing that *A. affinis* lacks a stomach. (3) A comparison of relative gut length (RGL) in the four taxa with the expectation that the most herbivorous taxon (likely estuarine *A. affinis*) would exhibit the longest gut and the most carnivorous taxon (likely *As. californiensis* or *L. tenuis*) would possess the shortest gut. (4) A comparison of the microvilli surface area of the intestinal epithelium in the four taxa. We proposed a null hypothesis of no difference among these taxa because we had no expectations based on the few published studies of epithelial surface area of the intestine in related fishes with different diets. (5) A comparison of the activities of five digestive enzymes—trypsin, aminopeptidase, amylase, maltase, and lipase—among the four taxa. We expected that estuarine *A. affinis* would exhibit an "herbivorous" profile of digestive enzyme activity with low protease (trypsin and aminopeptidase) and high carbohydrase (amylase and maltase) activities, whereas kelp-forest *A. affinis*, *As. californiensis*, and *L. tenuis* would exhibit the opposite "carnivorous" profile with high protease and low carbohydrase activities. Based on recent findings showing relatively high lipase activities in herbivorous species (Drewe et al. 2004; German et al. 2004), we hypothesized that lipase activity would be highest in estuarine *A. affinis*. Overall, our investigation was designed to determine what it takes to be an herbivore, still an elusive question, especially in fishes with seemingly simple guts as in the atherinopsids.

Materials and methods

Fish collection

Adults of the four atherinopsid taxa were collected in southern California waters between March 1998 and July 2003. Specimens of estuarine *A. affinis* were collected by beach seine in upper Newport Bay (33°37′N; 117°56′W), kelp-forest *A. affinis* at two locations at Santa Catalina Island (Catalina Harbor 33°26′N; 118°31′W, and Goat Harbor 33°25′N; 118°23′W), *As. californiensis* by hook and line from the Newport Beach pier (33°36′N; 117°55′W) and about 100 m offshore

from Dana Point (33°27′N; 117°43′W), and *L. tenuis* at Doheny State Beach (33°28′N; 117°41′W) by hand during a spawning event and from the water column about 300 m offshore from Bolsa Chica State Beach (33°40′N; 118°04′W) using neuston and 1-m plankton nets. Fish for pH measurements were killed by a blow on the head, whereas the remaining specimens were euthanized individually with an overdose (>1 g l^{-1} seawater) of tricaine methanesulfonate (MS-222, Argent Chemicals Laboratory, Inc., Redmond, WA, USA). Standard lengths (± 1 mm SL) of fish were measured and reported under each part of the study as mean ± SD and ranges.

Gut content analysis

Specimens of estuarine *A. affinis* (114±11 mm SL, range 97–132, $n=15$), *As. californiensis* (195±23, 154–238, $n=15$), and *L. tenuis* (81±4, 77–89, $n=12$) were dissected on a cutting board kept on ice. The intestine of *L. tenuis* collected on the spawning beach (129±6, 118–138, $n=15$) contained little or no food and was omitted from the analysis. Gut contents from estuarine *A. affinis* and *As. californiensis* were pushed out and stored in 4% formaldehyde-saline solution, whereas those from *L. tenuis* were frozen inside of the fish and analyzed without fixation following dissection. The gut contents of each fish were suspended in water and analyzed under a dissecting microscope equipped with an ocular grid (10×10 mm^2) using a point-contact method similar to that of Jones (1968) as described by Smith (2002). If a gut item occupied an intersection of two reticle lines, it was counted as a contact. Contacts were totaled for all gut content categories, and the percentage of each category was determined for each individual fish. The results were expressed as proportions of animal, algal, and detrital material to provide a broad intraspecific and interspecific comparison of diet composition in the four taxa. Animal material was classified at the family level, algal material as green or red, and other organic items that could not be identified were labeled as detritus.

