MOLECULAR ECOLOGY

Molecular Ecology (2017) **26**, 831–848 doi: 10.1111/mec.13983

Transcriptomic imprints of adaptation to fresh water: parallel evolution of osmoregulatory gene expression in the Alewife

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Abstract

Comparative approaches in physiological genomics offer an opportunity to understand the functional importance of genes involved in niche exploitation. We used populations of Alewife (*Alosa pseudoharengus*) to explore the transcriptional mechanisms that underlie adaptation to fresh water. Ancestrally anadromous Alewives have recently formed multiple, independently derived, landlocked populations, which exhibit reduced tolerance of saltwater and enhanced tolerance of fresh water. Using RNA-seq, we compared transcriptional responses of an anadromous Alewife population to two landlocked populations after acclimation to fresh (0 ppt) and saltwater (35 ppt). Our results suggest that the gill transcriptome has evolved in primarily discordant ways between independent landlocked populations and their anadromous ancestor. By contrast, evolved shifts in the transcription of a small suite of well-characterized osmoregulatory genes exhibited a strong degree of parallelism. In particular, transcription of genes that regulate gill ion exchange has diverged in accordance with functional predictions: freshwater ion-uptake genes (most notably, the 'freshwater paralog' of Na^+/K^+-ATPase α-subunit) were more highly expressed in landlocked forms, whereas genes that regulate saltwater ion secretion (e.g. the 'saltwater paralog' of NKAα) exhibited a blunted response to saltwater. Parallel divergence of ion transport gene expression is associated with shifts in salinity tolerance limits among landlocked forms, suggesting that changes to the gill's transcriptional response to salinity facilitate freshwater adaptation.

Keywords: *Alosa pseudoharengus*, functional genomics, gene expression, gill, ion regulation, Na^+/K^+ATPase α1 paralogs, osmoregulation, RNA-seq

Received 26 January 2016; revision received 15 November 2016; accepted 18 November 2016

Introduction

Advances in sequencing offer powerful ways to identify the functional role of genes involved in niche exploitation among nonmodel species (Stapley *et al.* 2010), promising to yield insight into the mechanistic basis of adaptation (Whitehead 2012; Cheviron *et al.* 2014; Velotta *et al.* 2016). For example, linking variation in genome-wide transcriptional regulation to physiological functions and fitness in a comparative framework offers a nuanced understanding of the mechanisms of adaptive evolution (Whitehead *et al.* 2012). Populations

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of aquatic organisms that have invaded novel salinity habitats are well suited for studies integrating functional genomics and physiology in an adaptive context: fresh water (FW) and seawater (SW) represent opposing physiological challenges, and adaptation to a novel salinity requires evolutionary changes to well-characterized osmoregulatory systems and their underlying molecular machinery (Scott *et al.* 2004; Scott & Schulte 2005; McCairns & Bernatchez 2010; Lee *et al.* 2011; Whitehead *et al.* 2011, 2012; DeFaveri & Merilä 2014; Kozak *et al.* 2014; Velotta *et al.* 2014, 2015; Brennan *et al.* 2015).

For teleost fishes, maintenance of osmotic balance in FW or SW is accomplished by distinct biochemical mechanisms that regulate the exchange of Na^+ and Cl^- ions with the environment (McCormick & Saunders 1986; Evans *et al.* 2005): FW animals actively take in Na^+ and Cl^- ions that are lost passively from a dilute environment (ion uptake), while SW animals must secrete excess salt accumulated in the body (ion secretion). These osmoregulatory processes take place at a variety of organs, most prominently the gills. Gene expression pathways at the gill that regulate acclimation to salinity (e.g. cell volume regulation, cell stabilization, immediate early signal transduction) are accordingly targets of selection during adaptation to new salinity regimes (Whitehead *et al.* 2011, 2012; Kozak *et al.* 2014; Taugbøl *et al.* 2014; Brennan *et al.* 2015). Moreover, genome-wide surveys of allele frequency variation between FW and SW forms of several teleosts suggest that salinity acclimation genes are under selection in FW, including Na^+/K^+-ATPase, an ion pump that powers transmembrane transport of ions (Hohenlohe *et al.* 2010; DeFaveri *et al.* 2011; Shimada *et al.* 2011; Jones *et al.* 2012). What is not yet clear is whether expression of these loci repeatedly evolves in the transition to novel FW habitats. This is crucial for our understanding of the mechanistic basis of osmoregulatory evolution, as it will yield insight into the genes and pathways that are predictably favoured by natural selection.

We explored the extent of repeated evolution of transcriptome-wide changes associated with transitions to a novel osmotic environment in multiple populations of Alewife (*Alosa pseudoharengus*). Alewives, which belong to a predominantly marine family (Clupeidae; Nelson 2006), exist in two distinct life history forms: an ancestrally anadromous form that migrates between SW and FW, and a landlocked form that is restricted to FW. In the northeastern United States, dams constructed during American-colonial development (*c.* 300–400 years ago) are thought to have landlocked populations as multiple independent isolates from a single genetically homogenous anadromous population (Palkovacs *et al.* 2008).

Previous research suggests that trade-offs in osmoregulation follow the transition to FW in landlocked Alewives; they are more tolerant of FW than anadromous Alewives, and are less tolerant of—and have reduced osmotic balance in—SW (Velotta *et al.* 2014, 2015; Fig. S1, Supporting information). Changes in the response of several molecular pathways have evolved in concert with changes in osmoregulation, including lowered activity in the gill of the active cation transporter Na^+/K^+-ATPase (NKA), reduced transcription of several ion transporters that facilitate SW ion secretion (Velotta *et al.* 2014, 2015) and reduced expression of β-thymosin (a cytoskeletal organizer; Michalak *et al.* 2014). Evolutionary divergence in gill transcriptome expression has been found between wild-captured Alewives from an anadromous Atlantic Ocean population and a landlocked population from Lake Michigan (Czesny *et al.* 2012), reflecting adaptation or acclimatization to different salinities.

In the present study, we examined the gill transcriptomes of FW- and SW-acclimated Alewives from an anadromous and two independently derived landlocked populations in order to elucidate the mechanisms underlying evolutionary divergence in osmoregulation and whether or not these mechanisms evolve in parallel. The analyses were conducted with three objectives, in which we (i) sequenced, de novo-assembled and annotated the Alewife gill transcriptome; (ii) performed RNA-seq (Wang *et al.* 2009) and differential expression analyses to identify how gill transcription is influenced by salinity acclimation and/or life history form divergence (Fig. 1A) and to characterize biological functions that have differentiated; (iii) conducted a targeted analysis of life history form divergence in transcription among genes with known roles in gill osmoregulatory function. We predicted that expression of genes involved in acclimation to salinity would largely differentiate in parallel in the landlocked populations.

Gill ion regulation is accomplished by a suite of well-characterized proteins that function in transmembrane and paracellular ion exchange. An understanding of how the genes that underlie these functions evolve in novel salinity environments will yield insight into the mechanistic basis of osmoregulatory adaptations. As such, we made explicit predictions for a suite of target genes involved in gill ion regulation (Fig. 1B): SW ion secretion genes should exhibit reduced responses to salinity change in landlocked Alewives, while genes involved in FW ion uptake should exhibit an enhanced response. We found that although transcriptome-wide responses to salinity primarily evolved in nonparallel ways among independent landlocked populations, targeted analysis of ion regulation genes revealed a large degree of parallelism in the magnitude and direction of

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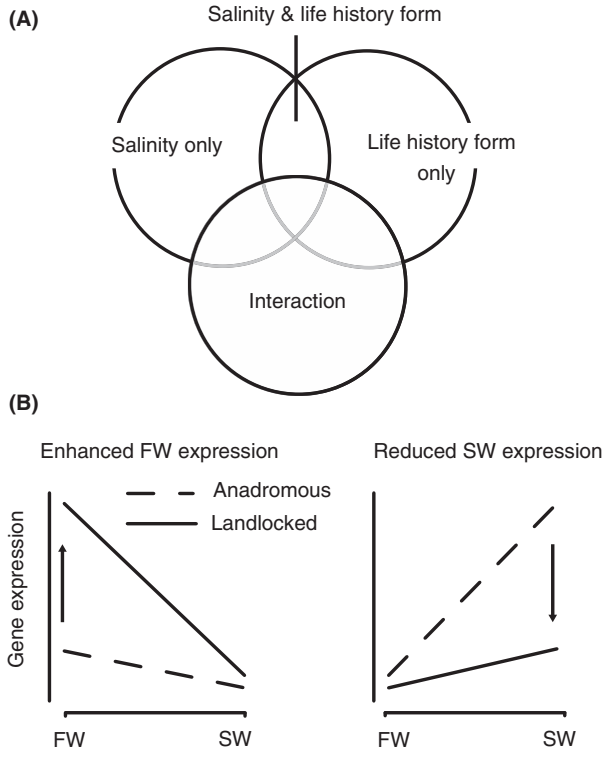

Fig. 1 Schematic representations of differential expression results and predictions. (A) Venn diagram of effects in a GLM testing for differences in transcriptome-wide gene expression between salinity treatments, Alewife life history forms and their interaction. Significant transcripts were categorized according to results of the model: salinity only, life history form only, salinity and life history form or a salinity × life history form interaction. (B) Plots of predicted patterns of differentiation among ion transport genes. Dashed lines indicate anadromous reaction norms; solid lines indicate landlocked reaction norms. The direction of the arrow represents the direction of predicted differentiation in landlocked compared to anadromous life history forms. Enhanced FW expression model: landlocked life history form exhibits greater upregulation in FW than anadromous life history form. Reduced SW expression model: landlocked life history form exhibits reduced upregulation in SW compared to anadromous life history form. [Colour figure can be viewed at wileyonlinelibrary.com]

transcriptional evolution. Our results suggest that changes to the regulation of a small suite of well-known osmotic effectors underlie repeated evolution of osmoregulatory function.

