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The effect of landlocking on intestinal aquaporin 1 expression in Alosa pseudoharengus (alewife)

Emily Funk
Cornell University, ef347@cornell.edu

Approval Page

Honors Thesis

The effect of landlocking on intestinal *aquaporin 1* expression in *Alosa pseudoharengus* (alewife)

Presented by:

Emily C. Funk

Honors Thesis Advisor: _____

Eric T. Schultz

Honors Academic Advisor: _____

Eric T. Schultz

Department of Ecology and Evolutionary Biology
The University of Connecticut
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Abstract

To determine the role of intestinal aquaporin 1 (*AQP1*), a passive water absorption channel, in the evolution of osmoregulatory physiology, I examined the differential expression of intestinal *AQP1* between anadromous and landlocked ecotype of alewife in response to seawater challenges. I cloned and sequenced *AQP1* from intestinal tissue of the alewife, following which I quantified the relative expression of *AQP1* in each ecotype using Real-Time qPCR. In response to an acute seawater (30ppt) challenge, the anadromous alewives showed an upregulation of intestinal *AQP1*, while the landlocked alewives did not show a significant increased in *AQP1* expression. After acclimation to seawater over 14 days, the anadromous alewives maintained a higher level of intestinal *AQP1* expression compared to the landlocked alewives. The reduced seawater response exhibited by the landlocked alewives provides evidence for osmoregulatory evolution towards a completely freshwater lifecycle through relaxed selection of intestinal *AQP1* water absorption mechanisms.

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Introduction

Osmoregulation is the process by which teleost fishes are able to maintain a constant internal fluid concentration independent of the environmental concentration and is necessary to prevent salt depletion in freshwater species and dehydration in saltwater species. In general, teleost fishes have an internal ion concentration, i.e. plasma osmolality, of approximately 10 parts per thousand (ppt), which is about one-third the concentration of seawater. Consequently, freshwater fish passively lose ions and gain water across the permeable membranes of the gills and skin because they occupy a habitat more dilute than their internal fluids. The opposite occurs in seawater fish; the concentrated environment of seawater causes passive gain of ions and loss of water. To counteract the passive loss of water, seawater fish drink significant amounts and absorb the ingested water through the intestine (Giffard-Mena *et al* 2007). Most teleost fish inhabit environments with relatively constant salinity and thus, only need to maintain the physiological mechanism necessary for osmoregulation in one halohabitat. However, some species are euryhaline and can inhabit a wide range of salinities, which requires the capacity to osmoregulate in both concentrated seawater and dilute freshwater environments. Consequently, the mechanism for water absorption across the intestine is active when the euryhaline fish occupies seawater to combat dehydration but is inactive when the fish inhabits freshwater because passive water loss desists (Raldua *et al* 2008). In this study, I examined the expression level of intestinal water transport proteins in the euryhaline fish *Alosa pseudoharengus* (alewife) to determine the physiological evolution occurring as populations become landlocked and specialized for freshwater habitats.

I was able to assess the variations in expression of intestinal water transport proteins because of the existence of two ecotypes of alewife, anadromous and landlocked. Anadromous

alewives live in seawater but migrate to freshwater to spawn, so they need to osmoregulate in both halohabitats. In Connecticut, dams built during the colonial period block passage to the sea and separate anadromous and landlocked alewife imposing a physical barrier to gene flow between populations. The lack of gene flow between populations led to the divergence in morphology and life history traits of a landlocked, freshwater ecotype (Palkovacs *et al*. 2008). These recent freshwater populations, derived from the anadromous form, do not need to osmoregulate in seawater. Over time, water uptake mechanisms of landlocked fish may exhibit a weaker response to seawater because of relaxed selection for water absorption. Relaxed selection occurs when the source of selection for a particular trait is removed by an environmental change rendering the characteristic obsolete as it no longer contributes to the organism's fitness (Lahti *et al* 2009). In the present case, the landlocking events caused the removal of seawater as a source of selection for water absorption mechanisms and hypoosmoregulation. The maintenance of any trait requires energy input and incurs a constitutive, inherent, cost in the form of energetic expenditure; in an environment where trait function is adaptive, the benefits outweigh the costs providing a net improvement of fitness. However, in the event of environmental change, the benefit could disappear, and the subsequent relaxed selection would lead to trait reduction because the constitutive cost of maintenance is no longer balanced by reciprocal benefit (Lahti *et al* 2009). If maintaining water absorption mechanisms is energetically costly, the evolution of a reduced seawater response would benefit the landlocked ecotype by allowing the allocation of energy elsewhere.

Through survival and plasma osmolality measurements, the landlocked alewife ecotype has been shown to have both decreased seawater tolerance and decreased hypoosmoregulatory ability. Salinity challenge experiments were conducted on seven alewife populations in

Connecticut, six were landlocked and one was an anadromous population. When subject to a 1ppt freshwater treatment, the seven populations showed no significant differences in survival; however, when exposed to 35ppt seawater, five of the landlocked populations exhibited greatly decreased survival compared to the anadromous population implying that landlocked alewives have reduced seawater tolerance (Velotta pers. comm. 2011). Further investigation was performed focusing on only two of the landlocked populations and the anadromous population; alewives from each population were subject to 1ppt, 20ppt, and 30ppt salinity challenge treatments, and across all salinities, the anadromous alewife exhibited significantly higher survivability than the landlocked form. Additionally, plasma osmolality of the salinity challenged alewife was measured to determine whether the decreased survival could be attributed to inadequate osmoregulation. Velotta *et al* (in review 2013) found that landlocked alewives possessed a higher plasma osmolality level when subject to seawater than the anadromous alewives. The greater increase in plasma osmolality of landlocked alewife in response to seawater suggests that the landlocked ecotype has reduced hypoosmoregulatory ability, for the elevated internal ion concentration reflects a failure to counteract the passive ion uptake and water loss to the seawater environment. The reduction in seawater tolerance and osmoregulation of the landlocked ecotype is evidence that physiological evolution for a freshwater lifecycle is taking place, which could be reflected in the genetic material of landlocked alewives. Because the intestine contributes extensively to osmoregulation by absorbing water, I expect the expression of genes controlling the level of intestinal water uptake to vary between ecotypes experiencing different halohabitats.

The fish intestine plays an important role in osmoregulation by facilitating water uptake; in fact, 70 to 85 percent of ingested seawater is absorbed across the intestinal epithelium (Grossel

et. al. 2011). The physiological mechanism that controls intestinal water absorption has been studied in relatively few species. However, it is understood that water is absorbed across the intestinal epithelium along a concentration gradient from an area with low ion concentration to one with high ion concentration because water can only move passively through the process of osmosis. Water cannot be actively transported to a given area, so concentration gradients are created using ions in order to facilitate the passive movement of water (Kim *et al* 2010). By drinking large amounts of saltwater, marine fish are able to obtain enough water to prevent dehydration as well as acquire the ions needed to generate the necessary concentration gradient. As the ingested seawater moves through the esophagus of a fish, sodium and chloride ions are absorbed, so the solute concentration of the water is greatly reduced and becomes isoosmotic with the internal fluids. After moving through the stomach, the water enters the intestine, where the majority of water uptake occurs. Water absorption is directly connected to sodium and chloride transport (Evans & Claiborne 2006) as these ions are actively pumped across the intestinal epithelium to establish a localized concentration gradient that drives the absorption of water (Kim *et al* 2010).

The passive transport of water across the intestine occurs through water transport proteins called aquaporins, which are located within the membrane of intestinal epithelial cells. Aquaporins are extremely selective channel proteins that only allow for the passage of water, while preventing the movement of essential small molecules out of the cell. The tetrameric structure of aquaporin is highly conserved among vertebrates; each monomer has six transmembrane α-helices surrounding two overlapping interhelical loops located within the membrane lipid-bilayer forming a single aqueous pore. The pore is large enough for water molecules to flow through but still impede the passage of larger solutes to ensure selective

passage of water. Additionally, charged regions of the pore prevent the transport of small, charged molecules, such as protons, through the aquaporin channel by disrupting hydrogen bonding (Heymann *et. al*. 1998). Aquaporins exist in nearly all living organisms ranging from vertebrates to invertebrates to bacteria (Agre *et. al.* 2002). The highly conserved aquaporin structure implies that aquaporin function is essential for water absorption and is a suitable candidate gene to investigate the evolution of osmoregulation. Several forms of aquaporin exist within the intestinal epithelium of fishes; in particular, aquaporin 1 (*AQP1*) has been shown to be directly involved in water uptake (Grossel *et. al.* 2011). *AQP1* has been previously investigated in several fish species, and the seawater-adapted fish have been shown to exhibit elevated *AQP1* expression in the posterior intestine when compared to their freshwater-adapted counterparts (Aoki *et al* 2003, Martinez *et al* 2005, Giffard-Mena *et al* 2007).

