J Comp Physiol B
DOI 10.1007/s00360-013-0780-1

ORIGINAL PAPER

Sequence and expression of an α-amylase gene in four related species of prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and species-level effects

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Received: 30 March 2013 / Revised: 19 August 2013 / Accepted: 24 August 2013

Abstract Partial α-amylase gene sequences were determined and α-amylase gene expression was quantified in four species of carnivorous, omnivorous, and herbivorous prickleback fishes (family Stichaeidae) to assess the effects of ontogeny, diet, and species on expression of this gene. Pairwise comparison of α-amylase nucleotide sequences revealed 96–98 % identity, and comparison of amino acid portions revealed 93–95 % similarity among the four prickleback species. Expression was determined using in situ hybridization and intensity of expression quantified using image analysis. Alpha-amylase expression level was compared in three feeding categories of the four species: (1) small, wild-caught carnivorous juveniles; (2) larger, wild-caught juveniles of the carnivorous species and the three that had shifted to herbivory or omnivory; and (3) larger, juveniles produced by feeding a low-starch artificial diet to small juveniles until they reached the size of the larger wild-caught juveniles. The results showed no dietary effect in any species but significant ontogenetic and species-level effects in *Cebidichthys violaceus*, as well as in the sister species *Xiphister mucosus* and *X. atropurpureus*. Based on a phylogeny for the Stichaeidae produced for this study using two mtDNA genes and one nuclear gene, the ontogenetic dietary shifts to herbivory/omnivory evolved independently in *C. violaceus* and in the clade containing the two species of *Xiphister*. All three of these species increased α-amylase gene expression with increase in size and had higher expression than *Anoplarchus purpurescens*, which is a member of a third, stichaeid clade comprising carnivores. These results show the importance of α-amylase in the herbivores and omnivores.

Keywords α-Amylase gene structure · α-Amylase gene expression · Prickleback fishes · Ontogenetic effects · Dietary effects · Species-level effects

Communicated by I.D. Hume.

Electronic supplementary material The online version of this article (doi:10.1007/s00360-013-0780-1) contains supplementary material, which is available to authorized users.

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Introduction

Alpha-amylase is a digestive enzyme that catalyzes the hydrolysis of starch, glycogen and related polysaccharides and occurs widely in plants, animals, and microorganisms (Mizutani et al. 2012). Alpha-amylase genes have been useful in evolutionary studies not only because of their high level of sequence variation (Hassan et al. 2002; Bouneau et al. 2003; Guinand et al. 2008), but also because the subsequent protein structures coded for by these genes are highly conserved (Janacek 1994; Krogdahl et al. 2005; Mizutani et al. 2012). For instance, although less than 10 % sequence identity exists between bacteria and mammals, the function of α-amylase is the same in these disparate groups of organisms (Nakajima et al. 1986; Mizutani et al. 2012). Alpha-amylase genes have been sequenced in a variety of vertebrates, including several fish species such as

Published online: 18 October 2013

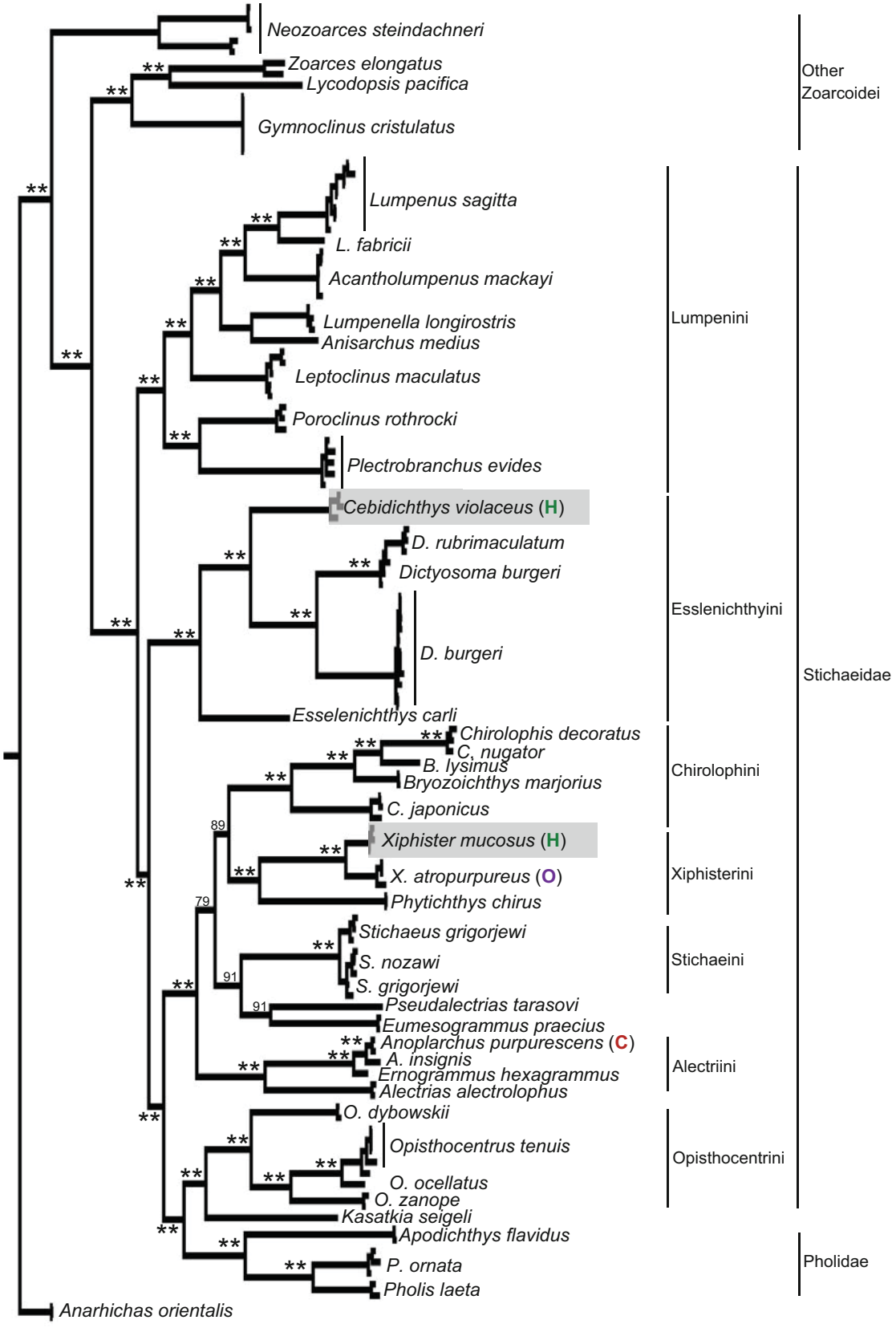
Pseudopleuronectes americanus (Douglas et al. 2000), *Lates calcarifer* (Ma et al. 2001), *Anguilla japonica* (Kurokawa et al. 2002), *Tetraodon nigroviridis* (Bouneau et al. 2003), and *Oryzias latipes* (Kasahara et al. 2007). Most fishes appear to possess at least two active α-amylase isoforms (Fernández et al. 2001; Krogdahl et al. 2005; Kushwaha et al. 2012), and up to three α-amylase genes are known to be present in fish genomes (Bouneau et al. 2003). The fish species in which α-amylase genes have been sequenced are mostly carnivores or omnivores, but herbivorous fishes are the taxa that face the particular challenges of gaining adequate nutrition from a diet rich in the substrate for α-amylase (i.e., starch; Horn 1989, 1998; Clements et al. 2009), thus leading to a need for more information on α-amylase genes in herbivores (Krogdahl et al. 2005).