Materials and methods

Animals and experimental procedures

Animals used in this study were subjects in a 2-week salinity challenge experiment previously published in Velotta *et al.* (2015). For the analyses in this study, we sequenced the gill transcriptomes of specimens from the FW (0 ppt) and SW (35 ppt) treatments (described below) and the final 2-week time point. Survival rates during this experiment varied with population and salinity treatment. Most notably, landlocked Alewife survival was 10–40% higher in FW and 5–20% lower in SW compared to anadromous Alewives. Relevant survival curves from Velotta *et al.* (2015) are redrawn in Fig. S1 (Supporting information) for clarity. Plasma osmolality of anadromous and landlocked Alewives was equivalent after 2-week exposures, suggesting that all populations acclimated (Velotta *et al.* 2015).

Young-of-the-year anadromous and landlocked Alewives were captured from their natal lakes in Connecticut (CT) in October 2011 using a purse seine. Anadromous Alewives were collected from Bride Lake (hereafter A-Bride) in East Lyme, CT, and landlocked Alewives were collected from Pattagansett Lake (hereafter L-Pattagansett; East Lyme, CT) and Rogers Lake (hereafter L-Rogers; Old Lyme, CT). Alewives were transported to the Conte Anadromous Fish Research Center (United States Geological Survey; Turners Falls, Massachusetts) in 190-L circular containers at 1 ppt salinity with aeration. We held fish in the laboratory at 1 ppt for 1 day, after which salinity was decreased to 0.5 ppt (rearing salinity). While rearing salinity has a higher concentration of solutes than the lake water from which they were collected (0–0.1 ppt; Velotta *et al.* 2015), such slightly elevated salinity minimizes mortality from pathogens (Stanley & Colby 1971; J. Velotta, S. McCormick, E. Schultz, personal observation). We segregated Alewives by site and held them in separate 1200-L recirculating tanks fitted with charcoal filtration systems for one month prior to experimentation. Fish were maintained between 14.5 and 16 °C water temperature, kept on an ambient photoperiod and fed ad libitum (Biotrout; Bio-Oregon, Westbrook, ME, USA). All animals were handled in accordance with the University of Connecticut's Institutional Animal Care and Use Committee (protocol A09-024).

Laboratory-acclimated Alewives from each site were subjected to replicate treatments of low-ion FW or full-strength SW. Both FW and SW treatments represented a considerable osmoregulatory challenge, as evidenced by excursions in plasma osmolality (mOsmol/kg) immediately after transfer, relative to levels at rearing salinity (Velotta *et al.* 2015). The FW treatment (0 ppt; mean conductivity = 19.9 ± 6.8 μS) was prepared by running filtered, dechlorinated tap water through a resin-filled cartridge (Culligan International Company, Rosemont, IL, USA). FW tanks were buffered with 0.2 ppm calcium carbonate (mean pH 6.4 ± 0.4). The SW treatment (35 ppt) was prepared by dissolving artificial sea salt (Crystal Sea Marine Mix, Marine Enterprises

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International, Baltimore, MD, USA) in filtered, dechlorinated tap water. Fish were fed to satiation once daily. Approximately 25 Alewives per site per salinity treatment were immediately transferred from rearing tanks to replicate 250-L recirculation oval tanks with charcoal filtration. After a 2-week exposure to FW and SW, we euthanized surviving Alewives and extracted gill tissue. Fish were euthanized in 250 mg/L tricaine methanesulphonate (MS-222; Argent, Redmond, WA, USA). Excised gill tissue was trimmed from the bone and then placed immediately in 1 mL of RNA*later* solution (Ambion; Life Technologies, Grand Island, NY, USA) at room temperature, incubated at 4 °C overnight and then stored at −20 °C.

Sequencing, de novo assembly and annotation of gill transcriptome (objective 1)

Massively parallel sequencing was conducted in January 2013 in order to quantify genome-wide transcriptional differences in the gills of FW- and SW-challenged anadromous and landlocked Alewives. Gill tissue from three individuals per population per salinity treatment was used for library preparation (18 individual libraries in total). We extracted gill RNA prior to sequencing using Trizol reagent following the manufacturer's instructions (Ambion, Life Technologies). Using Illumina's TruSeq RNA sample preparation kit (FC-122-1001/1002), mRNA from 1 μg of total RNA with RIN ≥8.0 was converted into a library of template molecules suitable for subsequent cluster generation and single-end read sequencing with Illumina HiSeq 1000. The libraries generated were validated using Agilent 2100 Bioanalyzer and quantitated using Quant-iT dsDNA HS Kit (Invitrogen) and qPCR. Individually indexed cDNA libraries were pooled, clustered onto a flow cell using Illumina's TruSeq SR Cluster Kit v3 (GD-401-3001) and sequenced to 101 cycles using two TruSeq SBS Kit -HS (FC-401-1002). Samples were randomized across six lanes of sequencing in order to minimize lane effects (Marioni *et al.* 2008). One library (A-Bride site, 0 ppt treatment) preparation failed, resulting in a total of 17 sequenced libraries.

We conducted a separate sequencing run on gill tissue pooled from three wild-caught L-Pattagansett fish (collected in September 2009) using a Roche 454 GS FLX Titanium sequencer (454 Life Sciences, Branford, CT, USA), which provides longer reads than Illumina. These data were included to improve the quality of full-length reference transcriptome assembly. We used an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) to extract total gill RNA for 454 sequencing (A260/A280 >1.8 as measured by spectrophotometry; NanoDrop; Thermo Scientific, Waltham, MA, USA), and then DNase-treated samples using a TURBO DNA-free kit (Life Technologies). mRNA selection was performed using Ambion's Poly(A)Purist MAG kit (Thermo Fisher Scientific, Waltham, MA, USA) with a minimum of 30 mg of RNA per individual used.

Low-quality sequenced reads (Phred-scaled quality score <35 and minimum read length of 45 bp) were filtered from Illumina and 454 libraries with Sickle (Joshi and Foss 2011), and adaptor sequences were removed using the FASTX-Toolkit. Because individuals were pooled during transcriptome assembly, we employed conservative quality control thresholds in order to minimize the potential influence of sequence error variation. Two Illumina libraries yielded low read quality (both from L-Rogers site, 35 ppt treatment; Table S1, Supporting information), resulting in a total of 15 usable libraries (1–3 individuals per salinity per population). We in silico-normalized each library prior to assembly using the *insilico_read_normalization.pl* function in Trinity (Haas *et al.* 2013). Quality control of the reads and normalization yielded approximately 230 million Illumina reads and 350 000 454 reads. Trimmed read lengths averaged 68 bp for Illumina libraries and 252 bp for the 454 library (Fig. S2, Supporting information). Lower-than-average Illumina reads lengths (<100 bp) reflect conservative quality control measures.

We de novo-assembled separate transcriptomes for Illumina and 454 data, which we then clustered into a single assembly. To assemble Illumina reads, we used two publically available and top-performing short-read transcriptome assembly packages: Trinity (minimum contig length of 350 bp, single-end reads; Grabherr *et al.* 2011) and SOAPdenovo (minimum contig length of 350 bp, single-end reads; Xie *et al.* 2014). We assembled the 454 reads using Mira, an algorithm that is optimized for longer reads and lower sequencing depth. Trinity and SOAPdenovo assemblies were clustered with the Mira assembly using the UCLUST utility from the USEARCH package (95% identity and 90% coverage), producing two independent transcriptomes that were similar in terms of transcript number and length (Table S2; Fig. S3, Supporting information). To compare assemblies, we annotated (UBLAST package in USEARCH; Edgar 2010) each against the REFSEQ protein database (NCBI; weak *E*-value of 0.0001 and strong *E*-value of 1e-9). Based on this comparison (Table S2, Supporting information), we used the Trinity/Mira hybrid assembly as a reference for RNA-seq analysis.

We used ENTAP (https://github.com/SamGinzburg/WegrzynLab) to assign functional significance to transcripts. The ENTAP software package is a comprehensive pipeline for nonmodel transcriptome annotation. The first portion performs similarity searches against select public repositories via USEARCH's UBLAST package, with

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BLASTX equivalent *E*-values of 10^{-9} for strict matches and 10^{-4} for weak matches (Edgar 2010). Two databases were selected for comparison: NCBI's curated REFSEQ protein and NCBI's nonredundant (nr) protein database. We searched for matches with all species represented in these databases in order to maximize the number of annotated Alewife transcripts. Insect and bacterial contaminants were identified by sequence alignment and filtered from the results. XML files with optimized similarity search matches were generated as input to BLAST2GO and used to perform Gene Ontology term assignment for the Biological Process and Molecular Function categories (Conesa *et al.* 2005). All terms were normalized to BLAST2GO level two to improve comparisons. Annotated transcripts were classified as informative, unknown or unaligned: (i) informative transcripts are those with a significant alignment to a functionally characterized protein; (ii) unknown transcripts align to a protein with an unknown function; and (iii) unaligned transcripts are those without any significant alignments.

