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Authors
Mundy, Paige C
Jeffries, Ken M
Fangue, Nann A
et al.

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Differential regulation of select osmoregulatory genes and Na^+/K^+-ATPase paralogs may contribute to population differences in salinity tolerance in a semi-anadromous fish

Paige C. Mundy[a,*], **Ken M. Jeffries**[a,b], **Nann A. Fangue**[c], **Richard E. Connon**[a,*]

[a]Anatomy, Physiology & Cell Biology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, California, USA 95616.

[b]Department of Biological Sciences, University of Manitoba, 50 Sifton Road, Winnipeg, MB R3T 2N2 CA

[c]Wildlife, Fish & Conservation Biology, University of California, Davis, One Shields Avenue, Davis, California, USA 95616.

Abstract

The Sacramento splittail (*Pogonichthys macrolepidotus*) is a species of special concern that is native to the San Francisco Estuary, USA. Two genetically distinct populations exist and differ in maximal salinity tolerances. We examined the expression of 12 genes representative of osmoregulatory functions in the gill over a 14 day time course at two different salinities [11 or 14 PSU (Practical Salinity Units)] and revealed that each population showed distinct patterns of gene expression consistent with population differences in response to osmotic regimes. The relatively more salinity-tolerant San Pablo population significantly upregulated nine out of the 12 transcripts investigated on day 1 of 11 PSU salinity exposure in comparison to the day zero freshwater control. Three transcripts (*nka1a*, *nka1b*, and *mmp13*) were differentially expressed between the populations at 7 and 14 days of salinity exposure, suggesting a reduced ability of the relatively salinity-intolerant Central Valley population to recover. Additionally, a phylogenetic analysis of several Sacramento splittail Na^+/K^+-ATPase α1 sequences resulted in grouping by paralog rather than species, suggesting that different isoforms of this gene may exist. These findings, together with prior research conducted on the Sacramento splittail, suggest that the San Pablo population may be able to preferentially regulate select osmoregulatory genes, including different Na^+/K^+-ATPase α1 paralogs, to better cope with salinity challenges.

Graphical abstract

*Corresponding author at: 1089 Veterinary Medicine Drive, Davis, CA 95616, reconnon@ucdavis.edu.

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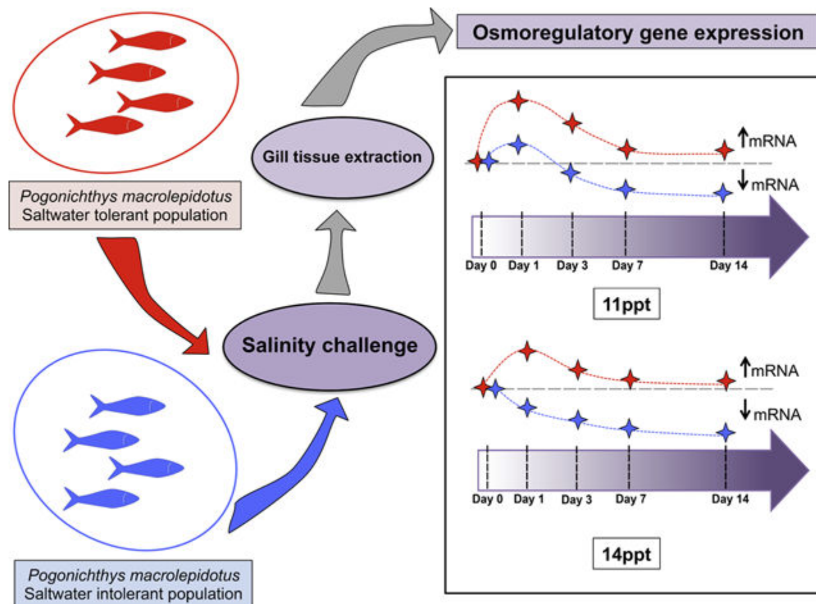

Keywords

Stress; osmoregulation; mRNA abundance; estuary; Sacramento splittail; Pogonichthys macrolepidotus