Measurement of gut pH and assay for pepsin activity

We measured gut pH and assayed for pepsin activity in *As. californiensis* and *L. tenuis* to determine whether these two species are stomachless. Specimens of *As. californiensis* (274±27 mm SL, range 248–329, $n=10$) and *L. tenuis* (150±9, 139–165, $n=7$) were dissected immediately after capture and the pH measured in four equidistant locations along the gut as described by Logothetis et al. (2001). Pepsin was assayed in the anterior and posterior halves of the gut of *As. californiensis* (198±14, 183–222, $n=6$) and *L. tenuis* (143±9, 129–155, $n=7$) following Logothetis et al. (2001).

Relative gut length

Standard length and gut length (± 1 mm GL) of all fish collected were measured and RGLs reported as the ratio of the two measurements ($RGL = GL \times SL^{-1}$). The fish used for this purpose include those used for all other analyses plus additional specimens. The sizes and numbers of fish used to determine RGL for each taxon were as follows: estuarine *A. affinis* (127±18 mm SL, range 97–165, $n=49$), kelp forest *A. affinis* (136±23, 105–182, $n=35$), *As. californiensis* (229±45, 152–303, $n=55$), and *L. tenuis* (138±12, 116–165, $n=60$).

The calculation of RGL was accompanied by an analysis of covariance (ANCOVA) using a general linear model (Minitab v. 13, State College, PA, USA) to determine whether GL increased similarly as a function of SL in the four taxa with the significance level set a priori at $P=0.05$. First, the results of the ANCOVA showed that GL increased significantly with SL in all four taxa. The four regression equations with GL as a response and SL as a predictor were as follows: estuarine *A. affinis*, $y = 0.7396x + 86.713$ ($R^2=0.111$, $F=5.87$, $P=0.019$); kelp-forest *A. affinis*, $y = 1.0719x - 24.239$ ($R^2=0.715$, $F=82.91$, $P<0.001$); *As. californiensis*, $y = 0.8249x + 81.949$ ($R^2=0.342$, $F=27.52$, $P<0.001$); *L. tenuis*, $y = 0.4484x + 27.24$ ($R^2=0.071$, $F=4.44$, $P=0.039$). Second, the results of the ANCOVA showed that the slopes of the resulting regression lines were not significantly different among the four taxa ($F=0.62$, $df=3$, $P=0.604$) indicating that our RGL comparisons were not affected by differing trajectories of increase in GL with increase in SL.

Microvilli surface area

Specimens ($n=5$) of estuarine *A. affinis* (127±17 mm SL, range 107–144), kelp-forest *A. affinis* (128±11, 110–140), *As. californiensis* (188±48, 152–273), and *L. tenuis* (139±9, 126–149) were dissected, and the gut of each was divided into three equal-length regions. From the center of each region, five 1–2-mm^2 pieces were cut and fixed in freshly prepared 2% formaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.2, overnight at 4°C. The fixed tissues were rinsed twice in 0.1 M PBS with 0.1 M glycine, pH 7.2, for 2 h each at 4°C and postfixed in 2% aqueous osmium tetroxide for 2 h at room temperature (RT = 22°C). The postfixed tissues were dehydrated in 50, 70, 90, 95, and 2×100% ethanol at 4°C for 30 min each and processed for embedding in 24% araldite and 19% eponate resin (Ted Pella Inc., Redding, CA, USA). Blocks were cut into 1-μm thick sections using a Reichert-Jung Ultracut-E microtome (Jena, Germany). The sections were stained with 1% toluidine blue, examined under a bright-field light microscope (Olympus BX60) and digitized into $1{,}024\times1{,}024$-pixel images using a monochrome, high-resolution cooled CCD digital camera (ORCA 100, C4742–95, Hamamatsu Co., Bridgewater, NJ, USA) connected to a C-IMAGING high-performance system for image analysis (Compix Inc., Imaging Systems, Cranberry Township, PA, USA). The images were used to select blocks with best quality tissue and to identify mucosal folds for microvilli measurements.