Based on the current evidence for the importance of *AQP1* in intestinal water absorption in fishes, I chose to investigate the evolution of osmoregulatory ability as populations become landlocked by quantifying the gene expression of intestinal *AQP1*. Gene expression is the level of DNA transcription into mRNA, and thus, *AQP1* expression governs the abundance of AQP1 proteins in an intestinal cell membrane and regulates the efficiency of water absorption. Previous studies have demonstrated the importance of *AQP1* for seawater acclimation within a single population; however, I aim to identify the interpopulation differences in *AQP1* expression of alewife (anadromous and landlocked) to reveal the potential evolutionary mechanisms involved in freshwater invasion. Studying the differential expression of *AQP1* across populations that inhabit disparate halohabitats allows for the assessment of physiological evolution in osmoregulation occurring in the freshwater environment as a result of relaxed selection for water absorption mechanisms.

Aquaporin 1 is primarily located within the epithelium of the intestine. The fish intestine is not clearly separated into multiple sections, and the morphological differences between the anterior and posterior portions of the intestine are only subtle. Despite the lack of demarcated sections, the anterior and posterior intestine exhibit differential expression of AQP1. In previous studies performed on sea-bass (Giffard-Mena *et. al.* 2007), Japanese eel (Aoki *et. al.* 2003), and European eel (Martinez *et. al.* 2005), the AQP1 expression of each intestinal segment was examined, and all investigations found AQP1 expression to be highest in the posterior intestine. This elevated expression of AQP1 suggests that the posterior intestine is the main site for water absorption; hence, I used the posterior intestine to study the effect of land-locking on AQP1 expression and, more broadly, osmoregulation. In order to locate the point of division between the posterior and anterior intestine, I used the pyloric caeca, intestinal appendages that increase the absorptive surface area of the intestine and extend off the anterior region, for reference. The Clupeidae family, which includes the alewife, possess long pyloric caeca making identification of the posterior intestine possible (Suyehiro 1942).

I am investigating the mechanism by which intestinal AQP1 aids in hypoosmoregulation by counteracting the passive loss of water to a concentrated seawater environment. I expect the landlocked alewife to exhibit lower expression of intestinal AQP1 when subject to seawater challenge as compared to anadromous alewife. A weakened response to seawater would indicate the occurrence of evolution in landlocked alewife living solely in freshwater toward a reduction in seawater osmoregulatory ability.

Materials and Methods

Experimental Animals

In July of 2012, I collected juvenile alewife from an anadromous population and a landlocked population. The anadromous alewives were collected from Bride Lake in East Lyme, CT, which connects to Long Island Sound via Bride Brook. The landlocked alewives were collected from Rogers Lake in Old Lyme, CT (Table 1). I caught the anadromous and landlocked alewives on two different days using a 116 foot by 16 foot purse seine with 1/8 inch thick mesh. For efficient collection, I first put a pool light out in the lake to attract the alewives to one area and then purse seined around the light. Using two 50-gallon barrels filled with 0.3ppt freshwater treated with stress coat (5ml/10gallons concentration), I transported approximately 400 alewives from each population to S. O. Conte Anadromous Fish Laboratory in Turners Falls, MA. The alewives were transferred to 1200 gallon holding tanks (four tanks total; two held anadromous and two held landlocked alewife) at 0.5ppt salinity and ~23^{o}C to acclimate to laboratory conditions.

Throughout my experiment, I followed standard husbandry practices to maintain proper tank conditions. The fish were kept under artificial light conditions with a natural photoperiod, and they were held at a temperature between 22^{o}C and 24^{o}C. The tank water quality was maintained through Biofilter recirculation. I regularly tested the ammonia concentration of each tank; if ammonia levels rose above 1ppm, I treated the water with ClorAm-X. During the acclimation period, I fed the alewives to satiation with pellet fish food.

Salinity Challenge Experiments

I conducted two salinity challenge experiments over two different time periods, and compared the responses of the anadromous versus landlocked alewives by quantifying *aquaporin 1* gene expression. One experiment was a short-term challenge performed over 48 hours, while the second experiment was a long-term challenge completed over 14 days. Throughout both

experiments, the water quality of the tanks was maintained with Biofilter recirculation, and ammonia levels were check regularly. The temperature of the water was kept between 22^{o}C and 24^{o}C. Before beginning experimentation, I sampled ten fish from the anadromous population holding tanks and eight fish from the landlocked population holding tanks; these samples served as the time zero condition.

After allowing acclimation to laboratory conditions for 5 days, I transferred 25 anadromous alewives to a tank at 0.5ppt salinity (freshwater control) and another 25 anadromous alewives to a tank at 30ppt salinity (salinity challenge). Similarly, I transferred 20 landlocked alewives to a tank at 0.5ppt and 21 landlocked alewives to a tank at 30ppt (Figure 1). Twenty-four h prior to transfer and throughout the experiment (48 h long), I withheld food from the fish. At 24 h after transfer, I sampled a subset of anadromous fish from both the freshwater control and salinity challenge tanks along with sampling a subset of landlocked fish from both the freshwater control and salinity challenge tanks. Again, at 48 h post transfer, I sampled a subset of alewives from each of the four treatment tanks (Table 2).

In the long-term experiment, I transferred 20 anadromous alewives from the holding tanks to a 0.5ppt salinity treatment tank (freshwater control) and a 30ppt salinity treatment tank (salinity challenge). Likewise, I transferred 20 landlocked alewives to freshwater control and salinity challenge tanks (Figure 1). I withheld food from the alewife for 24 h prior to transfer, and during experimentation, I fed the fish pellet fish food every other day at an amount equal to 10% of their body weight. At 4 d and 14 d post-transfer, I sampled eight anadromous fish from the freshwater control and salinity challenge tanks and eight landlocked fish from each treatment tank as well (Table 3).

All samples were euthanized with a lethal dose of MS-222. I took length and weight measurements of each sample and immediately placed the fish on ice. Subsequently, I dissected the posterior intestine of each sample by making a cut along the left side of the body. Beginning at the anus, I cut up to the lateral line and then cut along the body axis between the muscle and the body cavity ending at the gill arches. I carefully pulled the esophagus, stomach, and intestine out of the body cavity and cut off the posterior intestine after the pyloric caeca. Each posterior intestine was placed in RNAlater, which prevents degradation of the RNA, and stored at -20^{o}C.

Gene Cloning and Sequencing

The quantification of intestinal *AQP1* expression in salinity-challenged alewife was accomplished by Real Time qPCR. I sequenced the alewife *AQP1* gene sequence in order to design alewife-specific primers for *AQP1*. I extracted RNA from an alewife intestine sample and reverse transcribed the RNA to create cDNA. With standard PCR, intestinal *AQP1* was amplified from the cDNA using a set of primers designed based on 454 sequence data available for the alewife gill (forward primer: 5'-TGACGTCAGCTCAACTCCTG-3', reverse primer: 5'-CAACAAGCTCGGAGTGAACA-3') (Velotta pers.comm., 2012). A specific gene region, contig26383, of the alewife gill 454 sequence exhibited sufficient similarity to known *AQP1* sequences for other fish species, so the primers designed from this sequence were able to anneal to the alewife intestinal *AQP1*, which was then amplified with a thermocycler using standard PCR. Subsequently, I further amplified the *AQP1* gene using a StrataClone Kit, in which *AQP1* was inserted into a plasmid and transformed into an *E. coli* vector. The *E. coli* was grown on ampicillin medium to allow growth of only the transformed vectors, and X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) was used to perform blue-white selection of the vectors containing a recombinant plasmid. After selecting the *E. coli* vectors with recombinant plasmids

(white colonies), I isolated the plasmids from the vectors and sequenced them using standard Sanger sequencing methods. I identified the gene of interest, *AQP1,* from within the plasmid sequence determining the flanking sequences of the restriction site where *AQP1* was inserted; the region between the restriction site ends is the *AQP1* sequence. Using NCBI nucleotide BLAST tool, I confirmed that the obtained gene sequence was *AQP1*. From the intestinal *AQP1* sequence, I designed alewife-specific *AQP1* primers for Real Time qPCR using the Primer3 program (forward primer: 5'-GGCCACTTGACAGCCATAAG-3', reverse primer:5' CCAGTACACCCAGTGATTTGC-3').