INTRODUCTION

Salinity is known to contribute to habitat limitation and physiological adaptation in aquatic organisms, and fluctuations are an environmental factor that many aquatic organisms encounter. Events driven by climate change, such as extended drought and sea level rise with subsequent saltwater intrusion into estuaries, increase the risk of aquatic organisms encountering salinity as an environmental stressor (Knowles and Cayan, 2002; Martinho et al., 2007). Teleost fishes exhibit numerous physiological mechanisms to handle changes in environmental salinity. Euryhaline fish can tolerate a broad range of fresh- and saltwater environments, and have developed an array of physiological mechanisms with which to cope. Fish maintain water and salt balance during salinity challenges via osmoregulation, primarily at the gill. In fresh water (hypoosmotic environments), fish actively uptake salts (Na^+ and Cl^-) across the gills, whereas fish in salt water (hyperosmotic environment) must excrete salts across the gill (Dymowska et al., 2012). During this process, certain cellular pathways in the gill, such as those involved in ion exchange, cell volume and restructuring, and a general stress response, are activated (Dymowska et al., 2012; Evans et al., 2005; Hwang et al., 2011). These pathways are also known to be targets of selection during adaptation driven by salinity (Whitehead et al., 2011).

Integral to the osmoregulation process in fish is Na^+/K^+-ATPase (NKA), an ion pump that powers ion transport across the membrane and is abundant in the gill. There are three distinct subunits of NKA including α (main catalytic unit), β (provides structural support for protein folding and placement within the membrane) and ϒ (adapts to alter kinetic function)

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(Blanco, Gustavo & Mercer, 1998; Clausen et al., 2017). It is known that the NKA subunit α (NKAα) has paralogs in multiple fish species, exhibiting specific electrochemical properties that lead to differential ion transport in fresh water or salt water. Transcript abundances of different NKAα1 paralogs are known to change in response to salinity exposure in multiple fish species. NKAα1a and NKAα1b are often referred to as the freshwater and saltwater isoforms; observed in some species to be upregulated in fresh water and salt water, respectively, in a term called isoform switching (Richards et al., 2003). Jorgensen (2008) found in Rainbow trout (*Oncorhynchus mykiss)* and Atlantic salmon *(Salmo salar)* gill that three particular amino acid substitutions (Lys^{783}Asn, Val^{933}Asp and Ser/Thr^{960}Glu) between NKAα1a and NKAα1b give rise to the functional differences between isoforms. Specifically, the mutations result in a reduction of the Na^+ to ATP ratio from 3:1 in NKAα1b to 2:1 in NKAα1a, effectively altering the electrochemical properties such that NKAα1a is suited for ion uptake in fresh water and NKAα1b for secretion in salt water (Jorgensen, 2008).

Although this process has been well characterized in salmonid species, NKAα isoform switching applies across teleost species. It is hypothesized that NKAα1 isoforms have arisen from small scale gene duplication events and that parallel evolution has contributed to salinity tolerance in fish (Dalziel et al., 2014). Though characterized most notably in salmonids, Dalziel et al. (2014) predicted that this could be a conserved mechanism across teleosts. Outside of salmonids, the classical pattern of isoform switching in which NKAα1a is increased in fresh water and NKAα1b is increased in salt water can be found in several other teleost species including the cichlid Mozambique tilapia (*Oreochromis mossambicus)* and the freshwater perciform climbing perch (*Anabas testudineus*) (Ip et al., 2019; Tipsmark et al., 2002). The fashion by which NKAα1 isoform switching occurs can also vary by species outside of this classical pattern. For example, zebrafish (*Danio rerio*) are a freshwater stenohaline cyprinid model organism that have been found to perform isoform switching when introduced into a dilute environment (i.e. fresh water containing less than 10 μM Na^+), regulating α subunits during hypo-salinity exposure (Esbaugh et al., 2019). In European seabass (*Dicentrarchus labrax*), short and medium length saltwater acclimation does not appear to give rise to an apparent isoform switch; however, a longer term (2.5 years) exposure does (Blondeu-Bidet et al., 2016). Some euryhaline fish, such as the model organism Japanese medaka *(Oryzias latipes*), do not appear to exhibit clear isoform switching (Bollinger et al., 2016). Although medaka appear to have six separate NKAα1 isoforms, it was proposed that kinetic changes in Na^+ affinity during saltwater acclimation may be due to posttranslational modifications of the alpha subunit (Bollinger et al., 2016). This range of differences highlights the variability of osmoregulatory function across teleost species. Considering the increasing risk of salinity as an environmental stressor facing fish populations (Knowles and Cayan, 2002; Martinho et al., 2007), the variability in physiological function emphasizes the need to investigate species-specific mechanisms by which fish cope with salinity.