Ultrathin sections (80–90 nm) were cut from the same central part of each selected mucosal fold, mounted on honeycomb copper grids (Pelco 8GC 180 or 270, Ted Pella) and stained with 1% uranyl acetate and 2% lead citrate. Cross sections of 5–10 enterocytes with undistorted (i.e., cylindrical) microvilli were photographed using a transmission electron microscope (H-7000, Hitachi, Japan). Micrographs from each region were printed (III RC polycontrast paper, Kodak) and assembled into 1×2 m^2 montages. Suitable montages ($n=3$ per region and taxon, 36 montages in total) were used for measurements of microvilli surface area per length of the intestinal epithelium determined at the apical membrane of the enterocytes following a two-dimensional model developed by Frierson and Foltz (1992). In this model each microvillus is represented by a rectangle topped with a semicircle whose diameter (D) equals the width of its base and whose height (H) equals the distance between the base and the top point below the glycocalyx. For each montage, individual width ($\pm$0.1 mm D) and height ($\pm$0.1 mm H) of 70–240 microvilli (171$\pm$39 SD per region) and the length of the intestinal epithelium (IEL) that these microvilli occupied were measured ($\pm$0.1 mm IEL) using a micrometric caliper and ruler. Each measurement was taken twice and the average of the two measurements converted to micrometers based upon the photographic magnification. Microvilli surface area was calculated as MVSA (μm^2) = $(H\pi D) + (\pi R^2)$, where $R=0.5D$. For each fish, the sum of MVSA (μm^2) for each region was divided by IEL (μm) and averaged for the three regions. For each taxon, MVSA was reported as the mean MVSA per μm of IEL.

Digestive enzyme activity

Activities of two proteases (trypsin and aminopeptidase), two carbohydrases (amylase and maltase), and lipase were determined as described by German et al. (2004) with the following modifications. Specimens ($n=10$) of estuarine *A. affinis* (147$\pm$12 mm SL, range 134–165), kelp-forest *A. affinis* (116$\pm$3, 110–119), *As. californiensis* (249$\pm$12, 181–292), and *L. tenuis* (141$\pm$16, 118–165) were dissected and their digestive systems divided into liver (including pancreas, spleen, and gall bladder) and three equal-length regions of the intestine. The liver and the three intestinal regions were each weighed ($\pm$0.001 g RM) and homogenized in 1:5 (w:v) of 50 mM Tris–HCl buffer, pH 7.4, at 0–4 °C. The homogenates were centrifuged at 9,300*g* for 2 min at 2°C and the supernatants stored in aliquots (0.5–1.0 ml) at −80 °C. The assays for pancreatic trypsin, amylase, and lipase were carried out on homogenates of the liver and the three intestinal regions, whereas those for brush-border aminopeptidase and maltase only on the intestinal homogenates. All assays were performed in triplicate at 17 °C, a temperature corresponding to an average of the temperatures measured at the collection sites. The assay protocols are described in detail by German et al. (2004) and only briefly summarized here. Trypsin activity was determined after activation with enteropeptidase using 2 mM N α benzoyl-l-arginine *p*-nitroanilide hydrochloride (BAPNA) as a substrate and expressed in U (1 µmol *p*-nitroaniline liberated per min) per g of tissue. Amylase activity was measured using 1% soluble starch as a substrate and expressed in U (1 µmol glucose liberated per min) per g of tissue. Lipase activity was measured after activation with bile salts using 10 mM *p*-nitrophenyl-myristate as a substrate and expressed in U (1 µmol *p*-nitrophenol liberated per min) per g of tissue. Aminopeptidase activity was measured using 2 mM L-alanine-*p*-nitroanilide HCl as a substrate and expressed in U (1 µmol *p*-nitroaniline liberated per min) per g of tissue. Maltase activity was measured with 56 mM maltose as a substrate and expressed in U (1 µmol glucose liberated per min) per g of tissue. The activity of each enzyme in U g tissue^{-1} was used to calculate total standardized gut activity (TSGA, U g GM^{-1}) representing the sum of total regional activities [(U g tissue^{-1}) g RM] divided by the mass of the whole digestive system (GM, g) comprising the mass of the liver and the three intestinal regions. Standardization of the activities per gut mass allowed for taxa with differing gut lengths to be compared.