From the biochemical perspective, α-amylase activity in the digestive tracts of fishes (German et al. 2010), and vertebrates in general (Kohl et al. 2011), appears to be related to diet. Matches between digestive enzyme activity level and dietary substrate concentration are predicted by the Adaptive Modulation Hypothesis (AMH), which posits that an animal would not waste energy synthesizing digestive enzymes for substrates in low supply in their food (Karasov and Martínez del Rio 2007). In support of the AMH, herbivorous and omnivorous species exhibit higher α-amylase activity than carnivorous species (Hidalgo et al. 1999; Fernández et al. 2001), even when analyzed in a phylogenetic context (Horn et al. 2006; German et al. 2010). Moreover, fishes that undergo an ontogenetic shift from carnivory to herbivory also show an increase in α-amylase activity concomitant with their changing diet (Moran and Clements 2002; Drewe et al. 2004; German et al. 2004). For instance, in a study of four species of prickleback fishes (Stichaeidae), German et al. (2004) found significant increases in α-amylase activity in three species that shift their diets to herbivory or omnivory as they grow. Alpha-amylase activity in a fourth, carnivorous species from a separate prickleback clade remained unchanged during ontogeny. Interestingly, α-amylase activities in the herbivores and omnivore remained elevated, even when the fish were fed a low-starch, artificial diet in the laboratory, which indicated that the fish were "hardwired" to have elevated α-amylase activity. The German et al. (2004) study, as well as that by Chan et al. (2004) also on digestive enzyme activity in pricklebacks, implies that gene programs during ontogeny and evolutionary history may have a greater influence than proximate diet on α-amylase activity in these species. Furthermore, the studies by Chan et al. (2004), German et al. (2004), as well as others (e.g., German et al. 2010; Kohl et al. 2011) argue that elevated α-amylase activity may in itself represent a biochemical specialization for herbivory or omnivory in vertebrates, as opposed to simple plasticity, as might be inferred from the AMH. But, how is this "specialization" achieved?

The hypothesis that elevated α-amylase activity represents a biochemical specialization for diet heightens the interest in studying α-amylase at the molecular level, i.e., the sequences and expression of α-amylase genes in fishes with different diets. Concomitant increases in α-amylase mRNA concentration (i.e., expression; Peres et al. 1998; Douglas et al. 2000; Ma et al. 2001; Kurokawa et al. 2002) and enzyme activity levels (Zambonino Infante and Cahu 1994; Ribeiro et al. 1999) have been detected in fish larvae, suggesting that α-amylase is transcriptionally regulated. Moreover, α-amylase mRNA levels of *P. americanus* increase through metamorphosis when the larvae are herbivorous and then decrease after metamorphosis, when the fish switch to a carnivorous diet (Douglas et al. 2000). But, do expression levels of α-amylase genes also explain the differences in α-amylase activity among herbivores and carnivores?

The present study was undertaken to increase the understanding of digestive specializations in herbivorous and omnivorous fishes with a focus on α-amylase expression in the same set of four prickleback species in which α-amylase biochemical activity was studied by Chan et al. (2004) and German et al. (2004). We compared the sequences and expression levels of α-amylase in these four related herbivorous, omnivorous, and carnivorous species. All four taxa begin life as carnivores, and, as the juveniles reach ≥45 mm standard length (SL), *Xiphister mucosus* and *Cebidichthys violaceus* shift to an herbivorous diet (Horn et al. 1982, Setran and Behrens 1993). *Xiphister atropurpureus* appears to shift to omnivory at larger sizes (>75 mm SL; German et al. 2004; Saba 2004; Boyle and Horn 2006), whereas *Anoplarchus purpurescens* remains carnivorous throughout its life (Barton 1982). Because a molecular phylogeny was not available for the Stichaeidae, we generated a phylogeny for the family using two mitochondrial (*cytb* and *16s* rDNA) and one nuclear gene (*tmo4c4*; Fig. 1). Although the Xiphisterini and Esselenichthyini each include herbivores, *Anoplarchus purpurescens* is a member of an adjacent clade apparently consisting solely of carnivores (Fig. 1). These four species were ideal for the present study because they are phylogenetically

Fig. 1 Bayesian phylogenetic hypothesis for the family Stichaeidae ▶ based on the sequences of the *cytb*, *16s*, and *tmo4c4* genes. Ten million generations of Markov Chain Monte Carlo were performed with trees sampled every 200 generations and 15 % of early runs counted as burnin. The percentage of times that a particular node was recovered is listed as the Bayesian posterior probability. **≥99 %. Dietary affinities of species used in this study of amylase gene expression are as follows: *C* carnivore, *O* omnivore, *H* herbivore. *Gray shaded boxes* on tree show independent evolution of herbivory in *X. mucosus* and *C. violaceus*


Neozoarces steindachneri
Zoarces elongatus
Lycodopsis pacifica
Gymnoclinus cristulatus
Other Zoarcoidei
Lumpenus sagitta
L. fabricii
Acantholumpenus mackayi
Lumpenella longirostris
Anisarchus medius
Leptoclinus maculatus
Poroclinus rothrocki
Plectrobranchus evides
Lumpenini
Cebidichthys violaceus (H)
D. rubrimaculatum
Dictyosoma burgeri
D. burgeri
Esselenichthys carli
Esslenichthyini
Chirolophis decoratus
C. nugator
B. lysimus
Bryozoichthys marjorius
C. japonicus
Chirolophini
Xiphister mucosus (H)
X. atropurpureus (O)
Phytichthys chirus
Xiphisterini
Stichaeus grigorjewi
S. nozawi
S. grigorjewi
Pseudalectrias tarasovi
Eumesogrammus praecius
Stichaeini
Anoplarchus purpurescens (C)
A. insignis
Ernogrammus hexagrammus
Alectrias alectrolophus
Alectriini
O. dybowskii
Opisthocentrus tenuis
O. ocellatus
O. zanope
Opisthocentrini
Kasatkia seigeli
Apodichthys flavidus
P. ornata
Pholis laeta
Pholidae
Stichaeidae
Anarhichas orientalis
89
79
91
91

related, represent herbivory, omnivory, and carnivory, and also co-occur in the rocky intertidal habitat on the central California coast. To compare the structure of the α-amylase gene in the four prickleback species, partial α-amylase gene sequences for each species were determined and described in the context of the phylogenetic hypothesis for the Stichaeidae.

To distinguish the effects of ontogeny, diet, and species on α-amylase gene expression, intensity levels of expression were compared in three feeding categories in each of the four species: (1) small, wild-caught fish (30–40 mm SL = $w_{30–40}$) representing the carnivorous condition before *C. violaceus* and *X. mucosus* shift to herbivory, and *X. atropurpureus* to omnivory; (2) larger, wild-caught fish (60–75 mm SL = $W_{60–75}$) representing the natural diet condition of the carnivorous species and the three that have shifted to herbivory or omnivory; and (3) larger, laboratory-raised fish (60–75 mm SL = $L_{60–75}$) produced by feeding a low-starch artificial diet to small juveniles ($w_{30–40}$) until they reached the size of the larger wild-caught ($W_{60–75}$) fish. Comparisons between categories $w_{30–40}$ vs. $W_{60–75}$ allowed us to test for an ontogenetic effect, in $W_{60–75}$vs. $L_{60–75}$ for a dietary effect, and within categories $w_{30–40}$, $W_{60–75}$, and $L_{60–75}$ for species-level effects.