RNA-seq and functional enrichment analyses (objective 2)

We conducted an RNA-seq analysis (Wang *et al.* 2009) in order to determine whether gill transcript abundance was affected by salinity, life history form or both. Trimmed, non-normalized reads from individual Illumina libraries were mapped to the reference transcriptome using BOWTIE (Langmead *et al.* 2009). Across the 15 libraries, an average of 87% of reads mapped to at least one transcript in the assembly. We found substantial heterogeneity among populations in the proportion of transcripts to which reads mapped (Table S3, Supporting information). Reads from A-Bride mapped to 52% of assembled transcripts in the transcriptome, compared to 85% mapped from L-Pattagansett and 97% from L-Rogers. This discrepancy in mapping success may have arisen because of a population difference in the number of genes that are expressed and/or because of heterogeneity in post-quality-control library sizes. Because low read counts are a significant source of measurement error in differential expression analyses (Robinson & Smyth 2007), we filtered the transcriptome, retaining transcripts for which at least one individual from each combination of population and salinity had at least one mapped read. This is a conservative approach, because it effectively ascribes all mapping bias to library size effects rather than to potentially real patterns of gene expression.

We performed RNA-seq (Wang *et al.* 2009) and differential expression analysis using the filtered gill transcriptome as a reference. Expression values of each transcript for each Illumina library were computed from the results of the BOWTIE alignments using RSEM with default parameters via the *align_and_estimate_abundance.pl* function in the Trinity pipeline. We used the Bioconductor package EDGER (Robinson & Smyth 2007; Robinson & Oshlack 2010; Robinson *et al.* 2010) to assess differences in transcript abundance between life history forms and salinity treatments. First, the function *calcNormFactors* was used to normalize read counts among libraries. Model dispersion for each transcript was estimated separately using the function *estimateGLMTagwiseDisp* (McCarthy *et al.* 2012). EDGER analysis was performed in R (version 3.1.0).

Differences in normalized transcript abundance levels were tested using a generalized linear model (GLM), in which $\log_2$-transformed transcript abundance was the response variable, and factors included salinity, life history form and their interaction. GLM log-likelihood ratio tests (LRTs) were performed to assess significant differences in transcript abundance. For the effect of salinity, SW expression was compared to FW. For the life history form and interaction effects, A-Bride was compared to L-Pattagansett and L-Rogers separately. This approach was implemented to facilitate direct comparison between anadromous and each landlocked site. We controlled for multiple testing by enforcing a genome-wide false discovery rate (*q*-value) of 0.05 (Benjamini & Hochberg 1995). Within this framework, differentially expressed transcripts were classified as (Fig. 1A): (i) salinity only: transcripts exhibiting a significant salinity effect and no other effects; (ii) life history form only: transcripts exhibiting a significant life history form effect and no other effects; (iii) salinity and life history form: transcripts exhibiting both main effects, and not their interaction; (iv) interaction: transcripts exhibiting a salinity × life history form interaction. Among effect categories 2–4, we used Venn diagrams to identify transcripts significantly differentiated in both life history form contrasts (A-Bride vs. L-Pattagansett and A-Bride vs. L-Rogers). Among commonly differentially expressed transcripts, we correlated the $\log_2$ fold-changes in expression between A-Bride and each landlocked site using Pearson's correlations (*cor.test* function in R) to assess whether both landlocked sites were differentiated in the same magnitude and direction. To complement the GLM analysis, we performed multivariate ordination of $\log_2$-transformed and normalized transcript abundance via principal components analysis (PCA). PCA included only those transcripts exhibiting a life history form effect (categories 2–4 above) in order to assess whether differentiated expression patterns clustered among landlocked forms, reflecting parallelism. PCA was performed using the *prcomp* function in R with scaling.

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Enrichment analysis was used to identify biological functions that were overrepresented among differentially expressed transcripts. For this analysis, we used Gorilla (Eden *et al.* 2009), which implements hypergeometric testing to compare the frequency of Gene Ontology (GO) terms associated with genes in a target set to the frequency of GO terms in the background set (the transcriptome). We identified three target sets for our analysis: (i) transcripts exhibiting salinity-only effects; (ii) the combined set of transcripts exhibiting any life history form level differentiation (life history form only, salinity and life history form, interaction effects); and (iii) the combined set of transcripts commonly differentiated between life history form contrasts. We used the filtered gill transcriptome as the background set. In contrast to the functional annotation described in objective 1 above, annotation of GO terms for the enrichment analysis exclusively used the mouse (*Mus musculus*) protein database (via USEARCH, with an *e*-value of 1*e*-5, and retaining best hits; PYTHON scripts at https://github.com/SamGinzburg/WegrzynLab); this reduced the number of sequences that were assigned GO terms, but increased the number of GO terms represented. We examined GO terms for nominal significance ($P < 0.05$) and also applied a more conservative standard via FDR correction ($q < 0.05$) for multiple testing.

Differentiation in osmoregulatory genes (objective 3)

We included objective 3 to assess the extent of common patterns of evolutionary change in the expression of osmoregulatory genes. First, we screened the functionally annotated gill transcriptome for putative osmoregulatory genes (identified from: Bentley 2002; Evans *et al.* 2005; Hwang & Lee 2007; Evans 2008, 2010; Kültz 2012; Hiroi & McCormick 2012; Hsu *et al.* 2014) representing four GO processes: ion transport, osmosensing, tight junction regulation and water transport. We then determined which of these osmoregulation transcripts were differentially expressed, as revealed in RNA-seq (objective 2). We compared expression values between A-Bride and both landlocked populations using post hoc GLMs (implemented in EDGER as above). Population comparisons were made separately within FW and SW to isolate population-level effects within salinity treatments. We tested the predictions that SW genes would evolve reduced expression and FW genes would evolve enhanced expression (Fig. 1B) among transcripts identified with ion transport, tight junction regulation and water transport functions, because (unlike osmosensing genes) they exert predictable roles in FW or SW osmoregulation. Finally, we performed PCA of osmoregulatory gene expression patterns to test whether differentiation between life history forms reflects parallel evolutionary changes, as indicated by clustering of landlocked profiles.

Results

De novo assembly and functional annotation (objective 1)

The de novo Trinity/Mira hybrid assembly consisted of 221 474 transcripts and an N50 (a weighted median) contig size of 1130 bp. Filtration to eliminate mapping bias and contaminant sequences yielded a total of 76 657 transcripts, which consisted of 35 481 unique genes. The mean length of the transcripts was 1480 bp and that of the N50 was 1994 bp. The results of the combined annotation performed with ENTAP resulted in a functional annotation rate of 46%. We identified a total of 34 844 informative transcripts, 673 unknown transcripts and 41 140 unaligned transcripts. Unaligned transcripts may be those that have rapidly and dramatically differentiated in Alewives and related fishes, and may be useful in future studies assessing evolutionary divergence in this taxon. The top five species with which informative and unknown transcripts aligned were all fishes: *Astyanax mexicanus* (23%), *Danio rerio* (20%), *Esox lucius* (14%), *Stegastes partitus* (6%) and *Lepisosteus oculatus* (5%).

We associated 12 383 transcripts with at least one GO term. Of those transcripts, 10 415 had at least one Molecular Function and 10 056 had at least one Biological Process term, yielding a total of 40 004 Biological Process (Fig. 2A) and 25 646 Molecular Function (Fig. 2B) associations. The most frequent Biological Process associations were cellular process (25.0%), metabolic process (22.2%) and single-organism process (22.2%), and the most frequent Molecular Function associations were binding (50.0%) and catalytic activity (44.6%). Transporter activity, the third most frequently associated Molecular Function, was only 3.8% of the associations.

RNA-seq and functional enrichment analyses (objective 2)

A GLM identified a total of 6034 transcripts that were differentially expressed between salinities, life history forms and/or their interaction ($q < 0.05$). We found 918 transcripts to be significantly differentiated between FW and SW (LRT; $q < 0.05$), of which 390 exhibited a salinity-only effect (Fig. 1) and no other main effect or interaction. For the main effect of life history form, 1297 and 732 transcripts were significantly differentially expressed between A-Bride and L-Pattagansett and L-Rogers, respectively (LRT; $q < 0.05$). Of these, 940 and

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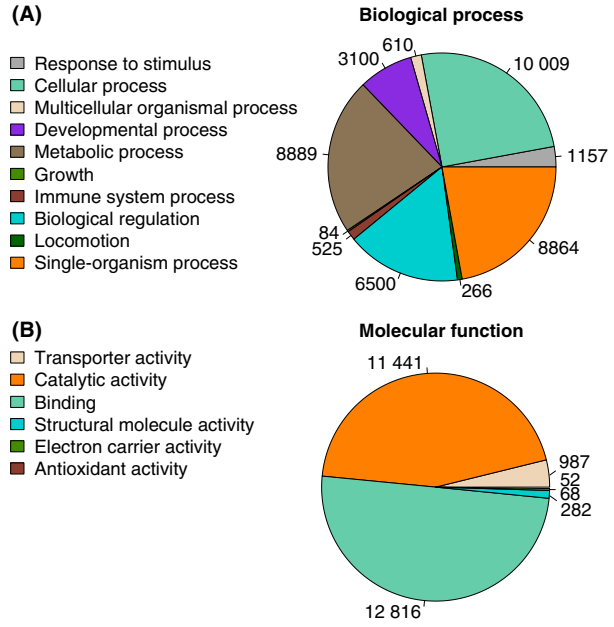