Statistical analysis

The means of the RGL, the microvilli surface area, and the TSGA for each of the digestive enzymes were compared among the four silverside taxa using one-way ANOVA followed by a Tukey's honestly significant difference (HSD) test with a significance level set a priori at $P=0.05$ (Minitab v. 13). Lipase TSGA values passed Levene's test for equal variances, whereas those for RGL and TSGA of trypsin, aminopeptidase, amylase, and maltase passed the test after log-transformation. All means presented are followed by their standard errors (SEM).

Mean TSGA for the five enzymes combined in each of the four taxa were analyzed by nonmetric multidimensional scaling (MDS) using PRIMER (v. 5, Plymouth, UK) to display graphically the overall patterns of digestive enzyme activity. Multivariate analysis of similarity (ANOSIM) was performed using PRIMER to test statistically the differences among the four taxa. The outputs from ANOSIM, called R statistics, are based on the differences in mean ranks among groups and within groups and give an absolute measure of the amount of difference on a scale of 0 (identical) to 1 (very different). Statistical significance is examined by $\leq$999 random permutations, and differences are considered significant at $P\leq0.05$. Because of the small number of groups (taxa), only 3–4 permutations were possible and with <9 permutations the significance levels cannot be less than 0.25 (Clarke 1993). Thus, the R values rather than the significance levels were used as a more accurate measure of the absolute separation of the groups.

Results

The proportions of animal, algal, and detrital material in the guts of the four taxa are shown in Fig. 1. Estuarine *A. affinis* were primarily herbivorous, feeding mainly on green algae (94.1%) and a small amount of animal (5.7%) and detrital (0.2%) material. Kelp-forest *A. affinis* (60–75 mm SL, $n=11$) collected at Catalina Harbor, Santa Catalina Island, were entirely carnivorous, feeding on a variety of zooplankton (Smith 2002). *As. californiensis* was omnivorous, feeding on animal (53%, mostly gammarid amphipods), detrital (35%), and algal (11%) material. *L. tenuis* was exclusively carnivorous, feeding mostly on mysid crustaceans.

The pH was alkaline in all four gut parts in the two taxa examined, with an overall mean of 7.9±0.2 in *As. californiensis* and of 8.1±0.1 in *L. tenuis*. No pepsin activity was detected in either anterior or posterior part of the gut of these two taxa. The alkaline pH and the absence of pepsin activity in *As. californiensis* and *L. tenuis* indicated that the two species are stomachless.

Mean RGL was significantly different among all four taxa (Fig. 2), ranging from the longest in estuarine *A. affinis* (1.43±0.04), followed by *As. californiensis* (1.20±0.03), then kelp-forest *A. affinis* (0.95±0.03), and to the shortest in *L. tenuis* (0.65±0.02).

Mean microvilli surface area per length of intestinal epithelium was significantly largest in estuarine *A. affinis* (7.3±0.2) and significantly smallest in *As. californiensis* (3.5±0.2), whereas those in kelp-forest *A. affinis* (5.3±0.1) and *L. tenuis* (4.9±0.1) were not significantly different from each other (Fig. 3). Differences in microvilli surface area were driven almost entirely by microvillar height because microvillar diameter was virtually identical in the four taxa.

The mean total standardized gut activities (TSGA, U g GM^{-1}) of the two proteases and the two carbohydrases but not lipase exhibited significant differences among taxa (Fig. 4). Trypsin TSGA was significantly highest in kelp-forest *A. affinis* (3.5±0.2), followed by estuarine *A. affinis* (2.3±0.3) and *L. tenuis* (1.9±0.2), which were not different from each other but significantly higher than that of *As. californiensis* (0.6± <0.1).