Real-Time qPCR

Real-Time qPCR can only be performed with DNA, so using an RNeasy Mini Kit, I extracted RNA from the dissected intestinal tissue. Following extraction, I used the Ambion TURBO DNA-free kit to treat the RNA with DNase enzyme to digest any contaminating genomic DNA. The purity of the RNA was determined using the NanoDrop spectrophotometer to measure the ratio of absorbance at 260nm and 280nm; if $A_{260/280}$ is close to 2.00, then the RNA is pure. Then, with the ABI cDNA Synthesis Kit, I reverse transcribed the purified RNA in a thermal cycler into cDNA, which I stored at -20^{o}C until it was needed for Real-Time PCR analyses.

To quantify the *AQP1* expression level in the posterior intestines of the salinity challenged alewives, Real-Time qPCR was performed with the alewife-specific *AQP1* primers (forward primer: 5'-GGCCACTTGACAGCCATAAG-3', reverse primer: 5'-CCAGTACACCCAGTGATTTGC-3'). Along with amplifying the target gene, *AQP1,* I also amplified a reference gene, *EF1a,* for each intestinal sample. The *EF1a* gene does not differ in expression level when the salinity is changed, so it can be used as a reference to determine the

relative expression of *AQP1* through correction of any variation between samples in initial cDNA concentration. I used previously designed alewife-specific *EF1a* primers (forward: 5'-GGTGGAAGGTTGAGCGTAAG-3', reverse: 5'-CACGGGTACAGTTCCAATAC-3') in the Real-Time qPCR analyses (Velotta pers.comm. 2012). I determined the efficiency values for each primer set, *AQP1* and *EF1a*, by performing a serial dilution (1:1, 1:10, and 1:100) with a random intestinal cDNA sample for each primer set and subsequently, generating a standard curve. Figure 2 illustrates the general procedure for serial dilution and efficiency calculation. I obtained threshold cycle values at each dilution using Real-Time qPCR and plotted these values against the cDNA starting concentration to create a standard curve for each primer set. The efficiencies of the *AQP1* and *EF1a* primers were calculated from the standard curves using the equation, $E = 10^{-1/slope}$. I ran the serial dilutions three separate times for both primer sets and took mean efficiency of the three different standard curves for each primer set, which I used in all *AQP1* expression level calculations (AppendixA Fig.1). The efficiency value was 1.99 for *AQP1* and 1.92 for *EF1a* primers.

I performed Real Time qPCR with the cDNA of each intestinal sample from both the acute 48 hour experiment and the long-term 14 day experiment to examine *AQP1* expression levels; each cDNA sample was run in triplicate. The reaction mixture contained 0.5µl of cDNA template, 0.5µl each of forward and reverse primer, 7.5µl of SYBR Green Master Mix, and 6µl of water creating a total reaction volume of 15µl. A negative control for both primer sets was run on each Real-Time PCR plate, in which all reagents except cDNA template were included in the reaction. The Real-Time PCR cycling conditions were as follows: 95ºC for 30 seconds, 95ºC for 3 minutes; 45 cycles of 95ºC for 20 seconds and 62ºC for 50 seconds; 95ºC for 1 minute, 55ºC

for 1 minute, and 55ºC for 30 seconds. I also ran a melt curve reaction on each plate following the PCR to ensure only one product was amplified.

To quantify *AQP1* expression of each sample, I used the Pfaffl method (Pfaffl 2001). Through use of Bio-Rad CFX Manager software, a plot of relative fluorescence units (RFU) of each sample versus cycle number is generated for each Real-Time qPCR run; the relative fluorescence units are a measure of the amplification of the target gene, *AQP1*, and the reference gene, *EF1a*. The Bio-Rad CFX Manager also sets a threshold line on each plot that corresponds to the point at which relative fluorescence and thus gene amplification becomes exponential. This threshold line remains fixed for each plot of RFU versus cycle number, and the point at which the relative fluorescence of each sample crosses the threshold line is the cycle threshold (Cq) value for that sample. From each sample, I obtained Cq values for both the *AQP1* and *EF1a* genes and used these values in the Pfaffl equation, $Ratio = \frac{(E_{target})^{\Delta Cq\ target\ (control-sample)}}{(E_{reference})^{\Delta Cq\ reference(control-sample)}}$, to calculate the relative expression of *AQP1* in each intestinal sample. In the equation, the E_{target} and $E_{reference}$ are the efficiency values for each primer set, and in the present case, *AQP1* is the target and *EF1a* is the reference. The ΔCq for the target and reference genes are determined by subtracting the Cq value of the sample from the Cq value of the control, which is a chosen sample that is run on every Real-Time qPCR plate in order to correct for between-plate variation. I used an intestinal sample from a Bride fish in the 30ppt salinity treatment sampled at 24 hours post-transfer as the control.

Statistical Analyses

For both the acute challenge and acclimation experiment, I used a linear mixed model to elucidate the differences in intestinal *AQP1* expression between alewife ecotypes in response to seawater exposure. Before applying the model, I natural log-transformed the expression values

obtained for *AQP1* in order to equalize the variance and obtain homoscedasticity among my data. Using R 2.15.2, I ran the linear mixed model with the lmer function (lme4 package). When analyzing the data for the acclimation experiment, the full model included ecotype, salinity treatment, time, and length as fixed variables, while tank was included as a random effect variable. The holding tanks for each ecotype and the experimental tanks for each ecotype by salinity combination were all assigned a distinct number identifier. After running the full model with a 4-way interaction and the subsequent reduced models with the non-significant variables eliminated (p-value > 0.05), I found no time effect in *AQP1* expression. Accordingly, I removed time as a variable and averaged the *AQP1* expression values from the 4 d and 14 d sampling points enabling me to examine acclimation differences between freshwater and seawater treatments. The linear mixed model with the combined data included ecotype, salinity treatment, and length as the fixed variables. For the acute challenge experiment, I analyzed the expression data with the linear mixed model for each salinity treatment, freshwater and seawater, separately. I included ecotype, time, and length as fixed variables and tank as a random effect in each model. I ran the full models with 3-way interactions for the acclimation experiment (ecotype x salinity x length) and the acute challenge experiment (ecotype x time x length); I reduced the models by sequentially eliminating the higher order, non-significant interactions (p-value > 0.05). After identifying the appropriate model size for each experiment, I tested for significance using the pvals.fnc function (language R package), which uses the Markov Chain Monte Carlo Method (MCMC) to calculate p-values from the linear mixed model.

In addition, I applied an analysis of variance (AVOVA) model to the acclimation experiment combined data to evaluate the differences in intestinal *AQP1* expression of each ecotype in response to the salinity treatments, freshwater and seawater. I applied the ANOVA in

R 2.15.2 using the aov function with ecotype and salinity treatment as predictor variables; I ran the full model with an ecotype by salinity interaction.

Results

Given the 454 alewife gill sequence available, I found a region of the cDNA, contig26383, that closely aligned to known *AQP1* sequences available for other teleost fishes in the NCBI database by running a nucleotide BLAST (Table 5). I designed primers from this gill sequence (contig26383) that successfully amplified *AQP1* from the alewife intestinal samples, and subsequently, I determined the sequence for the alewife intestinal *AQP1* gene. I obtained sixteen possible sequence contigs for *AQP1* and found that thirteen aligned together with 100 percent identity showing that the same gene was sequenced from each cDNA sample. The remaining three contigs contained too many unknown nucleotides to be useable. I selected a representative sequence, contig05, and performed a nucleotide BLAST search in NCBI, which generated alignments with known *AQP1* sequence from other fish species (Table 6). Three high scoring alignments exhibiting 77 percent sequence identity with the alewife contig include *Diplodus sargus*, *Rhabdosargus sarba*, and *Sparus aurata* AQP1 mRNA sequences. The corresponding e-values of 6.00E-84 indicate that the obtained alewife sequence is significantly similar to known AQP1 mRNA sequences (Table 6). I can confidently assert that the intestinal alewife sequence (contig05) is, in fact, the *AQP1* gene.