Fig. 2 Functional annotation of the Alewife gill transcriptome. The number of transcripts assigned to Gene Ontology (GO) Biological Process and Molecular Function terms is shown. All GO terms were normalized to level 2. [Colour figure can be viewed at wileyonlinelibrary.com]

348 exhibited only a life history form (Fig. 1) and no other effects. Thirteen percentage of these transcripts were commonly differentiated in both life history form contrasts (A-Bride vs. L-Pattagansett and A-Bride vs. L-Rogers; Fig. 3A). However, the majority of life history form-affected transcripts (69%) were exclusively differentiated between A-Bride and L-Pattagansett (Fig. 3A). A small number of all differentially expressed transcripts (~1%) exhibited both salinity and life history form main effects, of which 41% were differentiated between both life history form contrasts (Fig. 3B). We detected 195 and 4334 transcripts significant for a salinity–life history form interaction effect (LRT; $q < 0.05$) between A-Bride and L-Pattagansett and L-Rogers contrasts, respectively. Only two percentage of these interaction transcripts were differentiated commonly in both life history form contrasts, while 96% (about one-third of all differentially expressed genes) were differentiated exclusively between A-Bride and L-Rogers (Fig. 3C). For all commonly differentiated transcripts (overlapping regions of Venn diagrams; Fig 3), $\log_2$ fold-changes in expression between the anadromous and either landlocked population were highly correlated (life history form only: $r^2 = 0.98$; $P < 0.001$; salinity and life history form: $r^2 = 0.71$, $P < 0.001$; salinity × life history form: $r^2 = 0.97$; $P < 0.001$), indicating that life history form divergence in transcription generally occurs in the same direction and to the same degree in both landlocked sites (Fig. 3).

In a PCA of life history form differentiated genes, the first two axes explained 33% and 16% of total variance, respectively. The first six axes cumulatively accounted for 80% of the variance in gene expression. Transcript expression patterns clustered by life history form along PC2, and by salinity within population along PC1 and PC2 (Fig. 4A). Expression patterns of the single L-Rogers fish subjected to 35 ppt were divergent from all

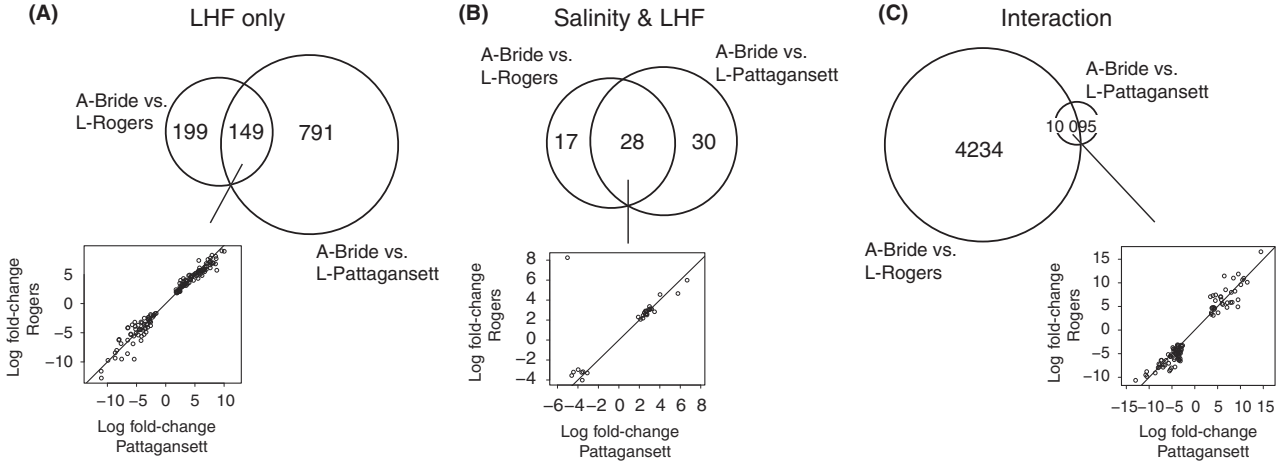

Fig. 3 Venn diagrams depicting the number of transcripts uniquely and commonly differentiated between Alewife life history form contrasts, shown separately for (A) life history form only, (B) salinity and life history form and (C) interaction effects (see Fig. 1A). Values represent the number of transcripts significantly ($q < 0.05$) differentiated according to a GLM (see 'Materials and methods'). Plots of $\log_2$ fold-changes in gene expression are shown for transcripts commonly differentiated between life history form contrasts. Diagonal lines represent 1:1 relationship. Lower plots demonstrate concordance in direction and magnitude of gene expression change among commonly differentiated transcripts.

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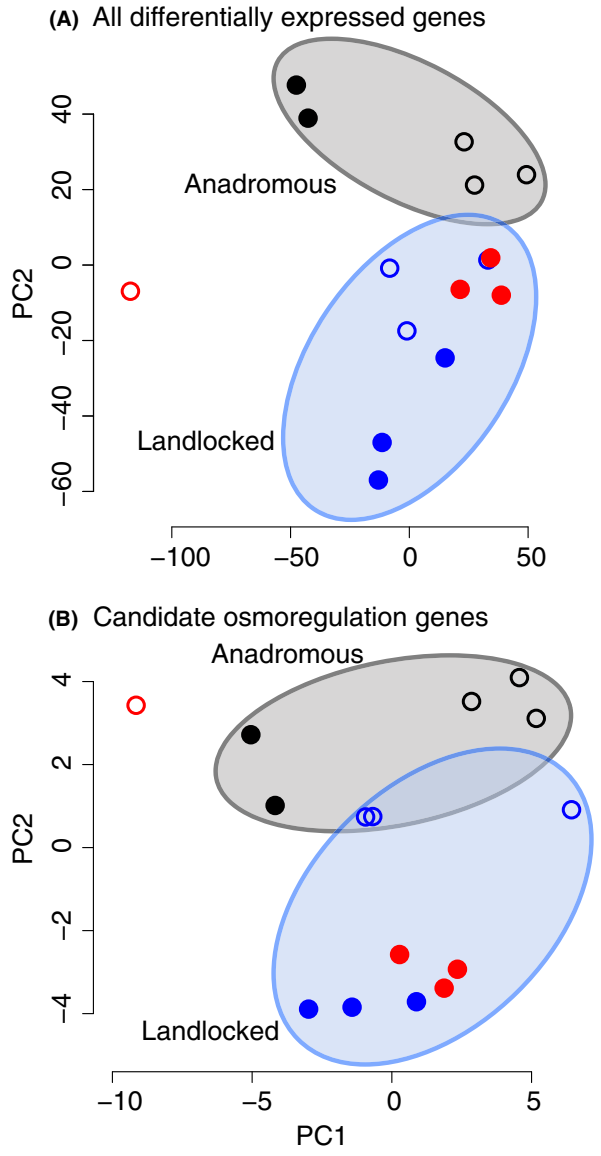

Fig. 4 Principal components analysis (PCA) of gene expression profiles for (A) all genes exhibiting life history form differences in expression (n = 5643) and (B) candidate osmoregulation genes (n = 41). A-Bride is coloured in black, and L-Pattagansett and L-Rogers are coloured in blue and red, respectively. Closed circles represent animals challenged in FW; open circles SW. Among both sets of differentially expressed genes, anadromous Alewives cluster separately from both landlocked populations. [Colour figure can be viewed at wileyonlinelibrary.com]

others. Moreover, expression patterns of landlocked fish in response to FW did not map together: responses of L-Rogers to FW were more distant from L-Pattagansett responses to FW, than to L-Pattagansett responses to SW.

Enrichment analysis identified a suite of functions overrepresented among transcripts with a conserved response to salinity, as well as those differentiated between life history forms. The portion of the transcriptome exhibiting a salinity-only effect was enriched in terms that are associated with osmoregulation. GO process term 'sodium ion export from cell' and component terms 'ATPase-dependent transmembrane transport complex' and 'sodium: potassium-exchanging ATPase complex' were nominally significant ($P < 0.001$; $q > 0.05$; Table S4, Supporting information). The life history form differentiated portion of the transcriptome was nominally enriched ($P < 0.001$; $q > 0.05$; Table S5, Supporting information) in processes reflecting post-transcriptional modification of RNA, translation and metabolic functions. Transcripts that were commonly differentiated in both life history form contrasts were nominally enriched for ion transport, including GO processes 'transepithelial ammonia transport' and 'potassium ion transport,' as well as the GO component 'cell junction' (Table S6, Supporting information).