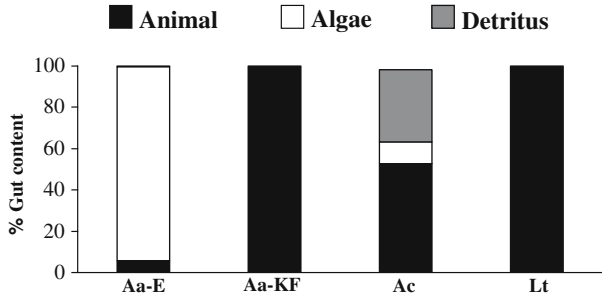

Fig. 1 Proportion of animal, algal, and detrital material in gut contents of estuarine *Atherinops affinis* (*Aa-E*), kelp-forest *A. affinis* (*Aa-KF*) (data from Smith 2002), *Atherinopsis californiensis* (*Ac*), and *Leuresthes tenuis* (*Lt*)

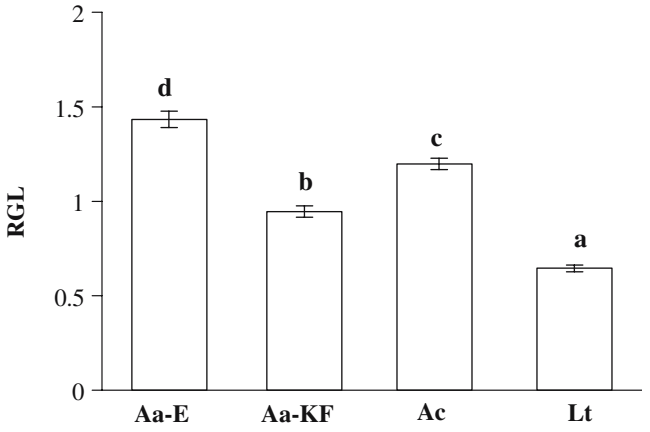

Fig. 2 Mean relative gut length (*RGL*) in estuarine *Atherinops affinis* (*Aa-E*), kelp-forest *A. affinis* (*Aa-KF*), *Atherinopsis californiensis* (*Ac*), and *Leuresthes tenuis* (*Lt*). *Vertical lines on bars* represent SEM ($n=35$–60), and *different letters* indicate significant differences revealed by among-taxa comparisons using one-way ANOVA and Tukey's test with a significance level of $P=0.05$ ($F=155.12$, $df=203$, $P<0.01$)

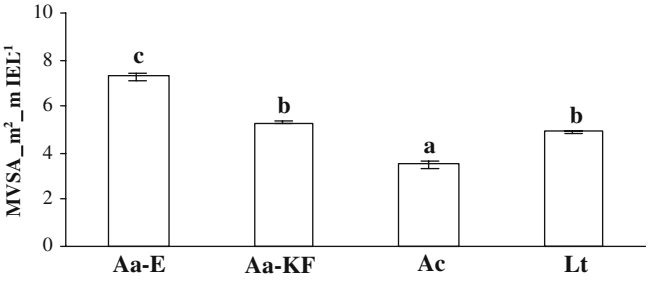

Fig. 3 Mean microvilli surface area (*MVSA*, μm^2) per length of intestinal epithelium (*IEL*, μm) in estuarine *Atherinops affinis* (*Aa-E*), kelp-forest *A. affinis* (*Aa-KF*), *Atherinopsis californiensis* (*Ac*), and *Leuresthes tenuis* (*Lt*). *Vertical lines on bars* represent SEM ($n=3$), and *different letters* indicate significant differences revealed by among-taxa comparisons using one-way ANOVA and Tukey's test with a significance level of $P=0.05$ ($F=126.02$, $df=11$, $P<0.01$)

Aminopeptidase activity was significantly higher in *As. californiensis* (0.62±0.07) than in estuarine *A. affinis* (0.37±0.03) but not statistically different from either kelp-forest *A. affinis* (0.42±0.02) or *L. tenuis* (0.56±0.09), which in turn were not significantly different from estuarine *A. affinis*. Amylase activity was significantly highest in estuarine *A. affinis* (14.7±1.7), followed by kelp-forest *A. affinis* (4.2±1.0) and *As. californiensis* (3.1±0.1), which were not different from each other but significantly higher than that of *L. tenuis* (1.4±0.2). Maltase activity was significantly highest in estuarine *A. affinis* (1.4±0.2), followed by that in kelp-forest *A. affinis* (0.6±0.1) and *As. californiensis* (0.4±0.1), which were not different from each other, and then by that in *L. tenuis* (0.3± <0.1), which was not different from that in *As. californiensis* but significantly lower than that in kelp-forest *A. affinis*. The similar TSGA for lipase in the four taxa ranged from 0.64±0.06 in kelp-forest *A. affinis* to 0.55±0.02 in *As. californiensis*. The MDS plot of TSGA of the five digestive enzymes combined (Fig. 5) revealed