Salinity stress is particularly relevant for species residing in estuary habitats, such as the San Francisco Estuary (SFE), California USA. The SFE is a system that has undergone significant modifications contributing to the decline in abundance of many native fishes, notably since the 1980s (Feyrer et al., 2003; Reid, 2003; Kimmerer, 2005; Sommer et al.,

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2007; Thomson et al., 2010). Specifically, due to climate change and anthropogenic manipulation of water flow, saltwater intrusion has affected the SFE (Knowles and Cayan, 2002; Cayan et al., 2007; Cloern and Jassby, 2012), thus it offers a strong system to investigate mechanisms by which aquatic organisms cope with salinity stress.

The Sacramento splittail (*Pogonichthys macrolepidotus*) is a cyprinid minnow native to the SFE, currently listed as a "species of special concern" by the California Department of Fish and Wildlife (CDFW), and previously listed as a threatened species until 2003 by the U.S. Fish and Wildlife Service (Moyle et al., 2004; Moyle et al., 2015). The status of species of special concern is a classification determined by the state of California that refers to populations exhibiting population declines that are high susceptibility to risk from any factor(s), which if realized, would result in their listing as threatened or endangered status. Particularly, this classification implies that anthropogenic threat factors are unlikely to drive a species to extinction by itself, but will contribute to an increased extinction risk, on the timeline of 100 years (Moyle et al., 2015). Sacramento splittail typically live 7–9 years and adults are known to migrate into tributaries of the SFE in the winter to spawn in their natal seasonal flood plains during March to April. There are two main populations of the Sacramento splittail, which have been identified as genetically distinct using microsatellite markers, the Central Valley (CV) population, and the San Pablo (SP) population (Baerwald et al., 2007, 2008; Mahardja et al. 2015). The populations differ in their migratory regions and spawning grounds; the CV population inhabiting the nearly fresh waters of the Sacramento and San Joaquin Rivers and tributaries, and the SP population inhabiting the Napa and Petaluma Rivers and tributaries, which are regions of higher salinity due to proximity to the San Pablo Bay (Feyrer et al., 2015) (Figure 1). The Napa and Petaluma Rivers experience greater fluctuations in salinity resulting from tidal influence as well as saltwater intrusion (Figure 1). Otolith strontium signatures from Sacramento splittail captured in their respective population's spawning site revealed that during the first three months of life, the CV population experiences salinities below 1 PSU whereas the SP population inhabit water that fluctuates in salinity from 1–10 PSU (Freyer et al., 2010). In laboratory studies, Verhille et al. (2016) observed that both populations were able to tolerate 14 PSU exposure for at least 14 days without losing equilibrium. The SP splittail were able to tolerate 16 PSU for at least 14 days; however, the CV splittail lost equilibrium within seven days of 16 PSU exposure.

Drought conditions resulting in low river flow into the estuary can increase salinity in the SFE above the tolerance limit of the species. This increase in salinity is predicted to contribute to reproductive isolation of the populations, restricting gene flow, as well as low to moderate degree genomic differentiation (R_{st} = 0.024–0.042, F_{st} = 0.064) (Baerwald et al., 2007; Mahardja et al., 2015; Jeffries et al., 2019).

Previous work has characterized physiological differences between the two populations of Sacramento splittail. In response to a 14 day exposure to either 11 PSU or 14 PSU, the CV population exhibited a slower recovery of plasma osmolality, suggesting that the CV population is less tolerant of elevated salinities (Verhille et al., 2016). This postulation was supported at the transcriptomic level through RNA-sequencing of gill tissue from Sacramento splittail (Jeffries et al., 2019). The SP population was found to have greater

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transcriptomic plasticity (3.6-fold more transcripts responded than the CV population), as well as a robust response of transcripts consistent with gill remodeling, when exposed to 14 PSU.

Gill remodeling is a morphological change in gill tissue, driven by cellular events, and often occurs in teleosts when faced with an osmoregulatory challenge. It is typically characterized by an increase in interlamellar cell mass and an increased number of ionocytes, with these changes occurring after the exposure to salt water (Blair et al., 2016). Ionocytes are specialized mitochondria-rich cells that account for about 1–10% of cell types in gill tissue, dependent on species and environment (Dymowska et al., 2012; Hwang and Lee, 2007). In order to increase the number of, or alter, cells in a tissue, genes responsible for proliferation and turnover are necessary, especially regarding alteration in the extracellular matrix. The extracellular matrix mainly consists of proteoglycans and fibrous proteins, and is commonly altered toward proliferation and remodeling of epithelial tissue (Pedersen et al., 2015). Thus, genes chosen to investigate gill remodeling in this study carry out functions related to proliferation, cell restructuring, and cell signaling.

