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## ABSTRACT

Predicting the success of a species' colonization into a novel environment is routinely considered to be predicated on niche-space similarity and vacancy, as well as propagule pressure. The role genomic variation plays in colonization success (and the interaction with environment) may be suggested, but has not rigorously been documented. To test an hypothesis that previously observed ecotype-specific polymorphisms between anadromous and landlocked alewife (*Alosa pseudoharengus*) populations are an adaptive response to osmoregulatory challenges rather than a result of allele sampling at founding, we examined multiple anadromous and landlocked (colonized) populations for their allelic profiles at a conserved region (3'-UTR end) of a  $\beta$ -thymosin gene whose protein product plays a central role in the organization of cytoskeleton. The putatively ancestral  $\beta$ -thymosin allele was prevalent in anadromous populations, whereas a newly derived allele was overrepresented in landlocked populations; a third allele was exclusive to the anadromous populations. We also conducted a complementary set of salinity exposure experiments to test osmoregulatory performance of the alewife ecotypes in contrasting saline environments. The pattern of variation and results from these challenges indicate a strong association of  $\beta$ -thymosin with colonization success and a transition from species with an anadromous life history to one with only a freshwater component. *J. Exp. Zool.* 321A:233–240, 2014. © 2014 Wiley Periodicals, Inc.

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George Gaylord Simpson reasoned that transitions between “adaptive zones” lead to rapid ecological diversification and evolutionary radiation (Simpson, '44). One of the most dramatic environmental transitions is the shift from marine to freshwater zones, at least as assessed by frequencies of the events on the macroevolutionary scale (Little, '90; Lee and Bell, '99). Although crossing the marine–freshwater boundary creates a formidable osmotic and physiological challenge (Alderice, '88) and hence a cross-colonization barrier, even to microbes (Logares et al., 2009), modern anadromous (sea-run migratory) fishes make the environmental transition at least once in their lifetime, and can potentially be pre-adapted to permanent colonization of fresh water (McDowall, '88; Schultz and McCormick, 2013). As recently as 10,000 years ago, postglacial colonization of freshwater environments was largely dominated by primitively anadromous fishes, as well as other marine fauna being trapped in inland lakes and underground caves (Bell and Andrews, '97). More recently, anthropogenic activity has accelerated freshwater invasions from marine or brackish habitats, not infrequently with dire consequences for ecosystems and conservation efforts (Lee and Bell, '99).

Striking morphological and life-history differences between anadromous and landlocked alewife (*Alosa pseudoharengus*) populations that have diverged in isolation for less than 300 years (Post et al., 2008) represent a rapid evolutionary change comparable with the consequences of domestication. Anadromous alewives migrate from the North American Atlantic coast into lakes and rivers above tidal influence to spawn in March–May, and young-of-the-year (YOY) alewives spend up to 6 months in freshwater before migrating back to the ocean in fall, whereas landlocked alewives are freshwater residents year-round. Landlocked alewives mature earlier, have slower adult growth, smaller gape and overall size at maturation, narrower spacing between gill rakers, and reduced fecundity relative to anadromous alewives (Graham, '56; Scott and Crossman, '73b; Palkovacs and Post, 2009). These changes are associated with a spectacular ecological success, as the alewife is the only alosid species that invaded the Laurentian Great Lakes, bypassed the natural barrier of Niagara Falls, and became a nuisance species reshaping the

food web in the ecosystem (Smith, '70; Post et al., 2008; Walsh et al., 2012). Alewives in the Great Lakes have also been blamed for the enigmatic spread of thiamine (vitamin B1) deficiency complex (TDC) and early mortality syndrome (EMS), both linked to enrichment in thiaminase, driving lake trout (*Salvelinus namaycush*) to near-extinction and affecting other salmonid populations as well (Marcquenski and Brown, '97; Fisher et al., '98; Fitzsimons et al., 2007; Fitzsimons et al., 2009).