Initially, prior to experiencing salinity challenges, the laboratory acclimated alewife in 0.5ppt freshwater, exhibited no significant ecotype differences in intestinal *AQP1* expression. In other words, the time zero conditions for both ecotypes, landlocked and anadromous, were equal in *AQP1* expression level. Following acute transfer to the freshwater control treatment maintained at 0.5ppt, the *AQP1* expression level in each ecotypes remained constant over 48

hours. Additionally, the ecotypes did not express any differences over time in intestinal *AQP1* level. Transfer to the freshwater control treatment did not elicit a change in the response of *AQP1* expression in either alewife ecotype (Fig. 3). However, when the alewives were exposed to the 30ppt seawater challenge for 48 hours, a significant ecotype by treatment interaction (linear mixed model: p-value = 0.041) was revealed showing that the two ecotypes responded differently to the seawater treatment. The anadromous alewives produced the expected seawater response by significantly increasing *AQP1* expression after transfer to the 30ppt salinity challenge. In contrast, the landlocked alewife did not display increased intestinal *AQP1* expression in response to seawater, but rather, maintained a constant expression level over the 48 hour time period (Fig. 4). The anadromous alewives displayed a greater upregulation of *AQP1* in response to the seawater treatment compared to the landlocked alewives.

To analyze the acclimation differences in *AQP1* expression between alewife ecotypes over a 14 day period, I applied two statistical approaches, a linear mixed model and an ANOVA. Over the duration of the experiment, I sampled alewives at 4 and 14 days; however, I found no significant effect of time on *AQP1* expression in either ecotype or salinity treatment, so I averaged the expression values for the 4 and 14 day samples of each ecotype-treatment group. I examined the acclimation differences between alewife ecotypes subject to 0.5ppt freshwater and 30ppt seawater salinities in the absence of a time component. The alewives acclimated to freshwater showed no significant differences in *AQP1* expression levels. Through application of the ANOVA model, the ecotypes acclimated to 30ppt salinity concentration produced a significantly different response in *AQP1* expression (p-value = 0.04) (Fig. 5). After acclimating to seawater, the anadromous ecotype maintained a higher level of intestinal *AQP1* expression than the landlocked ecotype. However, when I performed a linear mixed model analysis, which

accounts for the random effect of tank, the ecotype response difference was rendered insignificant (p-value = 0.16).

Discussion:

AQP1 expression was significantly increased by the anadromous alewives after an acute seawater challenge at 30ppt and was then maintained at a higher level after a seawater acclimation period, suggesting an enhanced ability for water absorption and thus, hypoosmoregulation by the anadromous ecotype. In comparison, the landlocked alewife did not upregulate *AQP1* expression in response to seawater challenge and possessed a lower expression level following the acclimation period. The observed difference in intestinal *AQP1* expression was expected as anadromous alewives frequently encounter seawater and must maintain water absorption mechanisms. However, the landlocked alewives living entirely in freshwater have been released from the pressure to hypoosmoregulate, and consequently the *AQP1* water absorption mechanism is undergoing relaxed selection leading a reduced seawater response. The divergent responses to seawater between the alewife ecotypes provide evidence for the occurrence of physiological evolution in osmoregulation resulting from landlocking events and adaptation for a completely freshwater lifecycle.

The elevated response in *AQP1* expression by the anadromous alewife when subject to seawater is consistent with previous studies on the effect of seawater acclimation within fish populations. Aoki *et al* (2003) examined differences between seawater- and freshwater-acclimated Japanese eels and discovered a higher rate of water absorption across the intestine of the seawater-acclimated eels, implying increased permeability of the intestinal epithelium. Correspondingly, intestinal *AQP1* was also found to exhibit higher expression levels in the seawater-acclimated eels (Aoki *et al* 2003). The correlation between increased water absorption

and increased *AQP1* expression is indicative of the importance of *AQP1* in seawater osmoregulation. Martinez *et al* (2005) observed a comparable increase in *AQP1* expression in the intestine of European eels acclimated to seawater. This increase in *AQP1* mRNA was accompanied by an increase in AQP1 protein abundance. Additionally, Giffard-Mena *et al* (2007) observed an elevated level of *AQP1* expression in sea-bass held in seawater compared to those acclimated to freshwater over three weeks. As evidenced by these investigations, intestinal *AQP1* expression is correlated with the protein quantity and water absorption rate showing that measurement of AQP1 mRNA abundance in the intestine is representative of water absorption ability.

The acclimatory increase of *AQP1* expression in fish exposed to seawater implies that the intestinal *AQP1* response is plastic and varies depending on the salinity environment. As anadromous fish species, alewife included, migrate between halohabitats, specific osmoregulatory genes are activated in the seawater environment, while they are inactive in the freshwater environment, and vice versa. Expression of plastic genes can be reduced or even lost in the event of elimination of the environmental cue that activates the genes (Lahti *et al* 2009). In my investigation, I observed a lower *AQP1* expression in the anadromous alewife when subject to the freshwater treatment compared to the seawater treatment. Furthermore, the anadromous alewife in the freshwater treatment expressed *AQP1* at a level equal to that of the landlocked alewife. The lowered *AQP1* expression when exposed to freshwater suggests that salinity elicits a plastic response by *AQP1*, where freshwater elicits a decrease and seawater elicits an increase in expression. In consequence, the absence of seawater as an environmental cue causes an evolutionary change in the landlocked alewives by reducing the expression of intestinal *AQP1* when exposed to seawater.

Compared to the landlocked form, the anadromous alewives possess enhanced hypoosmoregulatory ability through upregulation of *AQP1*; however, the landlocked alewives are still able to survive seawater challenge. The plasticity of intestinal *AQP1* could contribute to this retained seawater tolerance of landlocked alewife. Though the acclimation experiment revealed the appearance of elevated *AQP1* expression in response to seawater by the anadromous ecotype compared to the landlocked, the difference was not significant under the linear mixed model. The landlocked alewife still possess the ability to activate *AQP1* when exposed to the necessary environmental cue, seawater, reflecting the relatively recent isolation of alewife populations between 5000 and 300 years ago (Palkovacs *et al* 2008). When subject to an acute seawater challenge, the landlocked alewives are unable to react as efficiently as the anadromous form through immediate upregulation of intestinal *AQP1* expression; however, provided a longer time period for acclimation, the landlocked alewives are able to increase *AQP1* expression to an extent permitting seawater survival.

Further study of AQP1 has revealed the existence of multiple isoforms of AQP1 in several fish species providing the possibility for alternative protein function in addition to water absorption. An investigation conducted by Wook An *et al* (2008) revealed an increase in *AQP1* expression in black porgy when exposed to a freshwater challenge, which is opposite of the response that I observed in alewife. Because the AQP1 protein is a water channel allowing the bidirectional flow of water, Wook An *et al* (2008) proposed that AQP1 is acting as a water secretion channel in the intestine of freshwater-challenged black porgy, rather than as a water absorption mechanism. The use of AQP1 for water secretion would aid in the discharge of excess water passively gained from the dilute environment. Additionally, several studies, including those performed on Japanese and European eel by Aoki *et al* (2003) and Martinez *et al* (2005)

respectively, have shown the existence of different AQP1 isoforms, which could perform separate functions. One isoform could function in water absorption, while the other serves in water secretion. Aoki *et al* (2003) and Martinez *et al* (2005) found AQP1 to be apically located in the intestine of eels exhibiting increased *AQP1* expression in response to seawater, which supports its function in water absorption. A freshwater AQP1 isoform could possibly be located basally in the intestinal epithelium allowing for the secretion of excess water. Only subtle differences have been found between the sequences for each AQP1 isoform, which presents the possibility of amplifying both isoforms with the same primers when utilizing Real-Time qPCR to quantify expression levels (Aoki *et al* 2003, Martinez *et al* 2005). If one AQP1 isoform functions primarily in seawater and the other in freshwater osmoregulation, the intestinal *AQP1* expression differences between seawater- and freshwater-acclimated fish would be subdued.

My investigation into the mechanism of water absorption through *AQP1* in relation to osmoregulatory ability in alewife reveals a difference in intestinal *AQP1* expression levels between anadromous and landlocked ecotypes when exposed to seawater. The anadromous alewives, which are accustomed to seawater, exhibited the expected upregulation of *AQP1* in response to the seawater challenge. However, the landlocked alewives, which now possess a completely freshwater lifecycle, did not show the expected seawater response providing evidence for the occurrence of physiological evolution of the osmoregulatory system.

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Tables and Figures:

Table 1: The location, geographic coordinates, and population type of the two lakes (Bride Lake and Rogers Lake) where the experimental alewives were collected.

Body of Water	Location	Geographic Coordinates	Alewife Population
Bride Lake	East Lyme, CT	41.33ºN, 72.24ºW	Anadromous
Rogers Lake	Old Lyme, CT	41.36ºN, 72.30ºW	Landlocked

Table 2: The number of alewives in each of four experimental tanks (one freshwater control tank at 0.5ppt and one seawater challenge tank at 30ppt for both populations) at time 0 of the short-term experiment, and the number of alewives sampled from each tank at 24 hours and 48 hours post-transfer.