Transcript responses from the Jeffries et al. (2019) study were used to inform the design of the present study, such that select genes known to be important in osmoregulatory pathways in the gill were chosen for a focused investigation, including additional time points and salinities. We hypothesized that the SP population has the ability to upregulate genes specific to osmoregulation quicker and in a more robust manner than the CV population. We also examined whether the SP population may possibly transcriptionally regulate specific Na^+/K^+–ATPase α1 paralogs in a pattern consistent with isoform switching characterized in other fish species. We investigated mRNA abundance at 1, 3, 7, and 14 days of exposure to 11 and 14 PSU to assess whether the patterns of gene expression of each population would show distinct responses consistent with differing salinity tolerances to osmotic regimes, as well as conducted a phylogenetic analysis of several NKAα sequences.

METHODS

Fish collections and salinity exposures

Gill samples collected from individuals used in a previous study (Verhille et al., 2016), which examined the physiological response of the Sacramento splittail to salinity challenges, were used in the present study to evaluate mRNA abundance of key osmoregulatory genes. In brief, wild juvenile Sacramento splittail (>1 year) from the SP and CV populations were collected by the California Department of Fish and Wildlife.

The salinities of the capture locations in which these fish were collected ranged from 12.8 – 13.2 PSU for the SP population, and 0.1 – 5.5 PSU for the CV population. The fish were transported to University of California Davis, held in 150 L tanks in fresh water (0.4 PSU), and allowed to acclimatize to holding conditions for a minimum of 30 days before being subjected to salinity challenges. Juvenile Sacramento splittail were transferred to 20 L recirculating systems for salinity exposure. There were two replicate tanks (n=4–5 individuals per tank) for each population and exposure, totaling 12 tanks. Freshwater controls (0.4 PSU, as noted by time 0 from here on) were sampled 24 h after transfer for

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each population. Salinities were increased using Instant Ocean (Aquarium Systems, Mentor, OH) at a rate of 0.08‰ h^{-1} until they reached 11 or 14 PSU. Fish were maintained at targeted salinities and subsampled at 1, 3, 7, or 14 days, at which point they were euthanized in buffered tricaine methanesulfonate (MS-222) and sampled for gill tissue. Gill tissue was immediately frozen in liquid nitrogen and then stored at −80°C until use.

RNA extraction

Total RNA was extracted from gill tissue samples using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The extracted RNA was then tested for quality and concentration (ng μL^{-1}) using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Samples were accepted as being of sufficient quality having 260/280 and 260/230 ratios between 2.0 and 2.2 and 1.7 and 2.3, respectively. A subset of these same samples were processed using the same extraction protocol and were assessed using a Bioanalyzer and all had RIN (RNA Integrity Number) that ranged from 7.9–9.9.

cDNA synthesis

Complementary DNA (cDNA) was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen) according to the user's manual. Four μL of gDNA wipeout buffer was added to 1 μg total RNA to which water had been added to bring the total volume to 24 μL, and incubated at 42°C for 2 min. The Reverse Transcriptase (RT) master mix was then prepared by adding 5x Quantiscript RT Buffer, RT Primer Mix, and Quantiscript RT enzyme. Twelve μL of the RT master mix was used for each reaction. Reactions were incubated for 30 min at 42°C, followed by a denaturing step for 3 min at 95°C, and subsequently held at 4°C. Samples were then diluted 1:10 with nuclease-free water, and stored at −20°C until use.