The intensities of α-amylase gene expression (mRNA) were quantified using computer- assisted image analysis from in situ hybridization assays, a powerful tool to detect α-amylase mRNA concentrated in pancreatic tissue (Larsson 2001). Based on the study by German et al. (2004) of α-amylase biochemical activity in the same four prickleback species and using the same experimental design as in their study, we hypothesized the following outcomes for α-amylase gene expression: (1) an ontogenetic increase in *C. violaceus*, *X. mucosus*, and *X. atropurpureus*, and no ontogenetic increase in *A. purpurescens*; (2) expression levels of α-amylase that remain elevated in *C. violaceus*, *X. mucosus*, and *X. atropurpureus*, despite the consumption of a low-starch diet; (3) an increase in α-amylase expression in *A. purpurescens* consuming the low-starch diet, consistent with an increase in α-amylase activity levels in these fish (German et al. 2004); and (4) species-level effects, with *C. violaceus*, *X. mucosus*, and *X. atropurpureus* of separate, herbivore-containing clades, exhibiting higher levels of α-amylase gene expression than *A. purpurescens* from the carnivorous clade.

Materials and methods

Phylogenetic analysis for the Stichaeidae

The phylogenetic study employed sequences of three genes widely used in phylogenetics (Smith et al. 2008): *Cytochrome b*, *16s* rDNA, and *tmo4c4*. We obtained 1,090 bp of the *Cytochrome b* (*cytb*) gene, which codes for a transmembrane protein representing the main catalytic subunit of a respiratory chain enzyme (Esposti et al. 1993). We obtained 540 bp of the *16s* rDNA (*16s*) gene, which encodes a portion of the 30S small ribosomal subunit found in mitochondria. And, we obtained 480 bp of the nuclear gene *tmo4c4* (Streelman and Karl 1997), which codes for a portion of the protein titin that is involved in muscle assembly and resting tension. We obtained muscle samples from 34 species (n = 1–15 per species) within the family Stichaeidae, and eight outgroup taxa from the suborder Zoarcoidei, either directly from nature, or through museum collections (see supplementary Appendix 1). Total DNA was extracted using a Qiagen (Valencia, CA, USA) DNeasy DNA extraction kit following the manufacturer's instructions, and DNA concentration for each sample was quantified using spectrophotometry (Synergy H1, BioTek, Winooski, VT, USA). PCR amplification protocols generally followed Smith et al. (2008), except that annealing temperature was optimized for each primer set. For *cytb*, we used the following primers: *cytb*F_LA 5′-GTG ACT TGA AAA ACC ACC GTT G-3′, cytbR_HA 5′-CAA CGA TCT CCG GTT TAC AAG AC-3′ (Schmidt et al. 1998). For *16s* we used the following primers: 16srDNAF 5′-CGC CTG TTT ATC AAA AAC AT-3′, 16srDNAR 5′-CCG GTC TGA ACT CAG ATC ACG T-3′ (Palumbi 1996). For *tmo4c4* we used the following primers: tmo4c460F 5′-AA AAG AGT GTT TGA AAA TGA CTC GCT AAC G-3′, tmo4c4R 5′-CAT CGT GCT CCT GGG TGA CAA AGT-3′ (Streelman and Karl 1997). PCR products were visually inspected on agarose gels, cleaned with a Qiagen QIAquick PCR purification kit, and sent for sequencing at Retrogen Inc. (San Diego, CA, USA). Genes were aligned and concatenated in Codon Code Aligner software (Codon Code Corporation, Dedham, MA, USA). Phylogenetic relationships were generated using a mixed-model, partitioned Bayesian method as implemented in the software package Mr. Bayes version 3.1.2 (Huelsenbeck and Ronquist 2001). Codon partitions were chosen for *cytb* and *tmo4c4*, and a whole genome partition for *16s*, with the model GTR + I + G used for each partition, as determined using the Akaike Information Criterion (AIC) in the computer program Mr. Modeltest (Nylander 2004). Ten million generations of Markov Chain Monte Carlo (MCMC) were performed using a random starting topology with trees sampled every 200 generations, with 10 % of early runs counted as burnin. The retained 45,000 trees were used to construct a 50 % majority rule consensus tree. The percentage of times that a particular node was recovered in the analysis is interpreted as the posterior probability of the occurrence of that node (Huelsenbeck and Ronquist 2001). The analysis was repeated two additional times to confirm the tree topology and posterior probability values.

Fish collection, maintenance, and feeding

Juveniles of each of the four prickleback species were collected by hand and dipnet at low tide from rocky intertidal habitat on the central California coast near Piedras Blancas (35°40′N, 121°17′W) and, for *X. mucosus*, also from Diablo Canyon (Stillwater Cove, 35°12′N, 120°51′W). Forty small juveniles ($w_{30–40}$) of each species were collected and transported live out of water, as in our previous studies of prickleback fishes (Fris and Horn 1993; German et al. 2004), to the laboratory at California State University, Fullerton. Upon arrival, all fish were placed into 235-l tanks containing filtered, UV-sterilized, and circulating seawater held at 15 °C, within the upper part of the temperature range (9–17 °C) of the central California near-shore habitat (Horn et al. 1983). Ten individuals of each species were randomly selected and processed for either in situ hybridization (see below) or digestive enzyme assays (German et al. 2004), whereas the remaining 20 individuals were used for feeding experiments and placed individually into numbered 4.1-l compartments with nylon screen walls submerged inside 235-l tanks (10 compartments/tank) under a 12L:12D cycle as per Fris and Horn (1993). The lighting over the tanks was not uniformly positioned; thus, to eliminate a potential "tank effect", the compartments were rotated within the tanks, and one compartment from each tank was exchanged with the neighboring tank on a daily basis. After 2 days of quarantine, fish were fed manually three to four times a day to satiation with a low-starch artificial diet for marine fish larvae (BioKyowa B700 and C1000, Cape Girardeau, MO, USA) containing 55 % protein, 10 % minimum lipid, and less than 4 % carbohydrate (<0.5 % starch). Each feeding experiment was terminated after 3–4 months when fish reached sizes well beyond those at which *C. violaceus* and *X. mucosus* shift to herbivory in nature (Horn et al. 1982). Upon reaching the desired size, (65–75 mm SL: *X. atropurpureus*, *X. mucosus*, and *C. violaceus*, or 60–70 mm SL: *A. purpurescens*), ten individuals of each species ($L_{60–75}$) were euthanized with an overdose (>1 g l^{-1} seawater) of tricaine methanesulphonate (MS-222, Argent Chemicals Laboratories, Inc., Redmond, WA, USA) and processed for in situ hybridization (see below) and the remaining ten individuals used for analysis of digestive enzyme activity (German et al. 2004). Twenty juveniles of each of the four fish species that had reached the same size range of 60–75 mm SL in nature ($W_{60–75}$) were captured to represent fish consuming a natural carnivorous, omnivorous, or herbivorous diet. To provide adequate tissues for the DNA sequencing, two larger juveniles ($W_{80–180}$, 80–180 mm SL) of each species were collected at the Piedras Blancas site, euthanized at the site with an overdose of MS-222, measured (±1 mm SL), dissected, and frozen on dry ice. All handling of fish from capture to euthanization was conducted under approved protocol 99-R-10 of the Institutional Animal Care and Use Committee at California State University, Fullerton.