Although phenotypic plasticity cannot be ruled out as a determinant of morphological and life-history differences between distinct alewife ecotypes, anadromous, and landlocked alewife populations show signs of genetic divergence (Palkovacs et al., 2008; Czesny et al., 2012), while juvenile alewives raised in “common garden” conditions maintain morphological differences (Palkovacs and Post, 2008). We have previously reported single nucleotide polymorphism (SNP) in the 3'-untranslated region (UTR) of  $\beta$ -thymosin to be potentially ecotype-exclusive (Czesny et al., 2012).  $\beta$ -Thymosins, originally thought to be thymic hormones, are short (41–43 residue), ubiquitous polypeptides, with intracellular and extracellular activities related to organization of the cytoskeleton sequestering G-actin, angiogenesis, inflammation, wound healing, apoptosis, and cancerogenesis (Huff et al., 2001; Hannappel, 2007). Here, we use larger sampling to demonstrate that allelic distribution of  $\beta$ -thymosin 3'UTR SNPs across multiple populations is indeed associated with ecotype (anadromous vs. landlocked). As most landlocked populations represent independent freshwater invasions, this distribution presumably reflects nonrandom survival of  $\beta$ -thymosin genotypes in freshwater conditions. SNPs in 3'UTRs can alter transcriptional and/or translational regulation (Hobert, 2004; Ryan et al., 2010) and confer differential responses to abiotic stresses (Tao et al., 2012). We report that  $\beta$ -thymosin expression in landlocked populations is higher and more responsive to salinity changes than in anadromous populations, suggesting that the gene contributes to the spectacular success of alewife in freshwater environments.

## MATERIALS AND METHODS

### Collection Sites

Anadromous alewife samples were obtained from the Atlantic Ocean (43.35°N, 68.25°W; 43.51°N, 67.52°W; 43.56°N, 68.35°W; 2011), Bride Lake (East Lyme, Connecticut; 41.33°N, 72.24°W; 2011), Chowan River (Hertford, North Carolina, 36.16°N, 76.41°W; 2012), and Damariscotta Mills (Maine, 44.06°N, 69.52°W; 2012). Landlocked alewife were sampled in Lake Michigan (Wisconsin; 44.16°N, 87.24°W; 2008–2012), Lake Ontario (Olcott, New York, 43.40°N, 78.73°W; 2011), Finger Lakes (Dean Cove, New York, 42.74°N, 76.75°W; 2011–2012), Lake Champlain (Vermont, 44.53°N, 73.33°W; 2008), Pattagansett Lake (East Lyme, Connecticut; 41.37°N, 72.22°W; 2011), and Rogers Lake (Old Lyme, Connecticut; 41.35°N, 72.30°W; 2011). Additional specimens included, 20

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blueback herring (*Alosa aestivalis*) obtained from Chowan River (Hertford, North Carolina, 36.16°N, 76.41°W; spring 2012), plus two hickory shad (*Alosa mediocris*) and three American shad (*Alosa sapidissima*) from James River (Richmond, Virginia; 37.53°N, 77.43°W; 2001).

#### Short-Term Salinity Challenge

YOY anadromous and landlocked alewives were captured by purse seine from their natal lakes in coastal Connecticut in October 2011. Alewives were collected from three sites: an anadromous population from Bride Lake, and landlocked populations from Pattagansett and Rogers Lakes. We transported fish to the Conte Anadromous Fish Research Center in Turners Falls, Massachusetts in aerated 190 L cylindrical containers at a salinity of 2 ppt. Once in the laboratory, alewife were held at 2 ppt for 1 day, after which salinity was decreased by 0.5 ppt/day for 3 days, to a final acclimation salinity of 0.5 ppt. Alewives were segregated by site and held in separate 1,200 L recirculating oval tanks fitted with charcoal filtration systems for one month prior to experimentation. During this time fish were kept at ambient photoperiod throughout the experiment and water temperature was maintained between 14.5 and 16°C. We subjected laboratory-acclimated alewives to two salinity challenge treatments for 2 weeks: deionized freshwater (0 ppt) and seawater (35 ppt). Deionized freshwater and seawater salinity challenges were conducted consecutively on separate dates: freshwater challenge from 16 November to 1 December 2011, and seawater challenge from 20 December 2011 to 3 January 2012. In each treatment, approximately 25–35 alewives from each site were immediately transferred to one of two, 250 L recirculating oval tanks with charcoal filtration. Deionized freshwater was prepared by running filtered, dechlorinated tap water through a resin-filled cartridge (Culligan International Company, Rosemont, IL, USA). Seawater was prepared by dissolving artificial sea salt (Instant Ocean, Spectrum Brands, Madison, WI, USA) in filtered, dechlorinated tap water. At the end of each 2-week trial, we euthanized surviving alewives (mean survival was 79%) in 250 mg/L tricaine methanesulfonate (MS-222; Argent, Redmond, WA, USA) and measured them for total length and weight. Gill tissue was removed immediately from euthanized fish by excising the left gill arches and trimming the gill tissue off of the bone. Gill tissue was placed immediately in 1 mL of RNAlater (Ambion, Life Technologies, Grand Island, NY, USA), incubated at 4°C overnight, and then stored at –20°C prior to RNA extraction.