Time Point	**Bride 0.5ppt**	**Rogers 0.5ppt**	**Bride 30ppt**	**Rogers 30ppt**
0	25	20	25	21
24 h	10	8	10	8
48 h	10	10	10	10

Table 3: The number of alewives in each of four experimental tanks (one freshwater control tank at 0.5ppt and one seawater challenge tank at 30ppt for both populations) at time 0 of the long-term experiment, and the number of alewives sampled from each tank at 4 days and 14 days post-transfer.

Time Point	**Bride 0.5ppt**	**Rogers 0.5ppt**	**Bride 30ppt**	**Rogers 30ppt**
0	20	20	20	20
4 d	8	8	8	8
14 d	8	8	8	8

Table 4: The general forward and reverse primer sequences for *aquaporin 1* used to amplify the intestinal *aquaporin 1* with PCR. The alewife-specific primer sets for *aquaporin 1* and *EF1a* used for Real-Time qPCR analyses.

Gene	**Primer Sequence 5'-3'**
Aquaporin 1 (general)	Forward: TGACGTCAGCTCAACTCCTG Reverse: CAACAAGCTCGGAGTGAACA
Aquaporin 1 (alewife-specific)	Forward: GGCCACTTGACAGCCATAAG Reverse: CCAGTACACCCAGTGATTTGC
EF1a (alewife-specific)	Forward: GGTGGAAGGTTGAGCGTAAG Reverse: CACGGGTACAGTTCCAATAC

Table 5: Select sequence alignments from the BLAST results (NCBI database) retrieved using the alewife gill sequence (contig26383[1]) as the query sequence.

	Sequence ID	Max Identity (%)	Query Coverage (%)	e-value
Anguilla japonica AQP-1S mRNA for aquaporin 1, complete cds	gi|34013385|dbj|AB094502.1|	76.62	58	1.00E-135
Anguilla japonica AQP-1L mRNA for aquaporin 1, complete cds	gi|34013383|dbj|AB094501.1|	76.58	58	1.00E-135
Anguilla anguilla mRNA for aquaporin 1 (aqp1 gene)	gi|73852959|emb|AJ564420.1|	76.47	58	2.00E-134
Amphiprion metanopus aquaporin 1 mRNA, partial cds	gi|309252241|gb|HM768895.1|	76.44	58	2.00E-128
Sparus aurata aquaporin 1 mRNA, complete cds	gi|54401743|gb|AY626939.1|	76.44	58	2.00E-128
Diplodus sargus aquaporin 1 mRNA, complete cds	gi|358357311|gb|JN210582.1|	76.14	58	8.00E-126
Rhabdosargus sarba aquaporin 1 mRNA, partial cds	gi|334361414|gb|JF803845.1|	75.99	58	1.00E-124
Acanthopagrus schlegelii aquaporin 1 mRNA, complete cds	gi|225706605|gb|BT074725.1|	70.68	58	0.16
Hippoglossus hippoglossus aquaporin-1aa (Aqp1aa) mRNA, complete cds	gi|133779720|gb|EF451961.1|	75.53	58	2.00E-120
Cynoglossus semilaevis aquaporin 1 mRNA, complete cds	gi|225715939|gb|BT079392.1|	74.92	55	9.00E-119
Danio rerio aquaporin 1 mRNA, complete cds	gi|116805723|gb|DQ887675.1|	74.21	58	4.00E-111
Fundulus heteroclitus aquaporin-1 mRNA, complete cds	gi|410923810|ref|XM_003975326.1|	74.12	59	4.00E-110
Dicentrarchus labrax aquaporin 1 (AQP1) mRNA, complete cds	gi|209490728|gb|EU780153.1|	74	58	4.00E-110
Anabas testudineus aquaporin 1aa (aqp1aa) mRNA, complete cds	gi|410923812|ref|XM_003975327.1|	73.94	58	2.00E-108

[1]TGTTACATAGCCAACATATGTATTCTACATTTACTTACAAAAGTGGAGACAAGGAATCCAAGTGCACACAGAGTACATATATAAAAGGATACAATAAGCATTTACATAAAAAATAGT
TTACAAAAATATGTGATTCTACATCTAGAATACTGACAGTGAAGCCTGTTGTTTGAAACAGTAAAACATTATCTTACATTATCTGTAACAAACATGCCTTGTTTGAAAGGCTATTAACA
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GACATCGTAGTCTTTAGCCGGTCCGCTCATGAGCACCTTCATGCGGTCGGGGAAATCGTCCGTTTTGGATATAGCAGGAAGTCATACACGAGAGCAGCAGCCACGCCTCCACACATGG
GCCCGACCCAGTACACCCAGTGATTTGCGAAATTGCTCGTAACGACGGCAGGACCAAAGGACCGAGCAGGGTTGATGCCACACCCAGTGAAACTTATGGCTGTCAAGTGGCCCAGAG
CAACAGAGAGACCAATGGCCAGGGGGGCGGAACCTGTCACATCCCGCCGTCTTTTATCTGTGGTTGCTATGACACACAGGACGAGCTGGAAGGTGGCCAGTAGCTCGATGCCAATAC
CTTGACTTGGGGTCACACCAGAAAGACTGTTCACTCCGAGCTTGTTGTTGGTGTCCGGCCGCACTCCATAGATGATCCCGCTAGCCACGGTGGCTCCCAGCATCTGTGCCGCCATATAC
ATGACCGCGCGCAGCACGCTGATCTGACAACTGGCCAGCAAGCCCAGCGTGACGGCCGGATTCAGGTGGGCACCGCTGATGTGGCCCAAGCTCTGGGCCAGCGTGGCGATGGCCAGG
CCGAATGCCAGCGACGTCTTCACCTCATCCAGCTGGTCGTTGCCGATGGCGGCCGTGATGCTGAG

Table 6: Select sequence alignments from the BLAST results (NCBI database) retrieved using the alewife intestinal AQP1 sequence (contig05[1]) as the query sequence.

	Sequence ID	Max Identity (%)	Query Coverage (%)	e-value
Diplodus sargus aquaporin 1 mRNA, complete cds	gi|358357311|gb|JN210582.1|	77.41	96	6.00E-84
Rhabdosargus sarba aquaporin 1 mRNA, partial cds	gi|334361414|gb|JF803845.1|	77.41	96	6.00E-84
Sparus aurata aquaporin 1 mRNA, complete cds	gi|54401743|gb|AY626939.1|	77.83	94	6.00E-84
Anguilla anguilla mRNA for aquaporin 1 (aqp 1 gene)	gi|73852959|emb|AJ564420.1|	78.48	86	2.00E-83
Amphiprion melanopus aquaporin mRNA, partial cds	gi|309252241|gb|HM768895.1|	77.24	93	4.00E-80
Acanthopagrus schlegelii aquaporin 1 mRNA, complete cds	gi|133779720|gb|EF451961.1|	77.11	94	1.00E-79
Anguilla japonica AQP-1S mRNA for aquaporin 1, complete cds	gi|34013385|dbj|AB094502.1|	77.84	86	1.00E-79
Anguilla japonica AQP-1L mRNA for aquaporin 1, complete cds	gi|34013383|dbj|AB094501.1|	77.84	86	1.00E-79
Danio rerio aquaporin 1a mRNA, complete cds	gi|116805723|gb|DQ887675.1|	76.31	91	3.00E-76
Danio rerio aquaporin 1 mRNA, complete cds	gi|54401739|gb|AY626937.1|	76.31	91	3.00E-76
Centropristis striata aquaporin 1 mRNA, partial cds	gi|54401737|gb|AY626936.1|	76.34	93	3.00E-76
Fundulus heteroclitus aquaporin-1 mRNA, complete cds	gi|209490728|gb|EU780153.1|	76.27	93	1.00E-74
Cynoglossus semilaevis aquaporin 1 mRNA, complete cds	gi|295445029|gb|HM013715.1|	75.93	91	1.00E-73
Hippoglossus hippoglossus aquaporin-1aa (Aqp1aa) mRNA, complete cds	gi|339635337|gb|HQ185294.1|	76.03	93	5.00E-73
Dicentrarchus labrax aquaporin 1 (AQP1) mRNA, complete cds	gi|124269015|gb|DQ924529.3|	75.18	93	4.00E-68