cDNA sequencing

Total RNA was isolated from three fish for each species using the RNAwiz RNA isolation reagent (Ambion, Inc., Austin, TX, USA) and treated with DNase-free (Ambion) to remove contaminating DNA from the RNA samples. The total RNA was reverse-transcribed with heat denaturation of the RNA using the Retroscript kit (Invitrogen, Carlsbad, CA, USA). The enzymatic reaction was cleaned using the PCR Purification kit (Qiagen Inc.) and was followed by PCR. Degenerate primers [Amy1: 5′-GG(A/C) AGG AC(A/G) (T/G)CC AT(A/T/C) GT(T/C/G) CAC-3′. Amy3: 5′-CC(C/T) TCC TTC TG(A/G) CC(A/G) CC(A/G) GA(A/G) ATG-3′] were designed using a ClustalW (Thompson et al. 1994) alignment of three species, *P. americanus* (AF252633), *Anguilla japonica* (AB070721), and *L. calcarifer* (AF416651), whose cDNA sequences of α-amylase were obtained from GenBank. The PCR product was cloned into the pcr4TOPO vector using TOPO TA Cloning kit (Invitrogen) and grown overnight in LB plates with carbenicillin. Ten colonies were picked and inoculated into 3 ml of LB medium with carbenicillin and grown overnight in a shaking incubator at 37 °C. Plasmids were purified from the liquid culture using the Miniprep kit (Qiagen). A sample of the purified plasmid was digested with EcoR1 (Roche Applied Sciences, Laval, P.Q., Canada) to determine whether plasmids of the correct size were isolated. Once determined, inserts were sent to the Biotechnology Center at Utah State University for sequencing.

Tissue processing

The pyloric caeca region containing diffuse pancreas was dissected from ten juveniles of each of the three feeding categories and divided further into four 1-mm^2 pieces with a razor blade on a glass cutting board kept on ice. One piece of tissue per fish was placed in a processing cassette (HistoScreen, Richard-Allan Scientific, Kalamazoo, MI, USA), fixed in 10 % paraformaldehyde in 0.1 M phosphate buffer saline (PBS), pH 7.2, for 10–12 h at 4 °C, rinsed in PBS with 0.1 M glycine and 4 % sucrose for 2 h and again for 16 h at 4 °C, dehydrated in 50, 70, 90, 2× 100 % ethanol for 30 min each at 4 °C, impregnated in two changes of xylene for 30 min each, infiltrated in two changes of paraffin (Type 9, Richard-Allan Scientific) in a 60 °C vacuum oven for 30 min each, and embedded in paraffin at 57–60 °C. Blocks were stored at 4 °C until used. All reagents, solutions, and conditions used during tissue

processing, probe production, and in situ hybridization (see below) were RNase free.

Production of cRNA-digoxigenin probes

The amylase cDNA from *P. americanus* (Douglas and Gallant 1998) was cloned into pBluescript II SK (Stratagene, La Jolla, CA, USA) and chemically transformed using TOP10 Chemically Competent cells (Invitrogen) and grown overnight on LB plates with carbenicillin. Ten positive clones were inoculated into 300 µl LB medium with carbenicillin and grown overnight at 37 °C on a shaker. Fifty µl of the incubated picks was centrifuged at 10,000 *g* for 10 min at 25 °C. Cells were mixed with 30 µl of sucrose buffer (TE, 6 % sucrose, 0.1 % bromophenol blue dye) and 10 µl of phenol/chloroform/isoamyl alcohol 24:1 (Invitrogen), and centrifuged at 10,000 *g* for 10 min at 25 °C. The size of the resulting transformants was verified, and clones were streaked on LB plates with carbenicillin and grown overnight at 37 °C. The obtained cDNA plasmids were purified using Nucleobond Plasmid Purification kit (BD Biosciences, Palo Alto, CA, USA), linearized using restriction enzyme HindIII to generate both sense (S) and antisense (AS) probes, transcribed in vitro using DIG RNA labeling kit (Roche Applied Science) and relevant RNA polymerases (i.e., T3 for S and T7 for AS probes) as described by Murray et al. (2003). The full-size probes (1.2 kb) were hydrolyzed to 250 nucleotides at 60 °C for 28 min (Murray et al. 2003) and resuspended in 100 µl of in situ hybridization buffer (Murray et al. 2002), and stored at −80 °C until used.

Slide and section preparation

From each block, 7-µm serial sections were cut using a rotary microtome (HM325, MICROM Laborgerate, Germany) equipped with a disposable knife (Edge-Rite L, Richard-Allan Scientific) and mounted on two slides (6–8 sections per slide) treated with 3-aminopropyl triethoxysilane (TESPA, Sigma-Aldrich, St. Louis, MO, USA) as described by Jowett (1997) and Gawlicka and Horn (2006). Sections were dried onto the slides on a warming plate at 40 °C for 2 h and then baked overnight at 60 °C. Sections were deparaffinized in three changes of xylene for 5 min each, rehydrated by rinsing twice in 100 % ethanol, and again in 90, 70, and 50 % ethanol for 2 min each. The slides were rinsed twice in water for 2 min each and equilibrated in 50 mM Tris–HCl pH 7.5 for 5 min. The slides were rinsed in 0.2 M glycine–HCl buffer, pH 2.0, for 30 min and used to verify the presence of the pancreatic tissue by staining with 0.05 % acridine orange in the same buffer for 30 min in the dark followed by two rinses of 15 min each. The slides were mounted with 50 % buffered glycerin (Morel and Cavalier 2000) and examined under blue (470 nm) fluorescence. The pancreatic tissue appeared fluorescent green-orange, as it contained a high amount of RNA, and was detected on six out of ten blocks per species. The blocks with pancreatic mRNA were used in a random order for in situ hybridization assays until the three replications per species and category needed for statistical analysis were fulfilled.

In situ hybridization assays

Two sets of duplicate slides per each block were prepared a day before assay as described above. Each assay was limited to 24 slides maximum by the size of the hybridization chamber, thus allowing only 3–6 blocks to be analyzed simultaneously. The assays were conducted following the protocols developed by Murray et al. (2002, 2003) with the following modifications: the tissue equilibrated sections were digested with 2 µg proteinase K ml^{-1} 50 mM Tris–HCl, pH 7.5, at 37 °C for 30 min, rinsed in 2 mg glycine ml^{-1} 50 mM Tris–HCl, pH 7.5, at room temperature (RT, 22 °C) for 2 min, washed three times in 0.1 M triethanolamine (TEA, pH 8.0, for 5 min; TEA with 0.25 % acetic anhydride for 10 min; TEA for 5 min, all at RT), and once in 2× SSC for 5 min at RT. Following the last wash, sections were dehydrated (50, 70, 90 and 2× 100 % ethanol for 2 min each) and let dry at RT for 2 h. The first set of slides was assayed with the AS probe and the second with the S (control) probe. Fifty µl of probe (final concentration 0.5 µg $µl^{-1}$) was added to each slide and a cover slip placed on top. Slides were transferred on a stainless rack in a closed humid incubation chamber with the bottom lined with a filter paper infiltrated with 75 % formamide in 2× SSC, and hybridization was conducted overnight at 47 °C. The cover slips were removed by gently washing in 2× SSC at RT for 20 min and the slides incubated in 50 % formamide in 2× SSC for 60 min at 47 °C. The slides were rinsed three times in RNase buffer (10 mM Tris–HCl, pH 8.0, 0.5 M NaCl) at 37 °C (1st and 3rd for 5 min, and 2nd for 30 min with 10 mg RNase A ml^{-1} and 100,000 U RNase t1 ml^{-1} added to the RNase buffer) and washed with 2× SSC, 1× SSC, and 0.5× SSC at 47 °C for 30 min each. Slides were rinsed in a blocking buffer (0.1 M Tris pH 7.5, 150 mM NaCl, 1 % BSA, 10 % lamb serum, Sigma-Aldrich) at RT for 30 min, and digoxigenin was detected using alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Applied Science) diluted to 1:250 with the blocking buffer. 100 µl of the diluted material was applied to each slide, coverslips placed, and the slides transferred to a humid chamber with the bottom filter paper infiltrated with 0.1 M Tris pH 7.5 with 150 mM NaCl and incubated for 30 min at 37 °C. The coverslips were removed and the slides rinsed three times