#### Chronic Salinity Challenge

We obtained yearling (1+) landlocked alewives from Cayuga Lake in the Finger Lakes region of central New York in August 2012, and YOY anadromous alewives during their outmigration near Damariscotta Mills, Maine in September 2012. Alewives were transported to the fish culture facility at SUNY Buffalo State in New York. Alewives were separated by ecotypes and held in 16

757 L recirculating oval tanks. Tanks were arranged as two independent, recirculating systems consisting of eight tanks that shared common filtration and temperature control equipment. Landlocked and anadromous alewives were subjected to freshwater and seawater salinity challenge treatments in a similar fashion to common garden experiment 1, but with notable differences. Prior to the start of the salinity challenge period, half of all captured landlocked and anadromous alewives were acclimated to seawater (31–33 ppt) gradually over a 2-week period, while the other half remained in fresh water. For the seawater treatment, salinity was raised 3 ppt each day by adding artificial sea salt (Instant Ocean) until the final salinity was achieved. Alewives were maintained in freshwater and seawater treatments for a total of 10 weeks, during which time fish were kept at a density of 25–40 individuals per tank at 15°C and 12L:12D photoperiod. Dried Tetramin flakes were provided at a rate of 2% wet body weight per day. At the conclusion of the experiment, alewives were euthanized and processed as described above. Survival rates during the chronic salinity challenge exceeded 98% in both the freshwater and saltwater treatments, and all fish showed positive growth.

#### DNA Extraction and Sequencing

DNA was extracted from somatic tissues using Genra<sup>R</sup> Puregene Tissue Kit (Qiagen) according to the manufacturer's protocol and used for PCR amplification (Crimson Taq DNA Polymerase NEB) with the following primers: TGTGATTGTTGGTGCTTGCTCACC (forward) and TGGGCTGTACAGTCATCTGCGATT (reverse). PCR products were purified with ExoSap-It (Affymetrix) and sequenced with Prism 3730 Genetic Analyzer (ABI).

#### Gene Expression Analysis

RNA samples were extracted from gills with Trizol<sup>R</sup> Reagent (Ambion) according to the manufacturer's guidelines. Approximately 200 ng of each RNA sample was reverse transcribed using Quantitech Reverse Transcription kit (Qiagen) and thymosin expression was quantified with qRT-PCR using SYBR Green (ABI) reactions and StepOnePlus RT PCR System (ABI) with default cycling conditions. There were 8–10 biological replicates from each geographical location representing both salinity change treatments, with each RNA sample assayed twice for every experiment, and PCR reactions were repeated three times. The threshold cycle ( $C_t$ ) ratios between the target thymosin and the average endogenous control ( $\beta$ -actin) were calculated, and two-way ANOVA with repeated measures (PCR replicates) in R (v. 2.14.0) was used to test differences between ecotypes (anadromous vs. landlocked), water treatments (saltwater vs. freshwater), as well as ecotype  $\times$  treatment interaction.

#### Sequence Analysis

Sequences were aligned with Geneious Pro 5.5.6 and compared with GenBank using NCBI's BLASTN with default parameters.

Heterozygosity was not evaluated and all genotype counts are considered dominant based on the ABI 3730 GA call. An Atlantic salmon sequence (GenBank: BT059985.1) was used as an outgroup reference. Homology of 3'UTRs with microRNAs (miRNAs) was tested with BLASTN within miRBase (Griffiths-Jones, 2010; Kozomara and Griffiths-Jones, 2011), using E-value cutoff of 10, word size 4, match score +5, and mismatch penalty -4.