markedly different positions of estuarine and kelp-forest *A. affinis* (ANOSIM, $R=1$) and of these two taxa versus *As. californiensis* and *L. tenuis* (ANOSIM, $R=1$), which were similar to each other in position on the plot (ANOSIM, $R=0$).

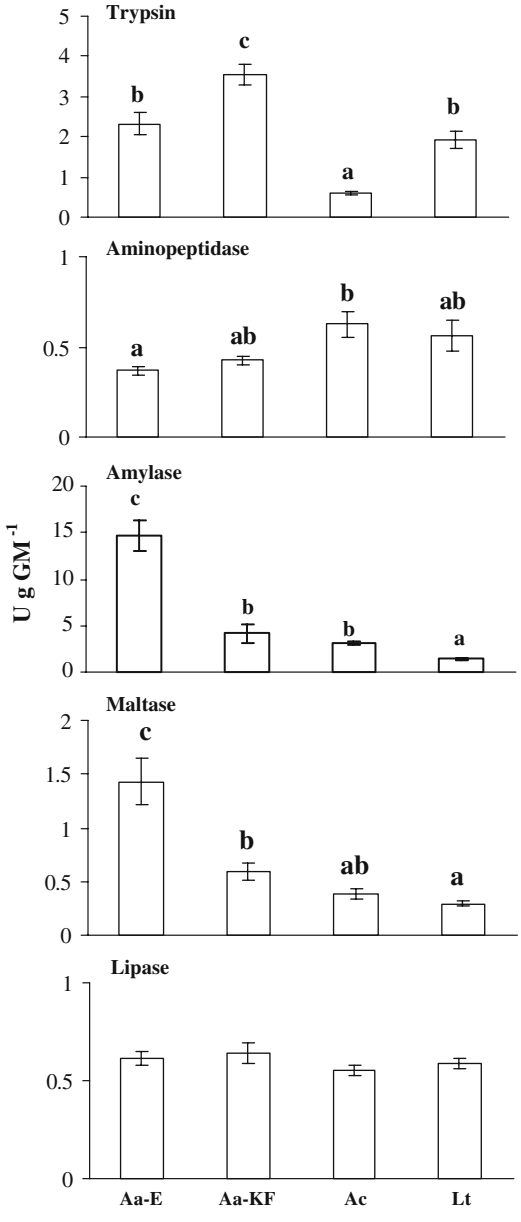

Fig. 4 Mean total standardized gut activity of digestive enzymes (*TSGA*, U g GM^{-1}) in estuarine *Atherinops affinis* (*Aa-E*), kelp-forest *A. affinis* (*Aa-KF*), *Atherinopsis californiensis* (*Ac*), and *Leuresthes tenuis* (*Lt*). *Vertical lines on bars* represent SEM ($n=10$). Among-taxa comparisons using one-way ANOVA and Tukey's test with a significance level of $P=0.05$ revealed significant differences (*different letters*) for all enzymes, except lipase (trypsin: $F=47.93$, $P<0.01$; aminopeptidase: $F=3.75$, $P=0.02$; amylase: $F=40.71$, $P<0.01$; maltase: $F=28.53$, $P<0.01$; lipase: $F=0.99$, $P=0.41$. $df=39$ for all enzymes)

Fig. 5 Nonmetric multidimensional scaling plot of total standardized gut activity of five digestive enzymes combined for each of the four taxa: estuarine *Atherinops affinis* (*Aa-E*), kelp-forest *A. affinis* (*Aa-KF*), *Atherinopsis californiensis* (*Ac*), and *Leuresthes tenuis* (*Lt*). The stress value of 0.0 indicates that the plot fits well (i.e., values <0.1) into two-dimensional space