Differentiation in osmoregulatory genes (objective 3)

We identified 48 transcripts with putative roles in gill osmoregulation whose expression varied with salinity, life history form and/or their interaction. Note that multiple transcripts of a single putative osmoregulatory gene (representing paralogs or isoforms) were reported when present and were numbered consecutively (Tables 1–5). Expression of 11 of these 48 transcripts varied only with salinity (Table 1), seven of which were related to ion transport and six of which were variants of the gene for the α1 subunit of NKA (*atp1a1*). Four of the six genes and all of the *atp1a1* variants were expressed more in FW than in SW. Expression of nine of the 48 transcripts varied only with life history form (Table 2), and the expression of the remaining 28 varied with life history form and salinity (Tables 3 and 4). In a PCA of osmoregulation genes (Fig. 4B), the first two axes explained 43% and 23% of the variance in gene expression, respectively. The first four axes accounted for over 80% of the cumulative variance in expression. Except for the divergent responses of the SW-challenged L-Rogers individual, life history forms separated along PC2, and responses to salinity separated along PC1 and PC2. Expression patterns of landlocked fish in FW grouped together.

The majority of differentially expressed osmoregulatory transcripts perform ion transporting (22 transcripts) or osmosensing (17 transcripts) functions, while several are involved in tight junction regulation (7 transcripts) and water transport (2 transcripts). Genes for ion transport proteins that perform SW ion secretion and FW ion-uptake functions exhibited salinity and life history form (Table 3), as well as interaction, effects (Table 4). Differentially expressed SW ion secretion genes

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Table 1 Candidate osmoregulatory transcripts with a significant salinity effect only. Table presents results of GLM testing for effects on gene expression. Putative genes with multiple unique transcripts (numbered consecutively), possibly representing paralogs or isoforms, appear in multiple rows. For each transcript, table indicates the gene symbol, its putative function, $\log_2$ fold-change (logFC) in expression between salinities, and raw *P* values. $P < 0.05$ is presented in boldface

Putative gene	Symbol	Function	logFC	*P*
aquaporin 3	*aqp3*	Water transport	−3.7	**<0.001***
ATPase, Na^+/K^+ transporting, alpha 1 transcript 1	*atp1a1*	Ion transport	−8.5	**<0.001***
ATPase, Na^+/K^+ transporting, alpha 1 transcript 2	*atp1a1*	Ion transport	−9.0	**<0.001***
ATPase, Na^+/K^+ transporting, alpha 1 transcript 3	*atp1a1*	Ion transport	−8.5	**<0.001***
ATPase, Na^+/K^+ transporting, alpha 1 transcript 4	*atp1a1*	Ion transport	−3.1	**<0.001***
ATPase, Na^+/K^+ transporting, alpha 1 transcript 5	*atp1a1*	Ion transport	−2.0	**<0.001***
ATPase, Na^+/K^+ transporting, alpha 1 transcript 6	*atp1a1*	Ion transport	−7.0	**<0.001***
claudin 1	*cldn1*	Tight junction	−2.3	**<0.001***
interleukin 22 receptor, alpha 2	*il22ra2*	Osmosensing	−1.8	**<0.001***
mitogen-activated protein kinase 8 interacting protein 3	*mapk8ip3*	osmosensing	1.7	**<0.001***
solute carrier family 9 (Na^+/H^+ exchanger), member 2	*slc9a2*	Ion transport	2.8	**<0.001***

Positive logFC values represent higher expression in SW relative to FW.
*False discovery rate (FDR)-corrected *P* value (*q*) < 0.05.

Table 2 Candidate osmoregulatory transcripts with a significant life history form effect only. Table presents results of GLM testing for effects on gene expression. Putative genes with multiple unique transcripts (numbered consecutively), possibly representing paralogs or isoforms, appear in multiple rows. For each transcript, table indicates the gene symbol, its putative function, $\log_2$ fold-change in expression (logFC) between populations, and raw *P* values. $P < 0.05$ is presented in boldface

			A-Bride vs. L-Pattagansett		A-Bride vs. L-Rogers	
Putative gene	Symbol	Function	logFC	*P*	logFC	*P*
aquaporin 3	*aqp3*	Water transport	3.1	**0.001***	1.4	0.08
claudin 15	*cldn15*	Tight junction	−2.5	**<0.001***	−1.2	0.08
claudin 3	*cldn3*	Tight junction	1.5	**0.001***	1.0	**0.03**
claudin 4 transcript 1	*cldn4*	Tight junction	3.2	**0.01**	−4.4	**<0.001***
forkhead box I3	*foxi3*	Osmosensing	3.8	**<0.001***	3.7	**0.001***
insulin-like growth factor binding protein 1	*igfbp1*	Ion transport	3.4	**<0.001***	1.0	0.2
potassium inwardly-rectifying channel, subfamily J, member 1 transcript 1	*kcnj1*	Ion transport	−2.2	**<0.001***	−1.5	**0.01***
mitogen-activated protein kinase 13	*mapk13*	Osmosensing	1.8	**0.001***	2.0	**<0.001***
notch 1 transcript 1	*notch1*	Osmosensing	2.2	**0.001***	2.3	**<0.001***
Rhesus blood group-associated B glycoprotein	*rhbg*	Ion transport	−5.2	**<0.001***	−4.4	**<0.001***

Each landlocked site was compared to A-Bride separately in the GLM. Positive logFC values indicate higher expression in landlocked relative to anadromous population.
*False discovery rate (FDR) corrected *P*-value (*q*) < 0.05.

included NKA α1 (*atp1a1*), $Na^+/K^+/2Cl^-$ cotransporter (NKCC; *slc12a2*), cystic fibrosis transmembrane conductance regulator homolog (CFTR; *cftr*) and potassium inwardly-rectifying channels (K_{ir}; *kcnj1* and *kcnj2*). Differentially expressed FW ion-uptake genes included Na^+/Cl^- cotransporter (NCC; *slc12a3*), H^+ transporting V-ATPase beta subunit (VATP; *atp6vb1*) and anion exchanger 1 (AE1; *slc4a1*). We identified transcripts that perform gill osmosensing functions across the four categories of differential expression (Tables 1–4). Two transcripts of the water transport channel *aquaporin 3* (*aqp3*; Tables 1 and 2) and several unique tight junction regulation genes (*claudins*; Tables 1, 2 and 4) were identified.

Post hoc analysis revealed that the expression of genes involved in gill ion transport and tight junction regulation has diverged between anadromous and landlocked Alewives in ways predicted based on their function in osmoregulation (Fig. 1B). Seven transcripts with putative FW ion-uptake functions were life history form

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Table 3 Candidate osmoregulatory genes with significant salinity and life history form effects. Table presents results of GLM testing for effects on gene expression. Putative genes with multiple unique transcripts (numbered consecutively), possibly representing paralogs or isoforms, appear in multiple rows. For each transcript, table indicates the gene symbol, its putative function, $\log_2$ fold-change (logFC) in expression between life history forms, and raw *P* values. $P < 0.05$ is presented in boldface

			A-Bride vs. L-Pattagansett		A-Bride vs. L-Rogers	
Putative gene	Symbol	Function	logFC	*P*	logFC	*P*
ATPase, Na^+/K^+ transporting, alpha 1 transcript 7	*atp1a1*	Ion transport	3.00	**<0.001***	3.04	**<0.001***
ATPase, H^+ transporting, lysosomal V1 subunit B1	*atp6v1b1*	Ion transport	5.77	**<0.001***	4.66	**<0.001***
potassium inwardly-rectifying channel, subfamily J, member 1 transcript 2	*kcnj1*	Ion transport	−4.44	**<0.001***	−3.21	**<0.001***
potassium inwardly-rectifying channel, subfamily J, member 1 transcript 3	*kcnj1*	Ion transport	−3.98	**<0.001***	−2.96	**<0.001***
notch 1 transcript 2	*notch1*	Osmosensing	2.12	**<0.001***	2.07	**<0.001***

Each landlocked site was compared to A-Bride separately in the GLM. Positive logFC values indicate higher expression in landlocked relative to anadromous population. All transcripts in table also exhibit a significant salinity effect ($q < 0.05$, not shown).
*False discovery rate (FDR)-corrected *P* value (q) < 0.05.

differentiated (five ion transport and two tight junction transcripts). Six of these transcripts were consistent with the enhanced FW expression model, and all but one was differentiated between A-Bride and both landlocked populations in the same direction (Table 5). Two of the ion-uptake transcripts of the *atp1a1* gene (transcripts 7 and 8) were homologous to the 'FW' isoform of NKA α1 (NKA α 1a) in other fishes (Dalziel *et al.* 2014) and were named accordingly (NKA α1a transcripts 7 and 8; Table 5). FW expression of NKA α1a transcript 7 was sevenfold higher in both landlocked populations compared to A-Bride (Fig. 5A). FW expression of transcript 8 of NKA α1a was also higher in L-Rogers than in A-Bride, but was not higher in L-Pattagansett (Fig. 5B; Table 5). Unexpectedly, the gene for the anion exchanger AE1 (*slc4a1*) was upregulated in SW in A-Bride relative to both landlocked populations (Fig. 5C; Table 5). FW expression of NCC (*slc12a3*) was higher in landlocked Alewives than in A-Bride (Fig. 5D; Table 5). FW expression of VATP (*atp6v1b*) was higher in L-Pattagansett fish, but not L-Rogers fish, relative to A-Bride (Fig. 5E; Table 5). Finally, two tight junction genes (*cldn 3* and *cldn 8*) were expressed more highly in FW in landlocked Alewives than in A-Bride (Fig. 5F, G; Table 5).