## RESULTS AND DISCUSSION

$\beta$ -Thymosin-12 in bony fishes is a paralog of  $\beta$ -thymosin-11 (84% sequence identity) and an ortholog of mammalian  $\beta_4$  (Low et al., '92). Strikingly, the 3'UTR sequence in thymosin  $\beta_4$  belongs to one of the most conserved noncoding sequences among at least 32 species of land vertebrates, ranging from primates to amphibians (Duret and Bucher, '97), as well as nonteleost bony fishes (sturgeons and gar), and cartilaginous fishes, but the conserved motif differs from teleost bony fishes (Edwards, 2012). Alewife, an invasive species from the Atlantic Ocean coast, exhibits ecotypic polymorphism of the 3'UTR (Table 1; chi square = 228.12,  $df = 2$ ,  $P < 0.0001$ ), which to our knowledge is unique. 3'UTR base pair positions 3 and 4 from the stop codon of the  $\beta$ -thymosin-12 are variable (Fig. 1) and all three alleles, CC, AC, and AT, are present in anadromous populations, with CC being most and AT least abundant (Table 1), but only AT allele present in the Great Lake landlocked populations (Table 1). However, Connecticut alewife populations that most likely represent freshwater invasions independent of the Great Lakes still contain a high frequency of the CC allele: 0.55 in Pattagansett Lake and 1.00 in Rogers Lake. The allele AC was not found in the landlocked populations. Other anadromous alosine species from the Western Atlantic Ocean (blueback herring [*Alosa aestivalis*], American shad [*A. sapidissima*], and hickory shad [*A. mediocris*]), have only allele CC (Fig. 1). These species are entirely anadromous in their natural state and have rarely if ever (Toole et al., '80; Smith, '85; Guest and Drenner, '91;

Owens et al., '98; Moyle, 2002; Winkelman and Van Den Avyle, 2002) become established as landlocked ecotypes as a result of deliberate introduction. Interestingly, this allele also is shared with Atlantic salmon (*Salmo salar*) in spite of divergence in other positions of the sequence (Fig. 1). This suggests that the CC allele is ancestral to the other two alewife alleles, presumably having been first mutated to AC, which in turn has given rise to the "freshwater-dominant" allele AT.

5'- and 3'-UTR sequences typically contain numerous *cis*-acting regulatory motifs that function either as target sites for RNA binding factors or interact directly with the translation machinery. In addition to proteins, UTR RNA-binding factors include other RNAs such as complementary natural antisense transcripts (Faghihi and Wahlestedt, 2009) and miRNAs (Doench and Sharp, 2004). The *in silico* miRBase analysis illustrated that relative to the CC allele, both AC and AT alleles lose sequence homology to at least one miRNA, namely miR-3617 (score 58,  $E$ -value = 9.3). Although this result should be treated with caution, as alewife miRNAs are yet unstudied and the tool does not predict miRNA target sites *par excellence*, miRNA sequences are highly conserved in metazoans (Lee et al., 2007; Ibanez-Ventoso et al., 2008), and their SNPs have been known to create, destroy, and modify miRNA target sites (Sun et al., 2009; Nicoloso et al., 2010; Gong et al., 2012).

When young alewives from each ecotype were transplanted to contrasting salinity environments for a 10-week-long exposure, measures of  $\beta$ -thymosin-12 expression in relation to the ecotype (anadromous vs. landlocked) differed significantly. Landlocked alewives maintained higher  $\beta$ -thymosin-12 expression (2-way ANOVA, short-term salinity challenge:  $F = 4.953$ ,  $P = 0.038$ ,  $df_{\text{Residual}} = 20$ ; chronic salinity challenge:  $F = 13.328$ ,  $P = 0.0022$ ,  $df_{\text{Residual}} = 16$ ; Fig. 2). Seawater tended to increase  $\beta$ -thymosin-12 expression in both groups, with the effect being more pronounced (yet not statistically significant,  $P > 0.05$ ) in landlocked fish in the short-term salinity challenge experiment, and anadromous fish in the chronic salinity challenge experiment.

Table 1. Thymosin genotype frequencies among anadromous and landlocked alewife populations.