[1]CAACAAGCTCGGAGTGAACAGTCTTTCTGGTGTGACCCCAAGTCAAGGTATTGGCATCGAGCTACTGGCCACCTTCCAGCTCGTCCTGTGTGTCATAGCAACCACAGATAAAAGACGG
CGGGATGTGACAGGTTCCGCCCCCCTGGCCATTGGTCTCTCTGTTGCTCTGGGCCACTTGACAGCCATAAGTTTCACTGGGTGTGGCATCAACCCTGCTCGGTCCTTTGGTCCTGCCGTC
GTTACGAGCGATTTCGCAAATCACTGGGTGTACTGGGTCGGGCCCATGTGTGGAGGCGTGGCTGCTGCTCTCGTGTATGACTTCCTGCTATATCCAAAAACGGACGATTTCCCCGACCG
CATGAAGGTGCTCATGAGCGGACCGGCTAAAGACTACGATGTCAACGGGGCAGAGGACCCCACAGGAGTTGAGCTGACGTCA

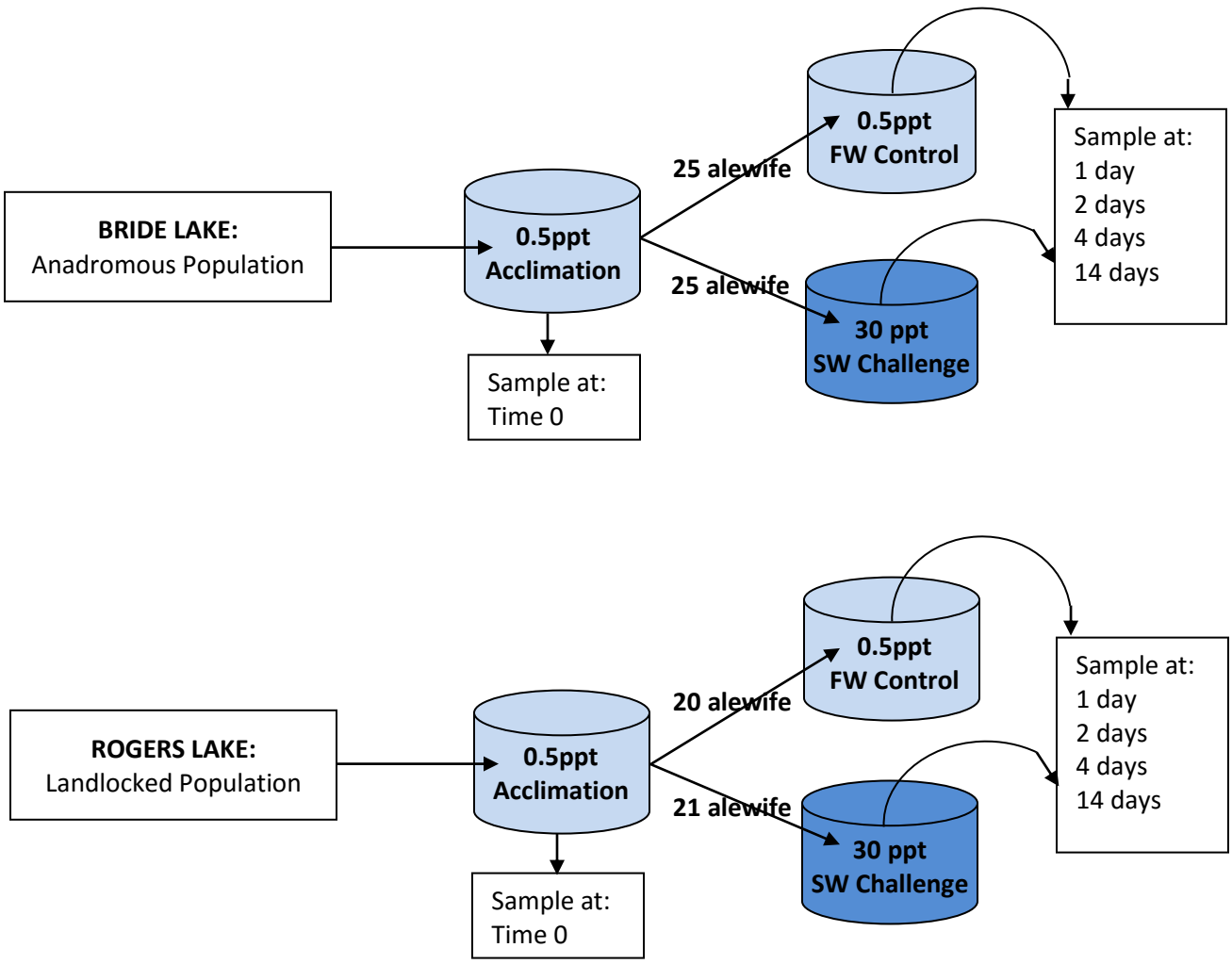


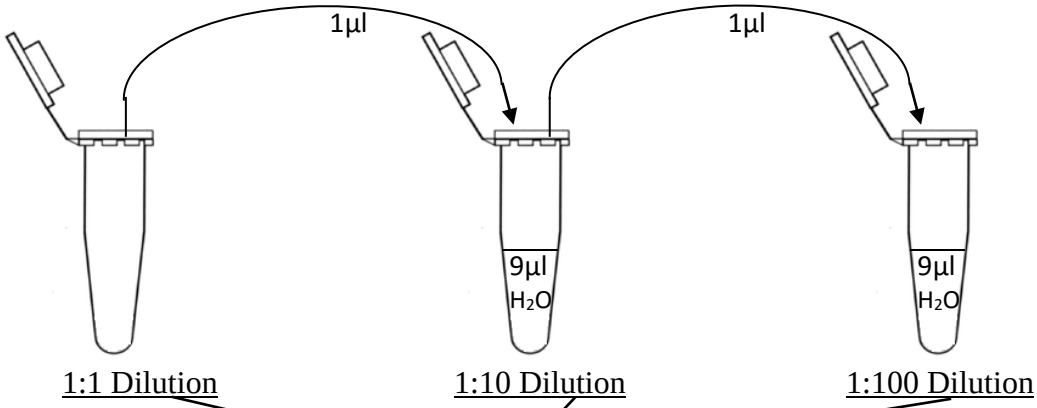
Figure 1: Experimental setup for salinity challenges: Anadromous and landlocked alewives were held in separate acclimation tanks, and then transferred to freshwater control tanks at 0.5ppt and seawater challenge tanks at 30ppt.

Step 1: **Serial Dilution**

1µl of cDNA and primers added to Real-Time qPCR solution

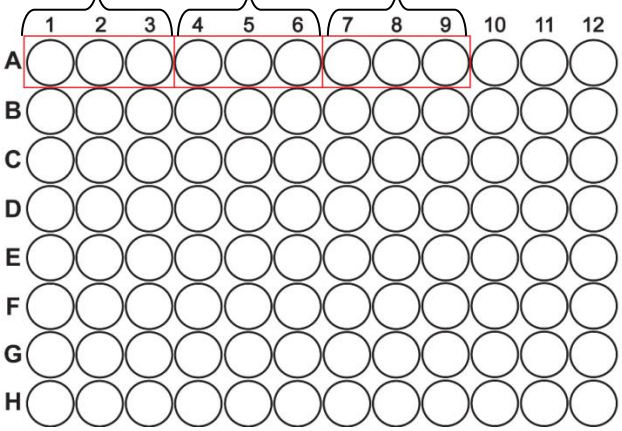
1µl of 1:1 dilution + 9µl of H_2O

1µl of 1:10 dilution + 9µl of H_2O

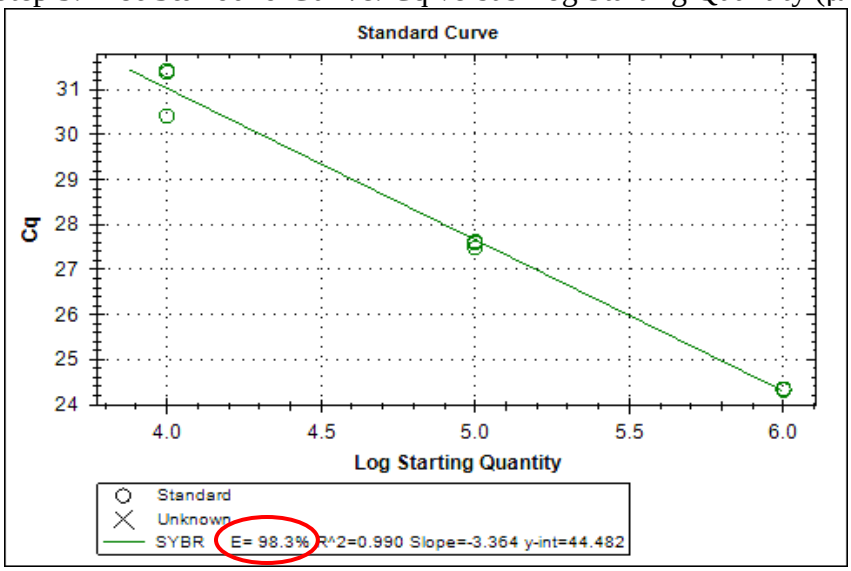


Step 2: **Real-Time qPCR**

The three dilutions are added to the 96-well plate in triplicate



Step 3: **Plot Standard Curve**: Cq versus Log Starting Quantity (µl)



The efficiency (E) of the primers is calculated from the slope of the standard curve using the following equation:

$$E = 10^{-1/slope}$$

Figure 2: The procedure for generating a standard curve, which was performed with both *AQP1* and *EF1a* primers to determine the efficiency values (E) for each primer set.