in 0.1 M Tris pH 7.5, 150 mM NaCl, 1 % BSA, and 1 % Tween 20 at RT for 10 min each. Alkaline phosphatase was detected using nitroblue tetrazolium (NBT, 100 mg ml^{-1}, Roche Applied Science) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 50 mg ml^{-1}, Roche Applied Science) as chromogenic substrates. The slides were rinsed in 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$ for 10 min at 37 °C and incubated in the same buffer containing NBT/BCIP for 10 min in the dark at 37 °C. This development time was optimized to allow the deep blue formazan reaction product to develop without producing any background staining. The reaction was stopped by washing in 50 mM Tris–HCl pH 7.5 for 2 min at RT. The slides were fixed in 4 % formaldehyde in 50 mM Tris–HCl for 5 min, rinsed in 50 mM Tris–HCl pH 7.5 for 5 min, and dehydrated by rinsing in 50, 95, and 100 % ethanol for 15 s each, followed by a xylene rinse for 15 s. The slides were mounted in Paramount (Fisher Scientific, Pittsburgh, PA, USA) without counterstaining to eliminate the risk of obscuring the hybridization signal. The blue precipitate was observed on the sections hybridized with AS probes, whereas those hybridized with S probe (control) appeared entirely unstained. To ensure reproducibility of the results required for quantitative in situ hybridization (Larsson 2001), the assay protocol was standardized so that all assays were conducted on sections of the same thickness using probes at the same concentration and same temperature and duration of hybridization.

Intensitometry by image analysis

The intensity of α-amylase gene expression, as detected through hybridization with α-amylase cRNA-DIG probe and visualized through immunoreaction with alkaline conjugated anti-DIG antibodies, was in a direct relationship to the amount of the α-amylase mRNA in the pancreatic cells. Sections with hybridization signal were examined under a bright-field light microscope (Olympus BX60) with a 40× objective and digitized automatically into 1,024 × 1,024-pixel images with 255-gray levels using a monochrome high-resolution cooled CCD digital camera (ORCA 100, C4742-95, Hamamatsu Co., Bridgewater, NJ, USA) connected to C-IMAGING high-performance system for image analysis (Compix Inc., Imaging Systems, Cranberry Township, PA). To account for cellular heterogeneity of the fish pancreas (Krogdahl and Sundby 1999) and ensure capture standardization required for image analysis (Larsson 2001), 12 images were captured per each fish (1–2 images per section, 432 images for all species and categories) and by the same operator at the same magnification, illumination, and linearity of the camera. For each image, a region of interest (ROI), corresponding to the area of the expression, was identified manually by intensity thresholding and mean gray intensity of each pixel of the ROI area measured automatically by linear calibration of gray level. The intensities for 12 images per fish were averaged, and the final mean intensity value was reversed from 0 (white = low expression level) to 255 (black = high expression level) to facilitate interpretation.

Statistical analysis

The mean intensities of expression ($n = 3$) were compared between $w_{30–40}$ and $W_{60–75}$ fish to test for an ontogenetic effect and between $W_{60–75}$ and $L_{60–75}$ fish to test for a dietary effect using a two-tailed t test with the significance level set at $P \leq 0.05$. Interspecific comparisons of hybridization intensity were made within each feeding category to test for species-level effects using one-way ANOVA (Minitab version 13, State College, PA, USA) followed by a Tukey's HSD multiple comparisons test with a family error rate set at $P = 0.05$. All data passed Levene's test for equal variances.

Results

Phylogenetic hypothesis for the Stichaeidae

The phylogenetic hypothesis for the family is presented in Fig. 1. Most nodes in the tree are supported with ≥99 % Bayesian posterior probabilities, with the lowest support

Table 1 Percent identity for the nucleotide sequences and percent similarity for the amino acid sequences (in parentheses) of the α-amylase gene portion from the four prickleback species and *Pseudopleuronectes americanus*

	X. mucosus	*X. atropurpureus*	*C. violaceus*	*A. purpurescens*	*P. americanus*
Xiphister mucosus	–	98 (98)	96 (95)	96 (93)	84 (85)
Xiphister atropurpureus		–	98 (95)	96 (94)	85 (86)
Cebidichthys violaceus			–	96 (94)	85 (86)
Anoplarchus purpurescens				–	86 (87)
Pseudopleuronectes americanus					–

Derived from pairwise nucleotide and amino acid (in parentheses) sequence alignment using ClustalW software. Species order reflects descending pairwise alignment scores