Primer design

Using previous mRNA sequence data from Sacramento splittail (NCBI accession SRP077066), genes specific to osmoregulation that were differentially expressed by a minimum of a 2-fold upregulation or downregulation in a previous study (Jeffries et al., 2019), were chosen for analysis by quantitative Polymerase Chain Reaction (qPCR). In the Jeffries et al. (2019) study, gill tissue mRNA from 6 individuals from each population (SP and CV) exposed to 14 PSU for 72 or 168 h were sequenced. The genes chosen for qPCR analysis in this study represented several functional categories relevant to osmoregulation including ion regulation, general stress, and extracellular matrix remodeling. Ion regulation genes included Chloride channel protein 2 (*clcn2*), Na^+/K^+-ATPase α1a (*nka1a*), and Na^+/K^+-ATPase α1b (*nka1b*). The chosen NKAα sequences, labeled as *Pogonichthys macrolepidotus atp1a1a* and *Pogonichthys macrolepidotus atp1a1b* in Figure 6A and 6B, were selected because their pattern of transcript expression from the Jeffries et al. (2019) study reflects that of the freshwater and saltwater NKAα1 isoforms. Specifically, in both populations, *atp1a1a* was downregulated during 14 PSU saltwater exposure, whereas *atp1a1b* was upregulated during the saltwater exposure (Figure 6B). The transcripts are thus referred for the rest of this study as their proposed paralogs of *nka1a* and *nka1b* (referring to their respective proposed isoforms of NKAα1a and NKAα1b). General stress genes

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included Early growth response protein 1 (*egr1*), 10kDA heat shock protein (*ch10*), and Peptidyl-prolyl cis-trans isomerase (*fkbp9*).

Extracellular matrix remodeling genes included Collagenase-3 (*mmp13*), Protein Wnt-4a (*wnt4a*), Mitogen-activated protein kinase 3–10 (*m3k10*), Bone morphogenic protein 2 (*bmp2*), Frizzled 5 (*fzd5*), and Secreted frizzled-related protein 1 (*serp1*). Primers and probe pairs were designed using the Roche Universal Probe Library Assay Design Center (Roche Life Sciences, Indianapolis, IN, USA) and adjusted using Primer Express to more appropriately fit the parameters of the instrumentation used (Table 1).

Quantitative Polymerase Chain Reaction

Quantitative qPCR was conducted using ThermoFisher Maxima Probe/ROX qPCR Master Mix (Waltham, MA). Primers were tested for efficiency against pooled cDNA from all samples that was serially diluted 1:10 six times in replicates of three. Amplification efficiencies ranged between 95.6 and 108.9%. The reactions were run on 384 well plates using the 7900 HTA FAST platform thermocycler (ThermoFisher). The cycling conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. Amplification data were analyzed using Sequence Detection Systems software (SDS v2.4; Applied Biosystems). Relative gene expression was calculated using the $2^{-\quad CT}$ method, relative to the reference genes 60s ribosomal protein L7 (*rl7*) and 40S ribosomal protein S9 (*rs9*) (Livak and Schmittgen, 2001). GeNORM algorithm was used in qbase+ software (Biogazelle, Zwijnaarde) to assure the stability of reference gene expression across samples.

Na^+/K^+-ATPase α1 phylogenetic analysis within Sacramento splittail

Seventeen transcripts of the Sacramento splittail gill transcriptome were annotated as “Sodium/potassium-transporting ATPase subunit alpha-1”. Although the annotation was the same, the expression patterns of these transcripts varied in that one set of transcripts was downregulated during exposure to 11 PSU whereas the other set of transcripts was upregulated (Figure 6B). To investigate similarity between the 17 sequences identified as NKAα1, all sequences were aligned using ClustalW (Waterhouse 2009). Phylogenetic analysis was performed using RAxML-HPC2 on XSEDE (8.2.10) via CIPRES Science Gateway (Miller et al., 2010). The data was visualized using Dendroscope (Huson and Scornavacca, 2012). Branches not supported by a bootstrap value of 50 (out of 100 iterations) were collapsed, thus we show 12 sequences in Figure 6B.

Na^+/K^+-ATPase α1 phylogenetic analysis across multiple species

In order to further investigate possible paralog differences, the 17 *P. macrolepidotus* NKAα1 sequences that were returned as “Sodium/potassium-transporting ATPase subunit alpha-1” were evaluated in a maximal likelihood analysis, including a range of teleost NKAα for comparison. The other teleost NKAα sequences used in the analysis were gathered in a combination of BLAST returns and manual selection. The representative sequences chosen for further qPCR analysis (*Pogonichthys macrolepidotus atp1a1a* and *Pogonichthys macrolepidotus atp1a1b*) were entered separately to tBLASTX2.4.0 (NCBI). Approximately 30 BLAST hits were selected from each return. Each hit was selected as the representative species from each group, chosen considering the most well annotated genome, max score,