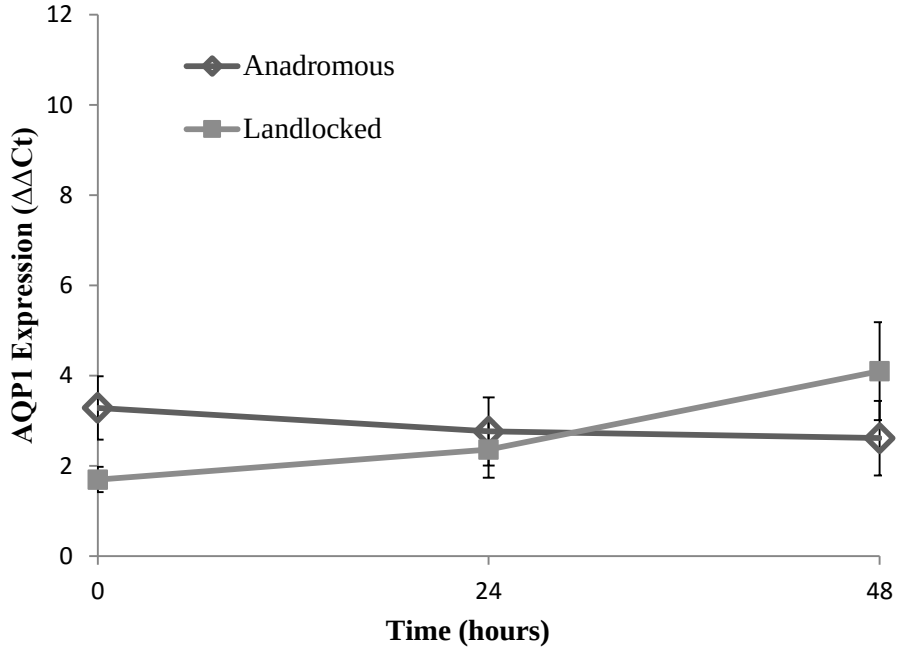


Figure 3: Intestinal *AQP1* expression of two alewife ecotypes (anadromous and landlocked) subject to 0.5ppt freshwater control treatment over 48 hours.

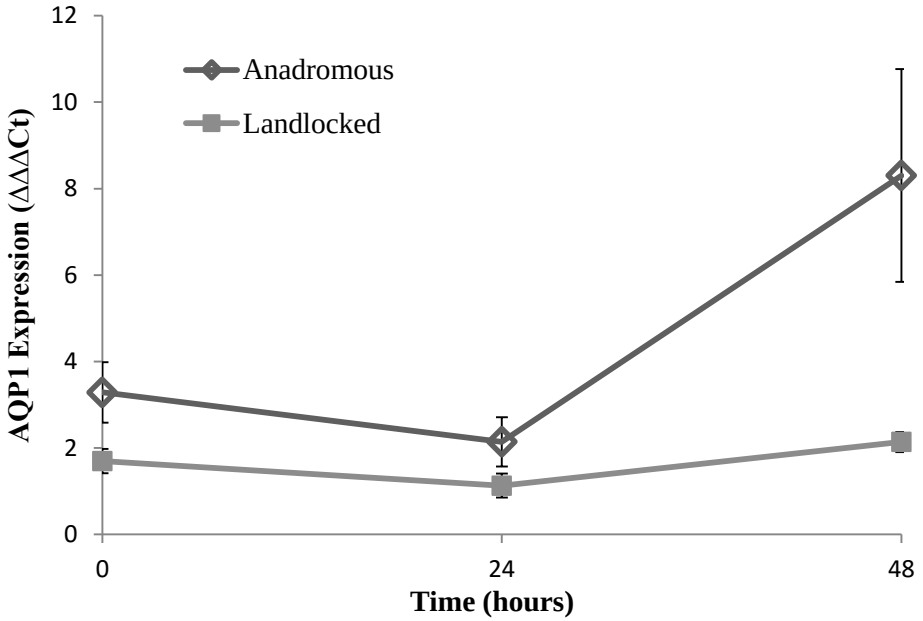


Figure 4: Intestinal *AQP1* expression of two alewife ecotypes (anadromous and landlocked) subject to 30ppt seawater challenge over 48 hours. Significant ecotype by treatment effect with p-value = 0.041 (linear mixed model).

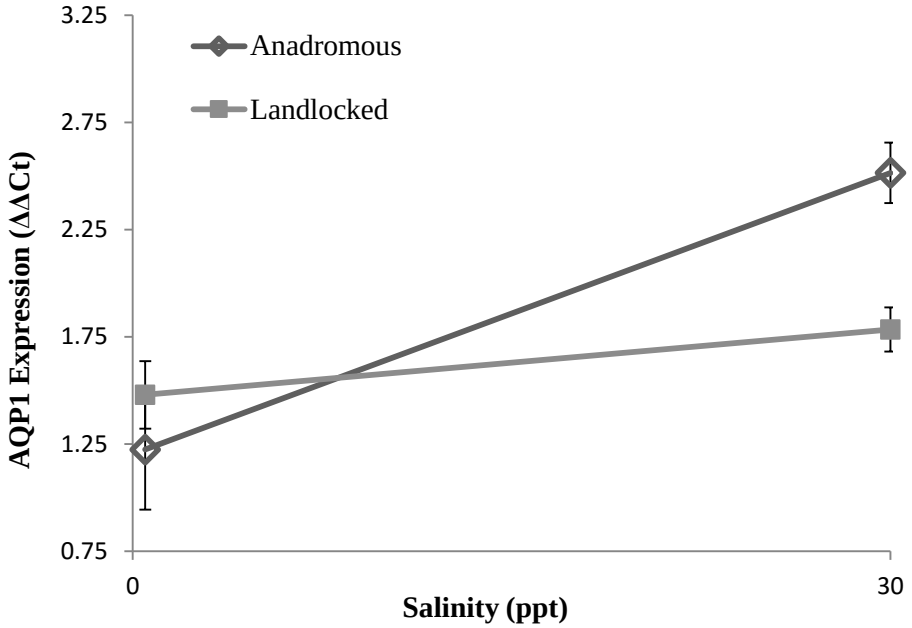
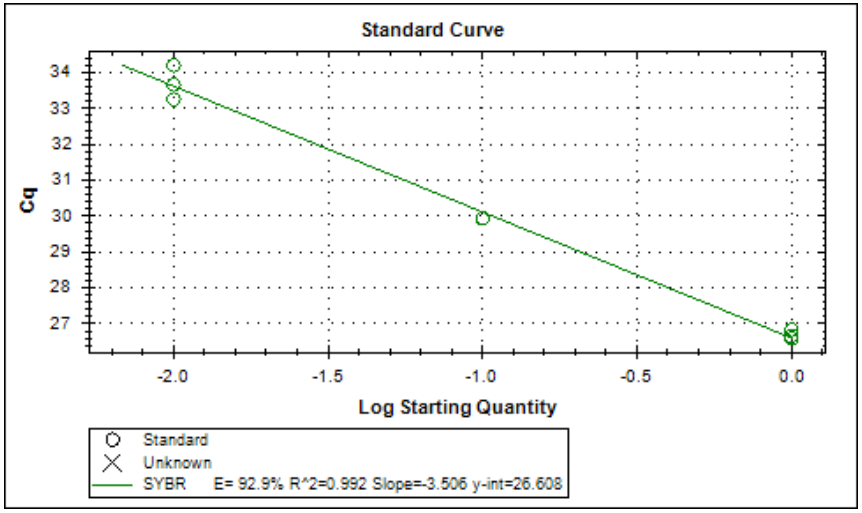
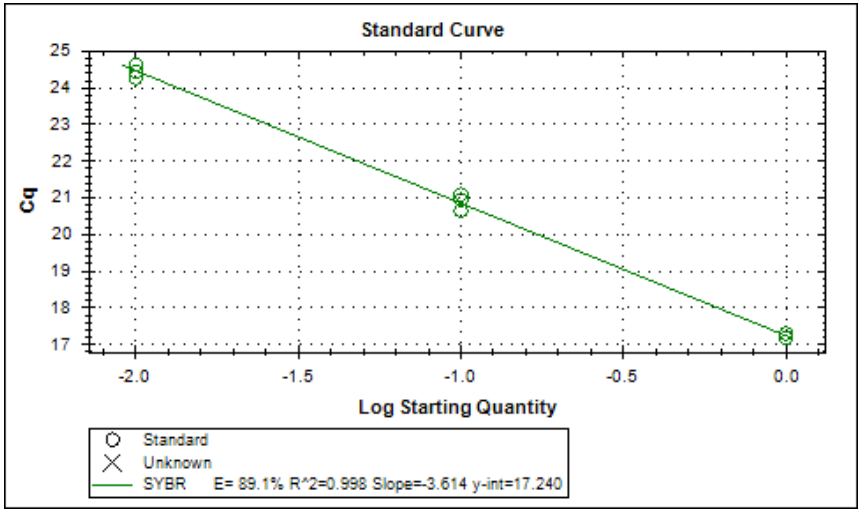