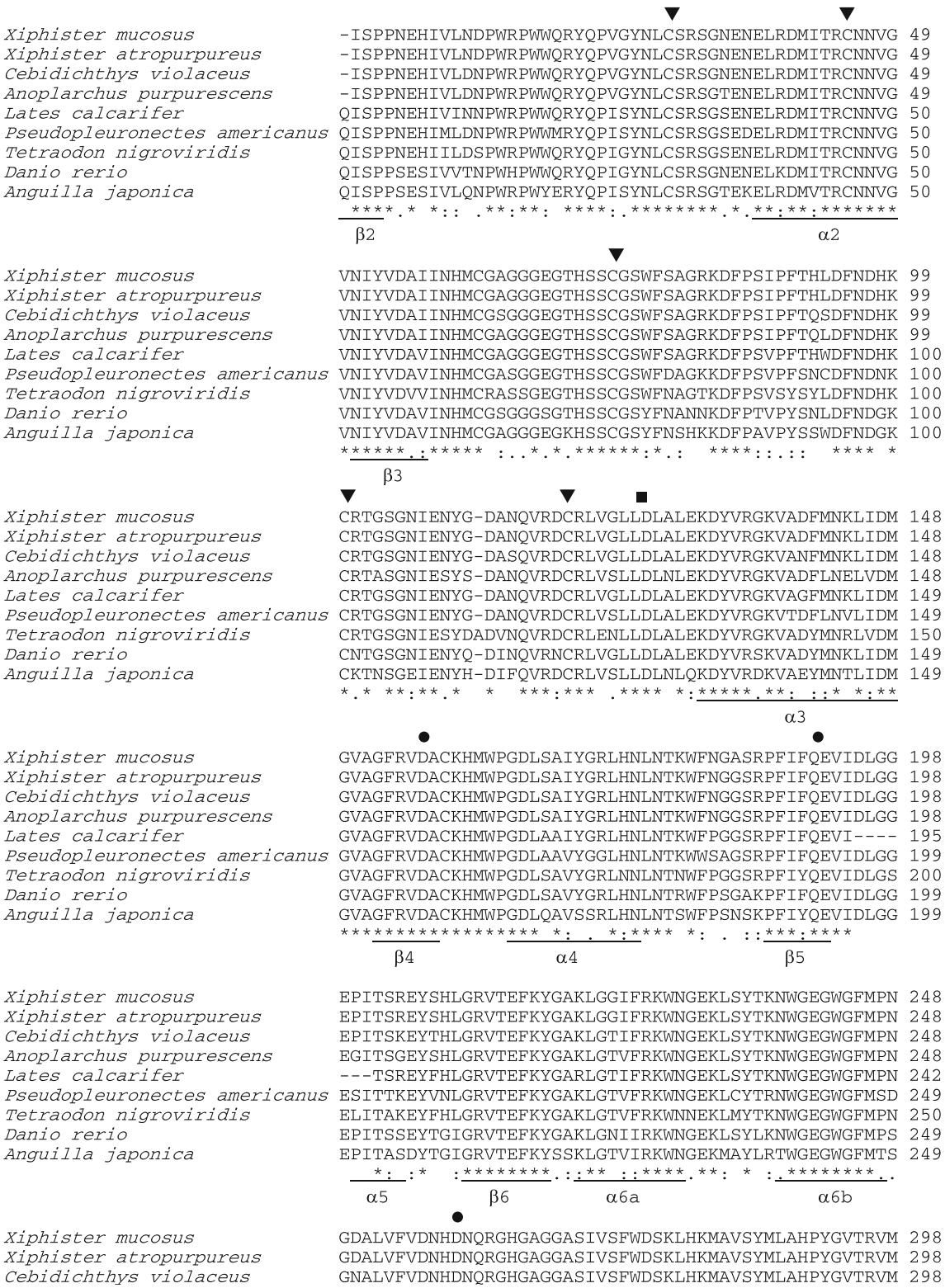


Fig. 2 Alignment of amino acid sequences of the α-amylase gene portion from the four prickleback species *Xiphister mucosus* (GenBank accession number DQ897652), *Xiphister atropurpureus* (DQ897654), *Cebidichthys violaceus* (DQ897655), and *Anoplarchus purpurescens* (DQ897653), and from *Pseudopleuronectes americanus* (AF252633) and four other species in GenBank: *Tetraodon nigroviridis* (AJ427289), *Lates calcarifer* (AF416651), *Danio rerio* (BC054669), and *Anguilla japonica* (AB070721). Alignment was used performing ClustalW software. *Asterisks* indicate amino acids conserved among all species, *colons* indicate amino acids conserved in almost all species, and *dashes* represent gaps introduced for alignment purposes. Active site residues are indicated by *circles*, the aspartic acid involved in calcium binding is indicated by *a square*, and cysteines are indicated by *arrowheads*. Positions of α-helices and β-sheets are *underlined*

```
Anoplarchus purpurescens       GNALVFVDNHDNQRGHGAGGASIVSFWDSKLHKMAVGYMLAHPYGVTRVM 298
Lates calcarifer               GNALVFVDNHDNQRGHGAGGASIVTFWDARLYKMAVGYMLAHPYGVTRVM 292
Pseudopleuronectes americanus  GNAVVFVDNHDNQRGHGAGGAAIVTFWDPRPHKMAVAYMLAHPYGVTRVM 299
Tetraodon nigroviridis         GNAVVFIDNHDNQRGHGAGGAAIVTFWDFRLHKMAVAYMLAHPYGVTRVM 300
Danio rerio                    DKALVFVDNHDNQRGHGAGGASIVTFWDARLYKMAVGFMLAHPYGFTRVM 299
Anguilla japonica              GNALVFVDNHDNQRGHGAGGSSVITFWDPRIYKMATGFMLAHPYGVTRVM 299
                               ..*:**:**************::::::*** : :***..:*******.****
                                     β7                              α7          β8
                                                                      ▼           ▼
Xiphister mucosus              SSFRWNRQIVNGKDQNDWMGPPSHGDGSTKSVPINADQTCGDGWVCEHRW 348
Xiphister atropurpureus        SSFSWNRQIVNGKDQNDWMGPPSHGDGSTKSVPINADQTCGDGWVCEHRW 348
Cebidichthys violaceus         SSFRWNRNIVNGKDQNDWMGPPSHGDGSTKSVPIKADQTCGDEWVCEHRW 348
Anoplarchus purpurescens       SSFRWNRYIVDGKDQNDWMGPPSHGDGSTKSVPINADQTCGDGWVCEHRW 348
Lates calcarifer               SSFRWNRHIVNGKDQNDWMGPPSHGDGSTKSVPINPDQTCGDGWVCEHRW 342
Pseudopleuronectes americanus  SSFRWNRHIVNGKDQNDWMGPPSHSDGSTKSVPINPDQTCGDGWVCEHRW 349
Tetraodon nigroviridis         SSFRWNRHIVNGKDQNDWMGPPSHPDGSTKSVPINPDETCGDGWVCEHRW 350
Danio rerio                    SSYRWDRNIVNGQDQNDWIGPPSNGDGSTKPVPINPDSTCGDNWVCEHRW 349
Anguilla japonica              SSFRWNRHIVDGKDQNDWMGPPSYPDGSTKPVLINPDTTCGDNWVCEHRW 349
                               **: *:* **:*:*****:****  *****.* *:.* **** *******


Xiphister mucosus              HQIKNMIIFRNVVNGQPHSNWWDNKSNQVAFGRGNRGFIVFNNDDWN- 395
Xiphister atropurpureus        RQIKNMVIFRNVVNGQPHSNWWDNQSNQVAFGRGNRGFIVFNNDDWN- 395
Cebidichthys violaceus         RQITNMVIFRNVVNGQPHSNWWDNQSNQVAFGRGNRGFIVFNNDDWN- 395
Anoplarchus purpurescens       RQITNMVIFRNVVSGQPHSNWWDNQSNQVAFGRGNRGFIVFNNDDWN- 395
Lates calcarifer               RQIKNMVAFRNVVNGQPHSNWWDNRSSQVAFGRGNRGFIIFNNDDWDL 390
Pseudopleuronectes americanus  RQITNMVKFRNVVDGQPHSNWWDNQSNQVAFGRGNRGFIVFNNDDWDL 397
Tetraodon nigroviridis         RQIKNMVIFRNVVNGQPHSNWWDNNSNQVAFGRGNRGFIIFNNDDWDL 398
Danio rerio                    RQIKNMVIFRNVVNGQPFSNWWDNGSNQIAFSRGSRGFIIINNDDWEL 397
Anguilla japonica              RQIRNMVIFRNVVNNQPISHWWDNGSNQVAFGRGNRGFIVFNNDDRHL 397
                               :** **: *****..** *:**** *.*:**.**.****::**** .
                                     α8
```

Fig. 2 continued

79 %. Based on a phylogeny constructed from 49 morphological characters, Stoddard (1985) suggested that the Xiphisterini included the members of the clade to which *C. violaceus* belongs, in addition to the two species of *Xiphister* and *P. chirus*. This relationship was not supported by our molecular phylogeny. Instead, the two species of *Xiphister*, along with *Phytichthys chirus*, formed a clade distinct from the one that included *C. violaceus*. The latter species is in a separate clade that includes *Esselenichthys carli* as the basal taxon, and the two species of *Dictyosoma* in more derived positions within the tree. We propose a name of "Esselenichthyini" for this clearly defined tribe that exists in a different part of the stichaeid phylogeny from the Xiphisterini (Fig. 1). These results strongly support the contention that herbivory evolved twice, independently, within the family Stichaeidae: once in the Xiphisterini (*X. mucosus*), and once in the Esselenichthyini (*C. violaceus*). *Anoplarchus purpurescens* was recovered within the Alectriini, a tribe whose monophyly appears supported in our analysis. Other recognized tribes (or subfamilies), including the Chirolophini, Lumpenini, and Stichaeini, were all well supported in our analysis.

Sequences

The pairwise comparison of α-amylase nucleotide portions (1,188 bp) showed 98 % identity between *X. atropurpureus* and both *X. mucosus* and *C. violaceus*; 96 % between *X. mucosus* and *C. violaceus*, and *A. purpurescens* and the three other prickleback species (Table 1). There was 84–86 % identity between the four prickleback species and *P. americanus*. In the pairwise comparison of amino acid portions of the α-amylase gene, *X. atropurpureus* had 98 % similarity with both *X. mucosus* and *C. violaceus*, and in all other comparisons between the prickleback species, there was 93–95 % similarity. The amino acid similarity with *P. americanus* was 85–87 %. Alignment of amino acid sequences with the four prickleback species and five other fish species revealed that the α-amylase gene is highly conserved (supplementary Appendix 2). The conserved structure is also supported by the amino acid alignment, which showed three common active site residues, the site of aspartic acid involved in calcium binding, and seven cysteines involved in disulphide bridge formation (Fig. 2).