Location	Total genotyped	SNP AT	SNP AC	SNP CC
Lake Michigan (landlocked)	96	96	0	0
Lake Ontario (landlocked)	10	10	0	0
Finger lakes (landlocked)	25	25	0	0
Lake Champlain (landlocked)	11	11	0	0
Pattagansett Lake (landlocked)	22	10	0	12
Rogers Lake (landlocked)	21	0	0	21
Atlantic Ocean (anadromous)	75	3	16	56
Maine (anadromous)	21	0	0	21
Bride Lake (anadromous)	28	2	2	24
Chowan River (anadromous)	50	1	4	45

<i>Alosa pseudoharengus</i>	5'-CATGA	AC	AT	CTTGTTGCCACTATGCACTGTACATT-3'
<i>Alosa pseudoharengus</i>	5'-CATGA	AC	AC	CTTGTTGCCACTATGCACTGTACATT-3'
<i>Alosa pseudoharengus</i>	5'-CATGA	AC	CC	CTTGTTGCCACTATGCACTGTACATT-3'
<i>Alosa aestivalis</i>	5'-CATGA	AC	CC	CTTGTTGCCACTATGCACTGTACATT-3'
<i>Alosa sapidissima</i>	5'-CATGA	AC	CC	CTTGTTGCCACTATGCACTGTACATT-3'
<i>Alosa mediocris</i>	5'-CATGA	AC	CC	CTTGTTGCCACTATGCACTGTACATT-3'
<i>Salmo salar</i>	5'-CATGA	AG	CC	CAAGCCTCCACTATGCACTGTACATT-3'

Figure 1. Sequence polymorphism within the 3'UTR β-thymosin of alewife (*Alosa pseudoharengus*), blueback herring (*A. aestivalis*), American shad (*A. sapidissima*), hickory shad (*A. mediocris*), and Atlantic salmon (*Salmo salar*).

This expression pattern suggests a strong *cis*-acting regulation of the gene, consistent with, for example, less efficient silencing of the “freshwater” (AT) allele and the increased rate of β-thymosin expression in landlocked populations. Unfortunately, most of the anadromous individuals used in the transplant experiments

turned out to have the CC genotype, and therefore genotype-specific expression responses to salinity within an ecotype could not be compared.

The unique patterns of β-thymosin sequence polymorphism and associated expression patterns may facilitate alewife freshwater invasions. Changes in the expression of β-thymosins are related to the differentiation of cells and intracellular sequestering of G-actin (Safer et al., '91). Actin filaments forming the cytoskeleton provide mechanical forces essential for compression or expansion during osmoregulatory changes in cell volume (Prager-Khoutorsky and Bourque, 2010), therefore, changes in these filaments may be critical during the transition from sea to fresh water. The intracellular actin cytoskeleton participates in a large variety of cellular activities such as locomotion, cytokinesis, intracellular transport processes, and phago- and exocytosis. Rapid sequestration activities require a high concentration (300–600 μM) of β-thymosin peptides that bind to monomeric actin or compete with other G-actin binding proteins (Mannherz and Hannappel, 2009). There is also ample evidence that β-thymosins have extracellular effects, such as modulating the rate of attachment and spreading of endothelial cells on matrix components (reviewed in Hannappel, 2007). β-Thymosins have additionally been implicated in the development and repair of injuries of the heart, brain, coronary vessels, and other organs in mammals (Vartiainen et al., '96; Bock-Marquette et al., 2004; Smart et al., 2007a,b).

Given their broad spectrum of functions and ubiquitous expression, β-thymosins emerge as interesting stress-responding molecules, potentially contributing to the spectacular colonization of freshwater by alewife. The first records of alewives in the Great Lakes are from Lake Ontario in 1873 (Miller, '57), and it is possible that their presence in the lake resulted from single or multiple invasion events. Ancestral anadromous populations may have gained access to Lake Ontario via the St. Lawrence River (Smith, '85). However, it is also possible that alewives invaded Lake Ontario and the Finger Lakes region of central New York via the Erie Barge Canal, which was completed in 1825