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lowest e-value, and largest query coverage percentage. Manually selected sequences included NKAα sequences known to alter expression in either fresh water or salt water (Supplementary Table 1), as well as a range of *Danio rerio* NKAα (as some isoforms were found in preliminary trees to be similar in sequence to Sacramento splittail NKAα). A sequence for Pacific purple sea urchin (*Strongylocentrotus purpuratus*) sodium/potassium ATPase alpha subunit (NM_001123510.1) was included for the purpose of rooting the tree. The sequences were aligned using ClustalW and the resulting alignment was then input into the Gblocks program (gblocks 0.91b) to select for conserved regions. A phylogenetic analysis was then performed on the trimmed alignment using RAxML-HPC2 on XSEDE (8.2.10) via CIPRES Science Gateway (Miller et al., 2010). Branches with a bootstrap value of 50 (out of 100 iterations) or below were collapsed. The tree with best bootstrap values was viewed using Dendroscope software (Huson and Scornavacca, 2012).

Statistical Analysis

To measure differences in expression of each gene between populations within a salinity exposure, the lm function in R was used to test the effects of time, population, and time*population interaction on gene expression. A two-way ANOVA was completed using this linear model and the anova function in R (version 3.3.2). As a post-hoc analysis, pairwise comparison was then conducted using lsmeans in R, using the Tukey's HSD method as p-value adjustment. The values are reported as mean ± SEM.

To measure differences of expression of each gene within a population and salinity exposure, the lm function in R was used to test the effect of time on gene expression (subsetted for one salinity exposure and population). Contrasts were measured via lsmeans multiple comparison test in R, using the contrast method trt.vs.ctrl to compare time 0 as control with times 1, 3, 7, and 14. The p-value was adjusted using the dunnetx method for 4 tests (time 0 versus 1, 3, 7, and 14).

RESULTS

Transcript expression

Significant differences between populations were detected in expression of both proposed paralogs of Na^+/K^+-ATPase α1 (*nka1a* and *nka1b*) at 14 days in the 11 PSU exposure, in which the SP population is upregulated and the CV population is downregulated ($t(96)= -2.85$ $p<0.05$; $t(96) = -3.46$ $p<0.01$) (Figure 2). Within the SP population, both *nka1a* and *nka1b* were significantly upregulated on day 1 and 3 at 11 PSU exposure in comparison with day 0 (*nka1a* day 1: $t(48)= 3.17$ $p<0.01$; *nka1a* day 3: $t(48)= 2.767$ $p<0.05$; *nka1b* day 1: $t(48)= 2.68$ $p<0.05$; *nka1b* day 3: $t(48)= 2.92$ $p <0.05$) (Figure 5). At day 14 at 11 PSU in the SP population, *nka1b* was significantly upregulated ($t(48)= 2.76$, $p<0.05$), and at day 7 at 14 PSU exposure, the SP population exhibited a significant increase in *nka1b* ($t(43)= 2.68$, $p<0.05$) (Figure 5). In the CV population, *clcn2* is downregulated at 7 and 14 days at 14 PSU exposure, in comparison to day 0 ($t(41)= -2.757$ $p<0.05$; $t(41)= -3.192$ $p<0.05$) (Figure 5).

There were no statistically significant differences between general stress genes investigated between populations (Figure 3). All general stress genes showed a significant increase in

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expression in the SP population at day 1 in comparison to day 0 at 11 PSU (*fkbp9* t(48)= 3.97 p<0.001; *egr1* t(47)= 3.126 p<0.05; *ch10* t(48)= 3.56 p<0.01) (Figure 5). At day 3, *fkbp9* was still significantly upregulated in the SP population in comparison to day zero (t(48)= 3.789 p<0.01) (Figure 5). At 14 PSU, *egr1* was significantly upregulated in the SP and CV population at day 3 in comparison to day 0 (SP t(44) = 3.74 p<0.01; CV t(41)= 2.932 p<0.05) (Figure 5). In the CV population, only *egr1* showed a significant increase at day 1 in comparison to day 0 at 11 PSU (t(47)= 3.13, p<0.05) (Figure 5). At 14 PSU, *egr1* was significantly downregulated at day 3 and 14 in comparison to day zero at (t(41)= 2.932 p<0.05, t(41)= −3.552 p<0.01) (Figure 5).