Ontogenetic effects

The intensities of α-amylase gene expression were compared between $w_{30–40}$ and $W_{60–75}$ fish within each species to test for an ontogenetic effect (Table 2). The $W_{60–75}$ fish of *C. violaceus*, *X. mucosus,* and *X. atropurpureus* showed significant ontogenetic increases in expression, whereas *A. purpurescens* did not show any significant ontogenetic changes.

Table 2 Within-species comparisons (two-tailed *t* test) of the intensity of α-amylase gene expression from the ontogenetic and dietary perspectives in the four species of prickleback fishes as determined by quantitative in situ hybridization

Cebidichthys violaceus	Xiphister mucosus	*Xiphister atropurpureus*	Anoplarchus purpurescens
Ontogenetic effect ($w_{30–40}$ vs. $W_{60–75}$)			
$t = -4.50$	$t = -20.41$	$t = -6.70$	$t = -0.39$
$P = 0.011$	$P < 0.001$	$P < 0.001$	$P = 0.718$
Dietary effect ($W_{60–75}$ vs. $L_{60–75}$)			
$t = 0.31$	$t = -2.75$	$t = 1.81$	$t = 0.66$
$P = 0.769$	$P = 0.051$	$P = 0.145$	$P = 0.547$

$n = 3$ fish for each species and category. $df = 4$ for all comparisons. See Table 3 for the intensity values

Dietary effects

The intensities of α-amylase gene expression were compared between $W_{60–75}$ and $L_{60–75}$ fish within each species to test for a dietary effect (Table 3). For all four species, *C. violaceus*, *X. mucosus*, *X. atropurpureus*, and *A. purpurescens*, no significant dietary effect was observed.

Species-level effects

The intensities of α-amylase gene expression were compared within each of the three feeding categories, $w_{30–40}$, $W_{60–75}$, and $L_{60–75}$, among the four species to test for species-level effects (Table 3). In the $w_{30–40}$ category, *A. purpurescens* showed a significantly lower mean level of expression than *C. violaceus*, *X. mucosus*, and *X. atropurpureus*. *Cebidichthys violaceus* had a significantly higher mean value than *X. atropurpureus*, but the value was not significantly different from *X. mucosus*, and the values for *X. mucosus* and *X. atropurpureus* were statistically indistinguishable. In both the $W_{60–75}$ and $L_{60–75}$ categories, *C. violaceus*, *X. mucosus*, and *X. atropurpureus* were not significantly different from each other, but all three species had significantly higher mean levels of expression than *A. purpurescens*.

Discussion

This study, to our knowledge, is the first to compare ontogenetic, dietary, and species-level effects on the expression of an α-amylase gene in closely related species of herbivorous, omnivorous, and carnivorous fishes. Results of the sequence analysis for the gene indicated its strong homology among the four target prickleback species and supported the general recognition that α-amylase genes are highly conserved in evolution. Results for α-amylase expression generally supported our hypotheses in that we observed significant ontogenetic increases in expression in the three members of the two herbivore-containing clades of pricklebacks but no ontogenetic change in the species from the adjacent carnivorous clade. Furthermore, in comparison with larger fish consuming their natural diet, no significant change in α-amylase expression was detected in any species when they were consuming the low-starch diet in the laboratory, suggesting constitutive expression of α-amylase. However, the three members in the herbivore-containing clades had significantly higher levels of α-amylase expression than the single species from the carnivorous clade, and the sister taxa, despite having different diets, were indistinguishable in this regard. Contrary to many previous studies involving α-amylase in fishes (Scheele 1993; Le Moullac et al. 1996; Peres et al. 1998; Huvet et al. 2003; Krogdahl et al. 2005), and the AMH (Karasov and Martínez del Rio 2007), the results here indicate that gene programming during ontogeny and evolutionary history exert greater influence than proximate diet on α-amylase gene expression in pricklebacks. Similar results were reported for *Odax pullus*, an herbivorous fish that consumes starch-rich red algae as a juvenile, and little starch as an adult, yet retains elevated amylase activity in adulthood (Skea et al. 2005). Within the Stichaeidae, *C. violaceus* maintains elevated rates of glucose transport across the intestinal epithelium regardless of diet (Buddington et al. 1987), further supporting the importance of soluble polysaccharides in the nutritional physiology of herbivorous pricklebacks. Thus, constitutive expression of α-amylase may represent a true dietary specialization for herbivory and omnivory in prickleback fishes.

Gene expression levels obtained in this study combined with enzyme activity data from previous research (German et al. 2004) suggest that α-amylase production is transcriptionally regulated in the three species from the herbivore-containing clades (Table 4), consistent with studies of fish larvae (Peres et al. 1998; Douglas et al. 2000; Ma et al. 2001; Zambonino Infante and Cahu 2001). However, α-amylase may be post-transcriptionally regulated in the

Table 3 Among-species comparisons of the intensity of α-amylase gene expression from the phylogenetic perspective in three feeding categories ($w_{30–40}$, $W_{60–75}$, and $L_{60–75}$) of the four species of prickleback fishes as determined by quantitative in situ hybridization

Cebidichthys violaceus	Xiphister mucosus	Xiphister atropurpureus	Anoplarchus purpurescens	ANOVA
Small wild-caught ($w_{30–40}$)				
104.3 ± 6.5 c	98.3 ± 1.2 bc	85.7 ± 3.3 b	64.0 ± 2.6 a	$F_{3,\ 8} = 20.65$ $P < 0.001$
Large wild-caught ($W_{60–75}$)				
134.0 ± 1.2 b	138.0 ± 1.5 b	132.7 ± 6.2 b	65.7 ± 3.4 a	$F_{3,\ 8} = 90.42$ $P < 0.001$
Large laboratory-fed ($L_{60–75}$)				
136.7 ± 8.4 b	130.3 ± 2.3 b	146.0 ± 4.0 b	71.0 ± 7.4 a	$F_{3,\ 8} = 31.38$ $P < 0.001$

Values are mean (±1 SE, $n = 3$) intensities of all pixels of expression. The intensities were evaluated using a reverse gray scale from 0 = white to 255 = black. Values within each category were analyzed with one-way ANOVA and Tukey's pairwise comparison test with a family error rate of $P = 0.05$. Values that share the same letter are not significantly different

Table 4 Patterns of α-amylase activity regulation from the ontogenetic and dietary perspectives

Effect	*Cebidichthys violaceus*	*Xiphister mucosus*	*Xiphister atropurpureus*	*Anoplarchus purpurescens*
Ontogenetic ($w_{30–40}$ vs. $W_{60–75}$):				
Expression	S↑	S↑	S↑	NS
Activity	S↑	S↑	S↑	NS
Regulation	T	T	T	T
Dietary ($W_{60–75}$ vs. $L_{60–75}$):				
Expression	NS	NS	NS	NS
Activity	NS	NS	NS	S↑
Regulation	T	T	T	PT

Deduced from the results of statistical comparisons of α-amylase gene expression levels (this study) and α-amylase activities (German et al. 2004), both obtained using fish subsampled from the same experiment. Within-species comparisons for the ontogenetic and dietary effects were performed between feeding categories and analyzed with a two-tailed t test with a family error rate of $P \leq 0.05$, and results were reported as significant (S) or not significant (NS), with arrows indicating the direction of change. $n = 3$ fish per species and category for expression levels, and $n = 10$ for activities. For each species, expression and activity changes occurring in the same direction indicate transcriptional regulation (T), whereas those occurring in different directions indicate post-transcriptional regulation (PT)

carnivorous *A. purpurescens* (Table 4). Evidence for this contention comes from an increase in α-amylase enzyme activity in *A. purpurescens* when this fish was consuming the low-starch diet in the laboratory (German et al. 2004), despite no apparent difference in α-amylase expression (this study). Moreover, in contrast to the other studied prickleback species, *A. purpurescens* may be more responsive to changes in starch content of the diet, as shown in some fish larvae (Zambonino Infante and Cahu 1994; Peres et al. 1996). Indeed, *A purpurescens* changed the activity of seven different digestive enzymes in response to the laboratory diet (not just α-amylase), and, therefore, *A. purpurescens* may just show more plasticity in overall digestive enzyme activity to diet than the other pricklebacks, which showed less change in all enzymatic activities (German et al. 2004).