Seven transcripts associated with SW ion secretion genes exhibited life history form differentiated expression (Fig. 6). All of these transcripts were expressed more highly in SW and were consistent with the reduced SW expression model (Fig. 1B; Table 5). One of these transcripts was analogous to NKA α1b, the 'SW' isoform of *atp1a1*, and was named as such (Dalziel *et al.* 2014; NKA α1b transcript 9; Table 5). In SW, expression of this transcript was significantly lower in both landlocked populations (Fig. 6A; Table 5). Two transcripts of *slc12a2*, the gene for NKCC, were expressed less in SW in both populations of landlocked Alewife than in A-Bride (Fig. 6B, C; Table 5). Both transcripts of *cftr* were expressed less in SW in both landlocked populations than in A-Bride (Fig. 6D, E, Table 5). SW expression of both transcripts of K_{ir} (*kcnj1*) was lower in L-Pattagansett, but not L-Rogers, compared to A-Bride (Fig. 6F, G; Table 5).

Discussion

In this study, we have demonstrated that colonization of FW has resulted in shifts in the response of the gill transcriptome to both fresh and saltwater. Our results suggest that while transcriptome-wide differentiation is primarily discordant among independent landlocked populations, evolved shifts in the transcription of candidate osmoregulatory genes exhibit a strong signal of parallelism. Among a suite of genes that function in ion transport and permeability across the gill, shifts in transcription diverged according to functional predictions (Fig. 1B): FW ion-uptake genes were more highly expressed in landlocked forms, while genes involved in SW ion secretion exhibited a limited response. Rapid, parallel divergence suggests that predictable shifts in gill gene regulation are repeatedly favoured by selection in the transition to FW. Divergence in gill ion transport gene expression is associated with divergence in osmoregulation, whereby landlocked forms exhibit enhanced FW tolerance and concomitant loss of SW tolerance (Velotta *et al.* 2014, 2015). These results suggest that evolutionary change in gill osmoregulation is a required early stage in the process of FW colonization.

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Table 4 Candidate osmoregulatory genes exhibiting a significant salinity × life history form interaction effect. Table presents results of GLM testing for effects on gene expression. Putative genes with multiple unique transcripts (numbered consecutively), possibly representing paralogs or isoforms, appear in multiple rows. For each transcript, table indicates the gene symbol, its putative function, $\log_2$ fold-change (logFC) in expression for the interaction term, and raw *P* values. $P < 0.05$ is presented in boldface

			A-Bride vs. L-Pattagansett		A-Bride vs. L-Rogers	
Putative gene	Symbol	Function	logFC	*P*	logFC	*P*
ATPase, Na^+/K^+ transporting, alpha 1 transcript 8	*atp1a1*	Ion transport	4.9	**<0.001***	1.1	0.3
bone morphogenetic protein 2 transcript 1	*bmp2*	Osmosensing	−5.5	**0.001**	−9.1	**<0.001***
bone morphogenetic protein 2 transcript 2	*bmp2*	Osmosensing	−0.7	0.4	−3.2	**0.001***
bone morphogenetic protein 3	*bmp3*	Osmosensing	−0.6	0.4	−3.4	**<0.001***
bone morphogenetic protein 4	*bmp4*	Osmosensing	−1.4	0.2	−3.7	**0.002***
bone morphogenetic protein 8a	*bmp8a*	Osmosensing	−0.3	0.6	−2.9	**0.001***
cystic fibrosis transmembrane conductance regulator transcript 1	*cftr*	Ion transport	−1.4	**0.04**	−2.9	**0.001***
cystic fibrosis transmembrane conductance regulator transcript 2	*cftr*	Ion transport	−1.6	**0.02**	−2.9	**0.001***
claudin 10	*cldn10*	Tight junction	−0.4	0.6	3.4	**0.001***
claudin 4 transcript 2	*cldn4*	Tight junction	1.3	0.06	3.2	**<0.001***
claudin 8	*cldn8*	Tight junction	−1.9	**0.05**	−4.0	**0.003***
FXYD domain-containing ion transport regulator 1	*fxyd1*	Ion transport	−0.2	0.9	4.3	**0.002***
FXYD domain-containing ion transport regulator 2	*fxyd2*	Ion transport	2.8	**<0.001**	6.2	**<0.001***
interleukin 17C transcript 1	*il17c*	Osmosensing	−4.1	**<0.001***	−1.2	0.3
interleukin 17C transcript 2	*il17c*	Osmosensing	0.0	0.9	6.4	**<0.001***
potassium inwardly-rectifying channel, subfamily J, member 2	*kcnj2*	Ion transport	−2.4	**0.02**	−4.1	**0.003***
mitogen-activated protein kinase-associated protein 1 transcript 1	*mapkap1*	Osmosensing	−1.1	0.1	−2.8	**0.002***
mitogen-activated protein kinase-associated protein 1 transcript 2	*mapkap1*	Osmosensing	−2.6	**0.01**	−4.8	**0.001***
notch 1 transcript 3	*notch1*	Osmosensing	−0.3	0.8	−3.9	**0.003***
notch 1 transcript 4	*notch1*	Osmosensing	−0.7	0.4	−3.5	**0.002***
serum/glucocorticoid-regulated kinase 2	*sgk2*	Osmosensing	−3.8	**<0.001***	−3.1	**0.002***
solute carrier family 12 ($Na^+/K^+/2Cl^-$ cotransporter), member 2 transcript 1	*slc12a2*	Ion transport	−3.2	**<0.001***	−6.1	**<0.001***
solute carrier family 12 ($Na^+/K^+/2Cl^-$ cotransporter), member 2 transcript 2	*slc12a2*	Ion transport	−2.7	**0.002**	−4.6	**<0.001***
solute carrier family 12 (Na^+/Cl^- transporter), member 3	*slc12a3*	Ion transport	−3.7	**0.002**	−5.8	**0.001***
solute carrier family 4 (anion exchanger), member 1	*slc4a1*	Ion transport	−2.3	**0.008**	−3.7	**0.001***
tumour necrosis factor receptor superfamily, member 11b	*tnfrsf11b*	Osmosensing	−0.1	0.9	3.9	**<0.001***

Each landlocked site was compared to A-Bride separately in the GLM. Positive logFC values indicate higher expression in landlocked relative to anadromous population.
*False discovery rate (FDR)-corrected *P* value (*q*) < 0.05.

Several thousand gill transcripts were differentially expressed between salinity treatments, life history forms or both and were classified into categories of differentiation (Fig. 1A): (i) salinity only, (ii) life history form only, (iii) salinity and life history form and (iv) salinity × life history form interaction. Hundreds of differentially expressed transcripts exhibited a salinity effect only, representing the set of genes that have a conserved role in acclimation to FW or SW. Several of these salinity-responsive genes function in ion and water transport and osmosensing (Table 1). Among life history form differentiated transcripts, expression profiles of landlocked populations clustered separately from the anadromous population in a PCA (Fig. 4A). The two landlocked populations, however, varied separately along PC2 (Fig. 4A), providing little support for parallel changes in gene expression. This is particularly evident in the single L-Rogers individual challenged in seawater, for which expression is divergent from all others. Moreover, for the majority of differentially expressed transcripts (95%), expression differences were detected between A-Bride and one, but not the other, landlocked population (Fig. 3), providing further support that independent colonizations of FW are marked by changes in a unique set of transcripts. Disparate changes in gene expression of the two landlocked populations are probably not reflective of local adaptation to water chemistry, as sites are nearly identical in salinity and

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Table 5 Evolution of the transcriptional response to osmotic challenge in ion transport genes. Table presents results of post hoc GLMs testing for population effects within each salinity treatment. For each transcript, table indicates the gene symbol and the $\log_2$ fold-change in expression (logFC) between populations. Each landlocked site was compared to A-Bride separately. Positive logFC values indicate higher expression in landlocked relative to anadromous population. Whether the differentiation in gene expression fits the predicted enhanced FW or reduced SW model (Fig. 1A), and whether the transcript is significantly differentiated in both population contrasts in the same direction (parallel), is reported

	logFC: Fresh water		logFC: Seawater				
Gene abbreviation	A-Bride vs. L-Pattagansett	A-Bride vs. L-Rogers	A-Bride vs. L-Pattagansett	A-Bride vs. L-Rogers	logFC: Salinity	Assigned model of differentiation	Parallel or nonparallel
atp1a1 (NKAα1a) transcript 7	3.0***	3.0***	5.2*	2.7*	−6.3***	Enhanced FW	Parallel
atp1a1 (NKAα1a) transcript 8	0.4	1.1**	5.2***	2.1	−8.2***	Enhanced FW	Nonparallel
atp1a1 (NKAα1b) transcript 9	0.9*	0.5	−2.0***	−3.1*	3.2**	Reduced SW	Parallel
atp6v1b1 (VATP)	5.8***	4.7***	1.2	0.3	4.8***	Enhanced FW	Parallel
cftr transcript 1	0.1	0.3	−1.3*	−2.6**	2.7***	Reduced SW	Parallel
cftr transcript 2	0.5	0.6	−1.1*	−2.3**	2.6***	Reduced SW	Parallel
cldn3	1.5***	1.0*	−0.3	−0.6	0.3	Enhanced FW	Parallel
cldn8	1.8**	1.8*	−0.1	−2.2	0.9	Enhanced FW	Parallel
knj1 (K_{ir}) transcript 2	−4.5***	−3.2***	−3.3***	−1.4	3.0***	Reduced SW	Nonparallel
kcnj1 (K_{ir}) transcript 3	−4.0***	−3.0***	−3.2***	−1.6	3.3***	Reduced SW	Nonparallel
slc12a2 (NKCC) transcript 1	1.7***	2.2***	−1.5**	−3.9***	3.3***	Reduced SW	Parallel
slc12a2 (NKCC) transcript 2	0.8	1.0*	−1.8***	−3.6***	2.7***	Reduced SW	Parallel
slc12a3 (NCC)	1.7*	1.4*	−2.0*	−4.3**	−0.5	Enhanced FW	Parallel
slc4a1 (AE1)	0.2	0.7	−2.0***	−3.0***	1.4*	None	Nonparallel