Significant differences between populations were detected in expression of *mmp13* at 7 and 14 days at 11 PSU, the SP population exhibiting higher expression than the CV population (Figure 4) (t(96)= −2.734, p<0.05, t(96)= −2.718 p<0.05). No differences between populations were found at 14 PSU. In the SP population, *wnt4a*, *m3k10*, *bmp2*, and *fzd5* were all significantly increased at 1 day exposure to 11 PSU in comparison to day 0 (*wnt4a* t(49)= 5.983 p<0.0001; *m3k10* t(49)= 5.031 p<0.0001; *bmp2* t(48)= 2.861 p<0.05; *fzd5* t(49)= 6.571 p<0.0001) (Figure 5). At day 3 at 11 PSU in the SP population, *wnt4a* was still upregulated (t(49)= 5.983 p<0.05). At 14 PSU in the SP population, *fzd5* was significantly upregulated at day 1 in comparison to day 0, and *m3k10* was significantly upregulated at day 3 at 14 PSU in comparison to day 0 (t(44)= 2.681 p<0.05) (Figure 5). In the CV population at 14 PSU, *m3k10* was significantly downregulated at day 3 in comparison to day 0 (t(43)= −2.850 p<0.05) (Figure 5).

Relationship of Sacramento splittail NKAα1 sequences

Out of the 17 transcript sequences annotated as “Sodium/potassium-transporting ATPase subunit alpha-1” from a previous Sacramento splittail transcriptomics study(Jeffries et al. 2019), there was a clear difference in mRNA abundance patterns showing that during exposure to 14 PSU, some transcripts were consistently downregulated at 3 and 7 days in both populations, and others upregulated at 3 and 7 days in both populations (Figure 6B). When the transcripts were aligned and analyzed for phylogenetic relationship, those that showed similar transcriptional patterns, as indicated as being grouped as “*nka1a*” or “*nka1b*” in Figure 6B, in some cases were more similar phylogenetically (Figure 6A). Specifically, sequences 15 and 17, and sequences 11 and 12 exhibited a bootstrap value of 100 at separation (Figure 6A). Although grouped by mRNA abundance pattern, it remains unclear if these sequences are splice variants or variants of the same gene.

Relationship of Sacramento splittail sequences of interest to other teleost NKAα1

The Sacramento splittail NKAα grouped by mRNA expression group. Outside of the other Sacramento splittail sequences, the *Pogonichthys macrolepidotus atp1a1a* sequences is closest in relation to *Danio rerio atp1a1.2* (NM_131687.1) and *Danio rerio atp1a1.5* (NM_178099.2). The *Pogonichthys macrolepidotus atp1a1b* sequence is located in a group separated by three branches from the *Pogonichthys macrolepidotus atp1a1a*. Outside of Sacramento splittail sequences, the closest sequences were *Carassius auratus atp1a1* (XM_026217736.1), *Sinoclocheilus rhinocerous atp1a1* (XM_06573961.1), and *Danio rerio atp1a1b* (AY008375.1).

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DISCUSSION

This study highlights population-specific responses to salinity stress in a species of conservation concern, the Sacramento splittail. Specifically, we focused on the cellular mechanisms of response to salinity stress in gill, an essential tissue in the osmoregulatory process. Fish gills consist of at least five different cell types and carry out functions critical to osmoregulation including ion transport, acid-base regulation, and ammonia excretion (Dymowska et al., 2012). In this study, differential mRNA abundance was used to evaluate osmoregulatory processes in the gill, including ion transport, extracellular matrix remodeling, and general stress pathways. We found that the SP population can better cope with salinity stress at the transcriptional level. At day 1 of 11 PSU salinity exposure, the SP population demonstrated the ability to most prominently upregulate transcripts specific to all categories of genes investigated (Figure 5). In contrast, the CV population did not exhibit a robust increase in mRNA abundance at day 1 of salinity exposure to either 11 PSU or 14 PSU, suggesting at the transcriptional level, that they may have a reduced ability to rapidly respond to salinity stress (Figure 5). The present study demonstrated a comprehensive temporal display of osmoregulatory capabilities in two populations of Sacramento splittail with differences in salinity tolerances.

Three transcripts, *nka1a*, *nka1b*, and *mmp13*, were differentially expressed between the populations at 7 or 14 days at 11 PSU (Figures 2, 4). In the CV population, there was a general lack of significant upregulation on day 1, followed by a pattern of downregulation on day 3 and 14. The pattern of transcriptional expression observed in the CV population suggests they have a reduced ability to initially respond to an osmoregulatory challenge. In contrast, the SP population exhibits a robust transcriptional response on day 1 of exposure, along with a pattern of transcription levels returning to those at 0.4 PSU. Gill tissue of fish sampled from the SP population has been found to be more transcriptionally plastic, exhibiting 1.4 and 3.6-fold more transcripts that responded to a salinity challenge than the CV population after three and seven days, respectively (Jeffries et al, 2019). Our results suggest that the SP population responds to an osmoregulatory challenge more rapidly that the CV population, and is able to return expression of the selected 12 transcripts assessed in the present study to pre-exposure levels by day 3 at 14 PSU.