Discussion

The results of this study revealed marked differences among the four taxa in all features of gut structure and function that we examined. These differences thus include both intraspecific and interspecific distinctions in the four externally similar silverside fishes. Mostly, our hypotheses or expectations with regard to diet, stomachlessness, gut length, and digestive enzyme profiles were supported. The hypotheses, however, of difference in lipase activity and no difference in microvilli surface area among the four taxa were rejected. Our gut content survey verified that estuarine *A. affinis* are largely herbivorous and that kelp-forest *A. affinis* are carnivorous, feeding mainly on zooplankton. We provided new data to show that *As. californiensis* and *L. tenuis* both are carnivorous species. Our analyses revealed that these same two species are stomachless, confirming, as expected, that they share this trait with *A. affinis*. All four taxa exhibited significantly different gut lengths with the estuarine *A. affinis* possessing the longest intestine. The finding that microvilli surface area of the intestinal epithelium is greater in estuarine *A. affinis* than in the three other taxa represents, to our knowledge, the first comparison of this feature among more than two closely related species. Remarkably, the herbivorous estuarine *A. affinis* exhibited both a longer gut and larger intestinal surface area than the carnivorous kelp-forest *A. affinis* and the two other species as well.

Digestive enzyme activities also revealed clear distinctions among the four taxa as might be expected between herbivores and carnivores. Kelp-forest *A. affinis* showed the highest trypsin activity, but the two other carnivorous taxa displayed the highest activity of aminopeptidase, the other protease we examined. In contrast, estuarine *A. affinis* exhibited by far the highest activities of amylase and maltase with the three other taxa showing much lower, somewhat similar activities of these two carbohydrases. The lack of difference in lipase activity

among the four silverside taxa remains unexplained although the values obtained are similar to published values for other fishes. Taken together, the activities of the five digestive enzymes clearly distinguished estuarine *A. affinis* from kelp-forest *A. affinis* and these two taxa from the other two silversides.

All three silverside species we studied may be capable of omnivory, but the case for *A. affinis* is by far the best documented. *A. affinis* has been called an "opportunistic omnivore" (Smith 2002). Indeed, our study may underestimate the dietary flexibility displayed by *A. affinis* within different estuarine habitats. While this species appears to be largely herbivorous in upper Newport Bay (Horn and Allen 1985; Smith 2002; this study), the fish consumes a wider array of food items in other California estuaries. At Bolsa Chica and in Anaheim Bay in southern California, the diet of *A. affinis* contains larger proportions of detritus and benthic invertebrates (Klingbeil 1972; Smith 2002). In Elkhorn Slough on the central California coast, macroalgae make up the largest dietary category, but a variety of planktonic and benthic invertebrates also are consumed (Barry et al. 1996). Kelp-forest *A. affinis*, however, appear to be more strictly zooplankton feeders (Quast 1968; Smith 2002).

The present study indicates that *As. californiensis* is mainly carnivorous, and that *L. tenuis* is probably a strict carnivore. We collected *As. californiensis* in coastal waters, and the species seems to be a less frequent visitor to estuaries in southern California than *A. affinis* (see Horn and Allen 1985; Valle et al. 1999; Allen et al. 2002). The fish is relatively common in Elkhorn Slough, and, there, its diet is almost as variable as that of *A. affinis*, comprising macroalgae, zooplankton, and a few other items (Barry et al. 1996). Nearer the mouth of the estuary, the diet of *As. californiensis* consists almost entirely of zooplankton. Much more dietary information is needed to establish the degree of omnivory in this species. Additional data on the food habits of *L. tenuis* are even more sorely needed as the data we presented here constitute about the only published information available on the diet of this species.