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

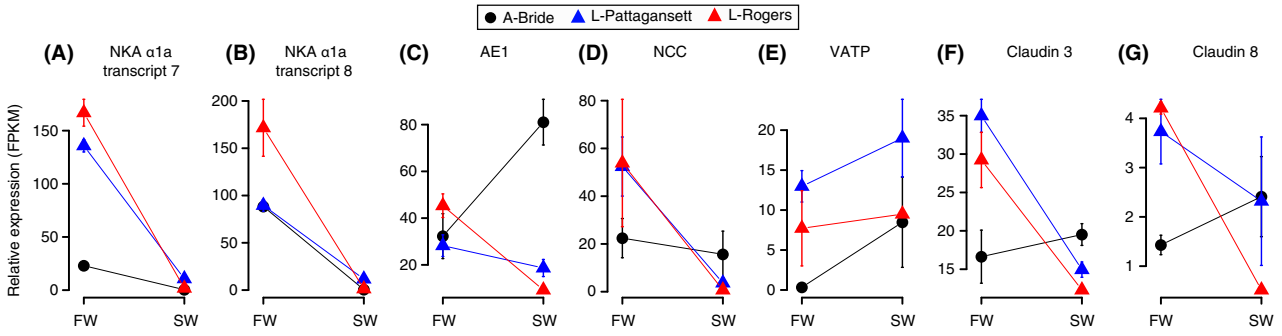

Fig. 5 Expression of ion-uptake genes in FW is generally enhanced in landlocked Alewives. TMM-normalized FPKM expression values are plotted (Robinson & Oshlack 2010). Protein names or abbreviations are listed for simplicity. NKA α1a: Na^+/K^+-ATPase (*atp1a1* gene) α1a; AE1 (*slc4a1* gene): anion exchanger 1; NCC (*slc12a3* gene): Na^+/Cl^- cotransporter; VATP (*atp61b1* gene): H^+ transporting V-ATPase β subunit; claudin 3 (*cld3* gene); claudin 8 (*cld8* gene). Results of post hoc GLMs testing for differences in gene expression are reported in Table 5. [Colour figure can be viewed at wileyonlinelibrary.com]

conductivity (Velotta *et al.* 2015). More likely, unique transcriptional changes are indicative of neutral evolutionary effects on gene regulation or genetic drift arising from small effective population size. Notably, expression differences were greatest between A-Bride and L-Rogers (Fig. 3C), which is consistent with evidence that L-Rogers has physiologically and genetically diverged more than L-Pattagansett from the anadromous ancestor (Palkovacs *et al.* 2008; Velotta *et al.* 2015). That the more genetically differentiated landlocked site also exhibits greater divergence in gene expression suggests that L-Rogers has been landlocked for a longer period of time. This result should be interpreted with caution because of limited replication in L-Rogers samples.

Despite the overwhelming signal of nonparallelism in gene expression, expression profiles of several hundred transcripts diverged concordantly from A-Bride in both landlocked populations (Fig. 3). It is particularly striking that transcripts whose expression has differentiated

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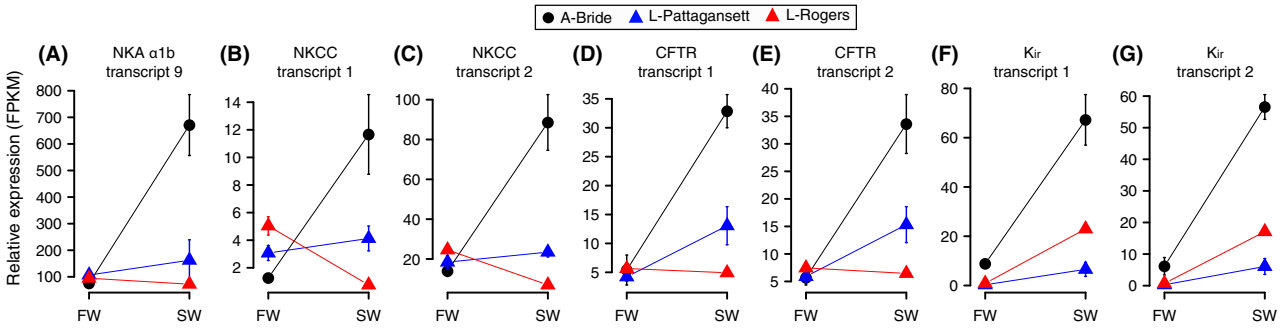

Fig. 6 Expression of ion secretion genes in SW is reduced in landlocked Alewives. TMM-normalized FPKM expression values are plotted (Robinson & Oshlack 2010). Protein names or abbreviations are listed for simplicity. NKA α1b: Na^+/K^+-ATPase (*atp1a1* gene) α1b; NKCC: $Na^+/K^+/2\ Cl^-$ cotransporter (*slc12a2* gene); CFTR: cystic fibrosis transmembrane conductance regulator (*cftr* gene); K_{ir}: potassium inwardly-rectifying channel, subfamily J, member 1 (*kcnj1* gene). Results of post hoc GLMs testing for differences in gene expression are reported in Table 5.

in both life history forms have done so in the same magnitude and direction, providing evidence for parallel adaptive changes in the colonization of FW (Fig. 3; lower panel). In particular, transcripts that are differentiated by both salinity and life history form (Fig. 3B, C) reflect those in which the response to salinity has evolved, and represent likely candidates in adaptation to FW.

Enrichment analysis revealed a diverse set of functions among differentially expressed transcripts. Among the set of transcripts exhibiting a conserved response to salinity, we found enrichment of ion-exchange functions, including the terms 'sodium ion export from cell' and 'sodium: potassium-exchanging ATPase complex' (Table S5, Supporting information), within which are genes with established roles in gill ion exchange, for example NKA *α1* and *α3*. Given the importance of NKA in establishing and maintaining electrochemical gradients (McCormick *et al.* 2003; Evans *et al.* 2005), it is not surprising that the expression of NKA α transcripts shows a conserved response to salinity. Several transcripts of NKA α1 show patterns of life history form differentiation (see below), suggesting that alternative variants of this enzyme may be more evolutionary labile than others. Transcripts that were differentiated in parallel were enriched with genes involved in ion transport across the gill (Table S6, Supporting information), providing evidence that the transition to FW is associated with the parallel evolution of the transcriptional regulation of ion exchange.

Parallel changes in expression of gill osmoregulatory genes

The expression of transcripts associated with water transport, osmosensing and ion exchange was differentiated between life history forms at the gill. While overall patterns of gene expression have differentiated in a nonparallel fashion, we detected signatures of parallelism in a small suite of osmoregulatory genes under FW conditions: FW expression profiles of both landlocked populations cluster tightly and independently of anadromous individuals (Fig. 4B). However, weak clustering of SW expression profiles among landlocked populations is inconsistent with parallelism. Most notably, a single L-Rogers individual in SW is highly divergent from all other expression profiles (Fig. 4B). This may reflect the fact that L-Rogers is more divergent from A-Bride than the other landlocked population (Palkovacs *et al.* 2008; Velotta *et al.* 2015); however, because of low replication in this treatment/population, it should be interpreted with caution. Further analyses that assess both transitory acute responses and acclimatory responses with greater replication within and among populations will be valuable.

Because ion transport and tight junction regulation genes exert predictable effects on FW and SW osmoregulation, we tested whether their expression diverged according to a priori models: enhanced FW expression and reduced SW expression (Fig. 1B). Among FW ion-uptake genes, six of seven fit an enhanced FW expression model (Fig. 5), whereby transcription was more sharply upregulated in FW among landlocked Alewives. Among these were two transporters—NCC (recently thought to be absent in the clupeid genome altogether; Hiroi & McCormick 2012) and VATP—both thought to play roles in Na^+ and Cl^- absorption in several euryhaline species (Hiroi *et al.* 2008). Higher VATP expression in FW is consistent with data from the FW-adapted marine copepod *Eurytemora affinis*, which exhibits parallel increases in VATP activity compared to SW forms (Lee *et al.* 2011). Our data, however, contradict other candidate gene expression analyses that found no difference in VATP expression between FW and SW

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forms (McCairns & Bernatchez 2010; Velotta *et al.* 2015). Such discrepancies may be due to quantification of different isoforms or paralogs of VATP. Given its purported importance in gill ion uptake, further investigation into the role of VATP expression and activity in adaptation to FW is needed. In addition to ion-uptake genes, we detected enhanced FW expression of claudins (*cldn3* and *cldn8*; Fig. 5F, G), which comprise a main component of strands that regulate ion permeability at cellular tight junctions and hence facilitate the retention of ions in FW (Karnaky Jr. 1991; Sonoda *et al.* 1999; Tipsmark *et al.* 2008). Overall, enhanced transcription of ion transport and tight junction regulation genes may be one mechanism by which landlocked forms have evolved improved FW tolerance.