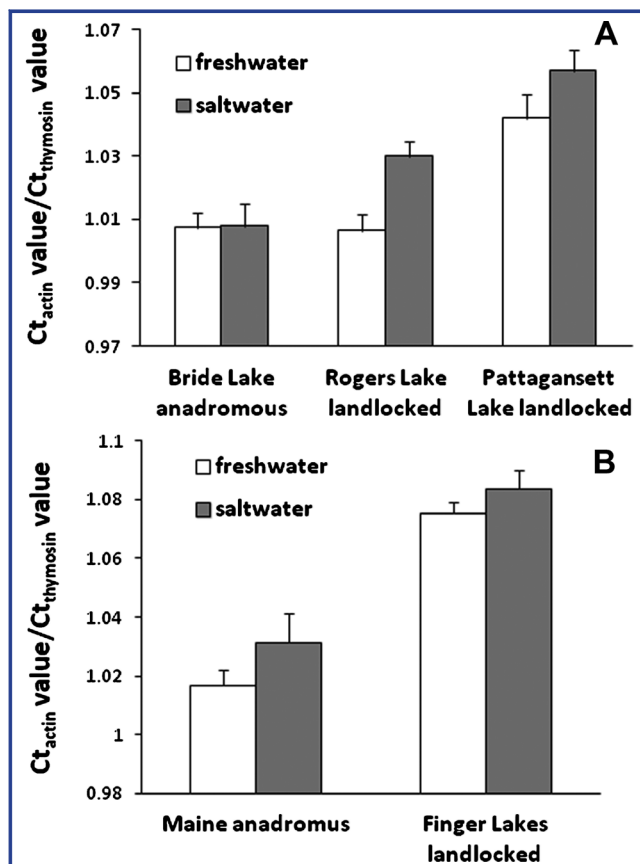


Figure 2. β-Thymosin-12 transcriptional activity during (A) short-term (2-way ANOVA,  $F = 4.953$ ,  $P = 0.038$ ,  $df_{Residual} = 20$ ) and (B) chronic salinity ( $F = 13.328$ ,  $P = 0.0022$ ,  $df_{Residual} = 16$ ).

(Smith, '85; Ihssen et al., '92). Alewives could also have been introduced into Lake Ontario unintentionally with attempted introduction of American shad, *Alosa sapidissima*, around 1870 (Bean, 1884), although this possibility was questioned by Smith (Smith, 1892). Alewives appeared next in Lake Erie in the 1931. Although alewives may have entered the Niagara River and eastern Lake Erie via the Erie Barge Canal, the consensus is that the most likely invasion route was from Lake Ontario to eastern Lake Erie via the Welland Canal (Scott and Crossman, '73a). This shipping canal, completed in 1824, bypassed the natural barrier of Niagara Falls and created a direct connection between Lake Ontario and Lake Erie. Alewives were next seen in Lake Huron in 1933, in Lake Michigan in 1949, and finally in Lake Superior in 1955, although they have never been abundant in the latter due most likely to its cold water temperatures (Smith, '85). Connecticut landlocked alewife populations were independently derived from an anadromous ancestral population anywhere from 5,000 to 300 years ago, according to genetic age estimates (Palkovacs et al., 2008). Dam construction during colonial development (ca. 300–500 years ago) is likely to account for the formation of these landlocked populations; dams are a ubiquitous feature of the Connecticut landscape, and there is no evidence for geological activity that can account for natural land locking within the time frame of genetic estimates (Palkovacs et al., 2008). Thus, independently derived Connecticut landlocked populations may be older than Great Lakes and Finger Lakes populations.

Given the evidence for serial successful invasion, our molecular results suggest that a  $\beta$ -thymosin-12 allele present at low frequency in ancestral anadromous alewife populations has been repeatedly selected in parallel, and in some cases fixed, in very recently and independently derived landlocked populations. Selection for the “freshwater”  $\beta$ -thymosin-12 allele, which appears to change  $\beta$ -thymosin-12 expression, may confer an advantage to alewives upon freshwater invasion. Given the role of  $\beta$ -thymosins in cell volume regulation, repeated selection for the “freshwater” allele among landlocked forms may result in improved freshwater osmoregulation, mediated through changes in  $\beta$ -thymosin-12 expression. Although largely correlative, this is the first study to suggest an adaptive genetic basis underlying the shift from marine to freshwater habitats in fishes, a shift that has facilitated widespread diversification.

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