Not surprisingly, we found that both *As. californiensis* and *L. tenuis* lack a stomach. We expected this outcome because Logothetis et al. (2001) had already established that *A. affinis* is stomachless, and our casual observations indicated that the digestive tracts of the two species were similar to that of *A. affinis*. Stomachlessness extends to other atherinomorph fishes including the hemiramphids (Tibbetts 1997) and may be a common trait of the entire series (E.A. Logothetis and M.H. Horn, unpublished data). Lacking a stomach, however, does not appear to restrict dietary flexibility or digestive ability in these fishes, at least for herbivory in *A. affinis*. Stomachlessness has arisen independently in numerous teleostean lineages and occurs in lampreys and hagfishes, the most basal of vertebrates, but the selective advantages of the condition remain obscure (Koelz 1992).

The gut lengths of the four silverside taxa match expectations based on the dietary profiles presented in this study, with the RGL of estuarine *A. affinis* 1.5× that of kelp-forest *A. affinis*, 1.2× that of *As. californiensis*, and 2.2× greater than that of *L. tenuis*. Although still relatively short among herbivorous fish species, the gut of *A. affinis* nevertheless equals or exceeds those of a few other marine herbivorous fishes either with or without stomachs (see Horn 1989). The differences in gut length shown by estuarine and kelp-forest *A. affinis* suggest phenotypic plasticity, but the degree to which gut length within each taxon is influenced by genetic differences versus environmental conditions remains unknown.

Few comparative studies have been conducted on microvilli surface area in fishes. Measuring microvilli surface area is much more labor intensive and time consuming than measuring gut length. The absorptive surface area of the intestinal epithelium can be increased either by lengthening the gut, increasing the folding of the intestinal mucosa, or by increasing the height (mainly) of the microvilli, or perhaps a combination of all three (Buddington et al. 1997). Given the claim (Frierson and Foltz 1992) that microvillar surface accounts for 90% of absorption surface area and that increasing this surface is a more efficient and flexible way to increase the absorptive area, fishes might be expected to exhibit this mode more often than by increasing gut length. Perhaps the most interesting aspect of our results on gut dimensions in the four silverside taxa is that estuarine *A. affinis* exhibited both increased gut length and increased microvilli surface area compared to the three other taxa. To our knowledge, this study is the first to compare microvilli surface area in related herbivorous, omnivorous, and carnivorous taxa, and therefore the first to show differences among fishes with these different dietary modes.

The distinctive patterns of digestive enzyme activities we found among the four silverside taxa emerged along the lines of differences expected between herbivores and carnivores. Estuarine *A. affinis* exhibited markedly higher activities in the two carbohydrases than the three other taxa but lower activities of the two proteases than at least one of the other taxa for each of these enzymes. These herbivore and carnivore profiles for digestive enzyme activities have been documented in other fishes including a siganid and a moronid (Sabapathy and Teo 1993) and estuarine *A. affinis* (Logothetis et al. 2001). Such a difference in digestive enzyme activities also has been demonstrated in species that switch from carnivory to herbivory or shift herbivorous diets with age (Moran and Clements 2002; Chan et al. 2004; Drewe et al. 2004; German et al. 2004). Also, an omnivorous sparid has been shown to possess higher amylase activity than four carnivorous members in the same family (Fernandez et al. 2001). In a study that departed somewhat from these expected profiles, Hidalgo et al. (1999) found that amylase activity but not trypsin activity was different among several species with different diets.

Our results on gut structure and function in the four silverside taxa call for deeper resolution of genetic structure, phenotypic plasticity, and phylogenetic relationships within the atherinopsine clade containing

these taxa. The striking degree of divergence in gut features we found between estuarine and kelp-forest *A. affinis* populations may reflect either genetic differences or phenotypic plasticity or possibly both. We have not emphasized comparisons from a phylogenetic perspective for the three atherinopsid species we studied because we did not include members of either species of *Colpichthys* in our investigation and because of the lack of consensus on sister taxa relationships within the Atherinopsini (see White 1985; Crabtree 1987; Dyer 1997). Our findings, however, identify some fertile areas for further comparative studies of feeding ecology, gut ultrastructure, and digestive physiology in these superficially similar but remarkably variable fishes.

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