Among the major transporters that contribute to ion secretion in the teleost gill, transcriptional responses to SW have diverged in ways consistent with a reduced SW expression model (Fig. 1B). NKA *α1b*, NKCC, CFTR and K_{ir}, which work in coordination within highly specialized ionocytes (McCormick 1995; Suzuki *et al.* 1999; Evans *et al.* 2005; Marshall & Grosell 2006; Hwang & Lee 2007; Christensen *et al.* 2012), all exhibit reduced responsiveness to SW in landlocked forms. Transcription of these genes is strongly upregulated in anadromous Alewives, but is weakly or not at all upregulated among landlocked Alewives from both populations (Fig. 6). This result supports previous work demonstrating that gene expression of NKCC and CFTR, as well as the enzymatic activity of gill NKA, is reduced in SW in landlocked Alewives (Velotta *et al.* 2014). Here, we demonstrate that expression of SW ion transporters has differentiated in parallel, and add K_{ir} and NKA α1b to the suite of genes for which expression has differentiated.

Reductions in SW tolerance among landlocked Alewives (Velotta *et al.* 2014, 2015) are associated with reductions in the transcriptional response to SW of gill ion secretion genes. Reduced transcriptional responses to SW among landlocked forms may be the result of directional selection if, for example, regulation of SW genes is constitutively costly even in FW (e.g. an energetic cost of the regulatory systems needed to detect and respond to SW; Auld *et al.* 2010) or if there are negative genetic correlations with other traits under selection in FW. We cannot conclusively discern, however, between a selective explanation and one resulting from slow deterioration via accumulation of deleterious mutations (i.e. drift via relaxed selection; Lahti *et al.* 2009).

We demonstrate that evolutionary changes to two paralogs of NKA (NKA α1a and α1b) conform to predictions based on their role in FW and SW gill ion exchange, respectively. Enhanced FW expression of α1a corresponded with reduced SW expression of α1b. NKA α1 paralogs have been identified in multiple fish species and have been shown to exhibit a high degree of salinity-dependent expression (termed 'isoform switching'); NKA α1a is upregulated in FW, while α1b is expressed more highly in SW (Richards *et al.* 2003; Shrimpton *et al.* 2005; Bystriansky *et al.* 2006; Nilsen *et al.* 2007; Larsen *et al.* 2008; Madsen *et al.* 2009; McCormick *et al.* 2009, 2013; Tipsmark *et al.* 2011; Urbina *et al.* 2013; Dalziel *et al.* 2014). Our results add to this growing body of literature suggesting that salinity-dependent paralogs have evolved in multiple, unrelated lineages. Distinctive electrochemical properties between NKA α1a and α1b are thought to maximize ion-exchange efficiency in FW or SW, respectively (Jorgensen 2008), suggesting that 'isoform switching' may help to facilitate movements between disparate salinities. Although similar patterns have been detected in salmonids (Bystriansky *et al.* 2006; Nilsen *et al.* 2007), this is the first study to demonstrate that transcription of NKA α1 paralogs in response to FW and SW evolves in parallel. Furthermore, reduced transcription of NKA α1b in SW-challenged landlocked Alewives may help to explain their reduced gill NKA activity (Velotta *et al.* 2014, 2015). This pattern is remarkably consistent with other, nonvertebrate species that have invaded FW (Lee *et al.* 2011). Given that modulation of NKA transcription and activity is central to the remodelling of the fish gill (Evans *et al.* 2005) and may be directly linked to salinity tolerance (McCormick *et al.* 2013), evolved differences in its transcriptional regulation are likely to be a primary way by which fish have adapted to FW.

Finally, a single water transport gene, aquaporin 3 (*aqp3*), exhibited enhanced expression in FW among landlocked forms, consistent with previous work in FW-adapted Mummichog (Whitehead *et al.* 2011). Life history form differences in *aqp3* expression may reflect differences in the flux of water or small molecules (i.e. glycerol or urea) across membranes (Cutler *et al.* 2007), and/or the sensing of cell volume changes in response to salinity (Watanabe *et al.* 2005). Consistent with this result, we identified life history form differentiation among genes with established roles in osmosensing (reviewed in Evans 2010; Kültz 2012), including those that (i) transduce the signal of osmotic stress to ion transporters (*mapk13*; Table 3; Leguen *et al.* 2001) and nearby cells and tissues (*il17c* and *tnfrsf11b*; Table 4; Fiol *et al.* 2006); (ii) contribute to the remodelling of gill epithelium in response to salinity stress (*bmp2*, *notch*, *foxi3a*; Table 4; Esaki *et al.* 2007, 2009); and (iii) regulate NKCC and CFTR activity via phosphorylation in response to salinity change (*sgk2*; Table 4; Evans 2010). Consistent with previous research (Shimada *et al.* 2011; Whitehead *et al.* 2011), our results suggest that adaptation to FW is accompanied by concerted changes in the cascade of

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responses to osmotic stress that lead to salinity acclimation.

The current analysis was aimed at assessing gene expression differentiation over an acclimation, rather than an acute, time frame. Previous research has identified population-level differentiation in gene expression responses at this timescale (Whitehead *et al.* 2011; Velotta *et al.* 2015). We note, however, that acute responses to osmotic stress are known to differentiate in other fishes (Whitehead *et al.* 2011); despite this, a full temporal response profile was not within the scope of this work. We elected to investigate a time frame in which differences between life history forms would be less subject to variability in timing and extent. Hence, our choice of sampling period is conservative, reflective of stable differences in expression. We note that there is potential for biased estimation of life history form differences, as selective mortality occurred before sampling (Velotta *et al.* 2015; Fig. S1, Supporting information). Future research will address how acclimatory and transitory gene expression profiles differ.

Summary and conclusions

Habitat transition to FW has resulted in shifts in both FW and SW tolerance limits in Alewives, which are strongly associated with transcriptional changes in osmoregulatory genes expressed in the gill. Although transcriptome-wide divergence in expression was generally discordant among landlocked populations, suggesting a strong signal of drift, genes that perform ionoregulatory functions were consistently differentiated in the same magnitude and direction in independent instances of landlocking. Many of the genes exhibiting evolved shifts in expression are under positive selection in FW-adapted populations of other fishes (Shimada *et al.* 2011; DeFaveri *et al.* 2011; Jones *et al.* 2012; Kozak *et al.* 2014), suggesting that modifications to the regulation of many well-known osmoregulatory pathways underlie adaptation to FW. Future studies that experimentally link changes in the expression of osmoregulatory genes to differences in osmoregulatory performance (e.g. via RNA interference experiments) will provide further support for the role of transcriptional evolution in FW adaptation.

Acknowledgements

The authors would like to thank Grace Casselberry, Jeffrey Divino, Emily Funk and Andrew Jones for help collecting Alewives. Mike O'Dea (1962–2014) and Amy Regish assisted with fish rearing and care. Nate Jue, Craig Obergfell and Center for Applied Genomics and Technology at the University of Connecticut provided technical assistance with sequencing and analysis. We thank Carl Schlichting, Mark Urban, Jeffrey Divino and three anonymous reviewers for providing insightful comments on earlier versions of this manuscript. Funding for Illumina sequencing was provided by the State of Illinois Department of Natural Resources (#CAFWS 74) and the University of Connecticut's Department of Ecology and Evolutionary Biology. Thank you to John Epifanio for help obtaining Illinois Department of Natural Resources funding. Any use of trade, product or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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J.P.V., S.C., S.D.M., P.M. and E.T.S. designed the research. P.M. and S.C. provided sequencing resources. J.P.V., J.L.W., L.K., R.J.O. and P.M. assembled the transcriptome. J.P.V., J.L.W. and S.G. annotated the transcriptome. J.P.V., L.K., R.J.O., P.M. and E.T.S.performed data analysis. J.P.V., S.D.M., P.M. and E.T.S.interpreted the results. J.P.V. and E.T.S.wrote the manuscript.

Data accessibility

Raw reads are deposited to NCBI's Short Read Archive: Illumina reads (SRP094709); 454 reads (SRR5085573). The unfiltered transcriptome assembly project has been deposited at DDBJ/EMBL/GenBank under the Accession no. GFCK00000000. The version described in this manuscript is the first version, GFCK01000000. The enTap transcriptome annotations are available in Dryad (https://doi.org/10.5061/dryad.1g304).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Details of samples used for RNA-seq analysis, including library size after quality control.

Table S2 Statistics for de novo assembled Alewife gill transcriptomes.

Table S3 Percentage of transcripts (N = 221 474) with non-zero read counts for each site.

Table S4 Enriched Gene Ontology (GO) terms associated with a salinity-only effect.

Table S5 Enriched Gene Ontology (GO) terms associated with life history form only, salinity and life history form, and interaction effects.

Table S6 Enriched GO terms associated with parallel evolution of responses.

Fig. S1 Survival of anadromous and landlocked Alewives in low-ion freshwater (FW; 0 ppt) and seawater (SW; 35 ppt) during 2-week salinity exposure experiments.

Fig. S2 Histograms of read lengths (base pairs; bp) for Illumina and 454 libraries.

Fig. S3 Frequency histogram of transcript lengths of Trinity/Mira and SOAPdenovo/Mira.

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