Figure 5: Intestinal *AQP1* expression in two alewife ecotypes (anadromous and landlocked) acclimated for 4 or 14 days to freshwater at 0.5ppt and seawater at 30ppt. There was no significant difference in *AQP1* expression over time between the two sampling periods (4 and 14 days), so expression values at each time point were average together. Ecotype by treatment interaction with p-value = 0.040 (ANOVA).

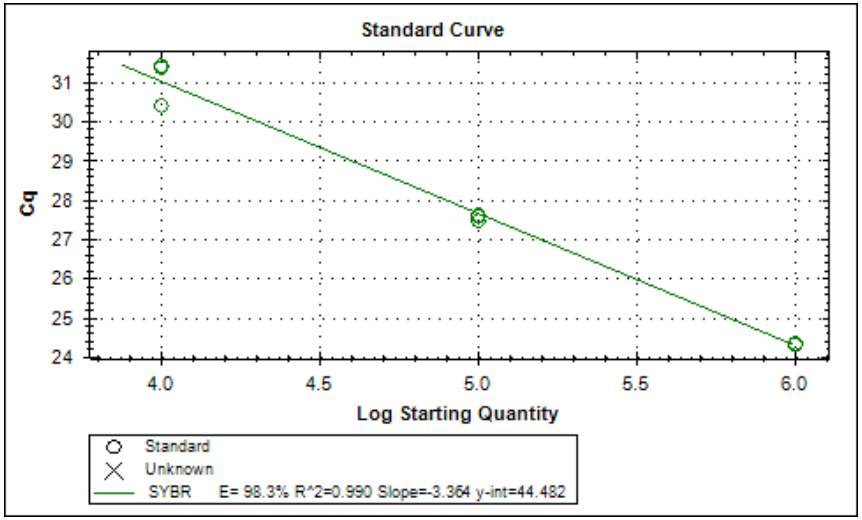
APPENDIX A



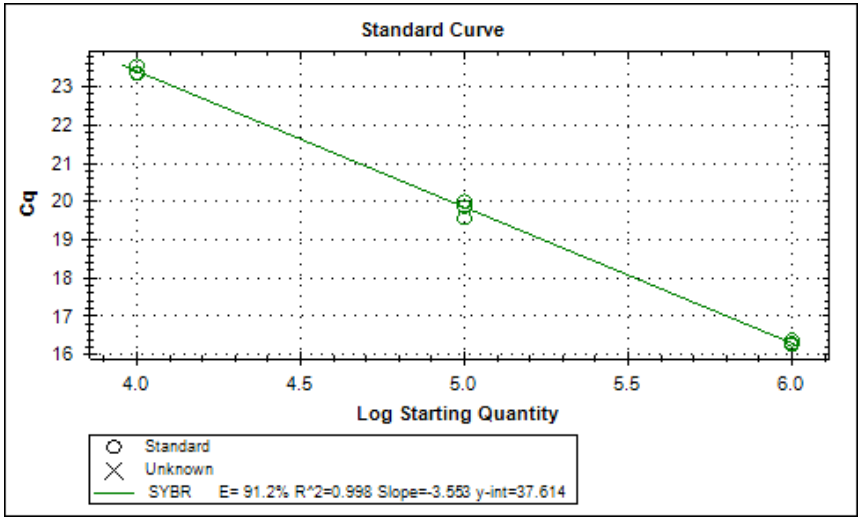
a) Efficiency = 92.9%



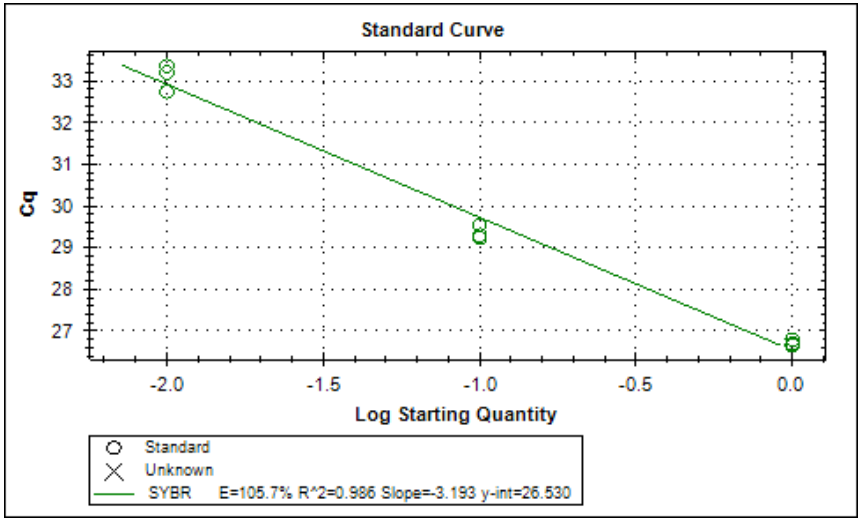
d) Efficiency = 89.1%



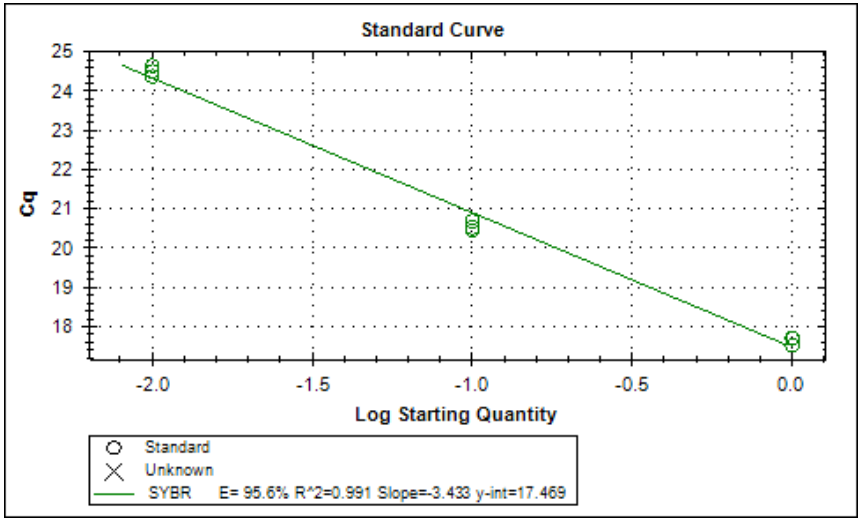
b) Efficiency = 98.3%



e) Efficiency = 91.2%



c) Efficiency = 105.7%



f) Efficiency = 95.6%

Figure 1: Three standard curves and efficiency values for *AQP1* primers (a-c) and three standard curves and efficiency values for *EF1a* (d-f) primers. The mean efficiencies for each primer set (1.99 for *AQP1* and 1.92 for *EF1a*) were used in all expression level calculations.