Based on our phylogenetic hypothesis, herbivory evolved independently in the clades containing *X. mucosus* (Xiphisterini) and *C. violaceus* (Esselenichthyini). This result suggests that the ontogenetic increase in α-amylase expression and activity associated with an ontogenetic shift towards a diet rich in starch also evolved twice. Within the Esselenichthyini, the ontogenetic changes in diet and α-amylase expression likely only evolved in *C. violaceus*. Evidence for this probability is provided by the fact that neither species of *Dictyosoma* show ontogenetic dietary shifts (D.P. German, unpublished dietary data on 80 individuals of the two *Dictyosoma* taxa) and that *Esslenichthys carli* reaches a small maximum size (i.e., 171 mm SL; Follett and Anderson 1990). Indeed, *E. carli* and its sister taxon, *E. laurae* (maximum size 97 mm SL; Follett and Anderson 1990), are rare in nature and in museum

collections, and thus, there is a lack of dietary information on these species. Only the larger taxa within the Stichaeidae—*C. violaceus* and *X. mucosus* reach maximum sizes of 760 mm SL and 580 mm SL, respectively (Eschmeyer et al. 1983)—are known to be herbivorous (German et al. 2004; German and Horn 2006), allowing for the expectation of carnivory in the two much smaller species of *Esselenichthys*. However, the story may be different in the Xiphisterini.

As the basal member of the Xiphisterini, *P. chirus* does undergo an ontogenetic shift in diet, consuming more algae as it increases in size (Cross 1981). *Phytichthys chirus* also shows an ontogenetic increase in α-amylase activity (D.P. German, unpublished data). Together, these data, along with the ontogenetic dietary shifts and increases in α-amylase expression in the two *Xiphister* species, suggest that the programmed ontogenetic increase in α-amylase expression and activity evolved early, in the common ancestor that gave rise to the entire Xiphisterini clade. Furthermore, in our updated phylogeny (Fig. 1), the common theme in the evolution of herbivory within the Stichaeidae appears to be the invasion of rocky intertidal habitats. The Esselenichthyini and Xiphisterini represent independent intertidal invasion events, as neither the Chirolophini nor the Stichaeini contain intertidal taxa. Thus, access to the abundant algal resources in the rocky intertidal zone may have engendered the evolution of herbivory (and omnivory) in the Xiphisterini and in *C. violaceus*. Interestingly, *A. purpurescens* is the primary intertidal taxon within the Alectriini, yet this species is carnivorous.

Results of the pairwise comparison of partial α-amylase gene sequences indicate a strong homology of the gene among all four target species. Identity scores for the gene sequences and similarity scores for the amino acid sequences showed that the sister taxa, *X. mucosus* and *X. atropurpureus*, were most alike and *C. violaceus* more like these sister taxa than *A. purpurescens*. Although the α-amylase gene and amino acid sequences of *P. americanus* were somewhat distant from those of the four target species, the still high identity of nucleotide sequences and high similarity of the amino acid sequences to those of the four prickleback species indicate that the *P. americanus* sequence was successful as a probe for the in situ hybridization assays.

Alpha-amylase genes in fishes consist of approximately 1,500 nucleotides, and the nucleotide sequences are highly conserved, with 70 % sequence identities (Krogdahl et al. 2005). In the present study, we were able to obtain partial sequences of 1188 bp for an α-amylase gene in the four prickleback species. β2, β3, β4, β5, β7, and β8 strands of the α-amylase-type $(\alpha/\beta)_8$-barrel are the most highly conserved amino acid sequences in well-defined regions of the gene, and the β4, β5, and β7 strands include the three active site residues, Asp, Glu, and Asp, respectively (Janacek 1997). The partial sequences we obtained for the prickleback species include most of the α-helices and β-strands, except for β1, α1, and part of β2. All three active site residues, as well as the aspartic acid involved in calcium binding and seven of the ten cysteines involved in the disulphide bridges found among various vertebrate amylases (Pasero et al. 1986; Janacek 1997), were present in the four prickleback species. The similar alignments of amino acids for α-amylase in these four species further showed that the gene is highly conserved.

A promising area to investigate in the future is variation in gene copy number (e.g., Perry et al. 2007; Sugino 2007; Axelsson et al. 2013), or isoforms, of α-amylase genes in relation to diet, especially in clades containing both herbivores and carnivores (e.g., the Stichaeidae). Many fish species, including the carnivorous *Tetraodon nigroviridis*, have more than one amylase gene within their genomes (Bouneau et al. 2003; Krogdahl et al. 2005), and *C. violaceus* appears to express more than one α-amylase gene at once (D.P. German, unpublished data). Humans also possess two copies of pancreatic α-amylase in their genomes (AMY2A and AMY2B) that are expressed simultaneously and show little sequence variation among them (Samuelson et al. 1988; Gumucio et al. 1988). How are these isoforms different? Other than different isoelectric points of the proteins (Ferey-Roux et al. 1998), we know little about functional differences among the different human pancreatic α-amylase isoforms, or how they might function differently in the digestive process. Given the diverse diets consumed by herbivorous fishes and variations in starch-type among different foods—for instance, green algae use amylose as their storage polysaccharide, whereas red algae use amylopectin (Painter 1983)—fishes may express different α-amylase isoforms that specialize in hydrolyzing different types of starch. Fishes clearly hydrolyze different types of starch at different rates (Zemke-White and Clements 1999; Krogdahl et al. 2005), and these differences likely have genetic underpinnings beyond differences in expression levels of a single gene. Such differences could have real relevance for *C. violaceus* and *X. mucosus*, which show seasonal variation in algal consumption, with more green algae consumed during the summer, and more red algae during the winter (Horn et al. 1982). Expressing different α-amylase isoforms based on seasonal algal consumption could provide an advantage for digesting the relevant starches inherent in their algal diets.

Acknowledgments We thank S. Douglas and J. Gallant at the Institute for Marine Biosciences in Halifax, Nova Scotia, for sequencing and providing us with the *P. americanus* probes and protocols for the in situ hybridization assays; A. Gawlicka, K. Boyle, and M. Saba for assistance in the field and laboratory; R. McCaman for help with sequence analysis; and A. Cohen and M. Tolmasky for

valuable comments on an earlier draft of the manuscript. Financial support was provided by a grant (OCE-9906857) from the National Science Foundation (M.H.H., principal investigator) and by a grant to California State University, Fullerton, from the Minority Scientist Development Program of the National Institutes of Health. Additional funding came from University of California, Irvine (startup funds to D.P.G), and through the Minority Science Program at UC Irvine (to A.E.S).

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