The 11 PSU exposure often resulted in greater mRNA abundance than the 14 PSU exposure in both populations, but particularly so in the SP population. This may be an artifact of the proximity to the fish's maximal salinity tolerance (16 PSU). It has been suggested that as stressors approach tolerance limits, it alters the expression patterns of various cellular pathways leading to reduced expression of transcripts not involved in survival mechanisms (Jeffries et al. 2018). At 11 PSU, the SP fish most likely can transcriptionally activate the pathways for osmoregulation at the gill. Although physical adverse effects were not seen in SP population at 14 PSU, it could be that they are diverting energy to different pathways and in other organs, such as cell maintenance and repair rather than restructuring at the gill.

Ion channels

Differences in mRNA abundance of genes encoding for proteins involved in gill remodeling were found between populations. The process of gill remodeling results in changes in

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ionocytes, including increased ionocyte density. Additionally, numerous subtypes of ionocytes exist and can be differentially expressed depending on the environmental state the organism is experiencing (Dymowska et al., 2012). Some functional categories of the subtypes include Na^{+} transporting/H^{+} excreting, Ca^{2+} transporting, Cl^{-} transporting/$HCO3^{-}$ excreting, and Na^{+}/Cl^{-} co-transporting (Dymowska et al., 2012). Considering this, the observed differences in ion channel-related gene expression in the gill should be expected. The ion channels evaluated in this study included *clcn2*, *nka1a*, and *nka1b*. Typically found in ionocytes, chloride channels participate in numerous functions including solute transport, pH regulation, and cell volume regulation (Dymowska et al., 2012; Jentsch et al., 2002). Although Chloride ion channel protein 2 (*clcn2*) mRNA abundance was not significantly different between populations, this ion channel was significantly downregulated in the CV population at 14 days of exposure to 11 and 14 PSU in comparison to day 0, suggesting an inability to return to pre-exposure levels (Figure 5).

The other two ion channel transcripts evaluated were α subunits of Na^{+}/K^{+}-ATPases. The pattern of *nka1a* and *nka1b* transcript expression in the SP population supports the possibility that the SP population has the ability to preferentially regulate NKAα during saltwater exposure. In the SP population, significant increases in both *nka1a* and *nka1b* can be seen days 1 and 3 of exposure to 11 PSU, whereas *nka1b* remains upregulated on day 14 at 11 PSU, and day 7 at 14 PSU (Figure 5). Although *nka1a* was initially upregulated on days 1 and 3 at 11 PSU, *nka1a* was not significantly different from day 0 freshwater control on days 7 or 14, and was not significantly different from day 0 at 14 PSU (Figure 5). This described pattern of expression in the SP population is not exactly consistent with other observed teleost species as described in the literature. For example, mummichog (*Fundulus heteroclitus*) have been shown to transiently increase *nka1a* expression after 1 day of saltwater exposure (transferred from brackish water). In this species, however, *nka1b* was not increased upon saltwater exposure (Scott et al., 2004) during the short period of exposure tested. The initial upregulation of *nka1a* at day 1 of 11 PSU exposure followed by a drop in expression to levels similar to freshwater control, paired with an upregulation of *nka1b* at day 7 or day 14 (14 PSU or 11 PSU, respectively), suggests that the SP population of Sacramento splittail may have the ability to preferentially differentially regulate NKAα during salinity exposure.

The CV population did not show the same pattern of NKAα transcript expression as the SP population. Neither *nka1a* or *nka1b* were significantly different from control, but exhibited a trend of decreased expression (Figure 2). In fish exposed for 14 days at 11 PSU, both proposed paralogs exhibited significantly different levels of mRNA abundance between populations (Figure 2). Further investigation to define the roles of the described *nka1a* and *nka1b* is necessary to appropriately interpret the physiological relevance of the presented findings. Because the SP population has the ability to maintain *nka1b* upregulation during exposure to salt water, this may be a contributing factor to their ability to better handle salinity stress.

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Phylogenetic analysis

Because it has been hypothesized that adaptations in salinity tolerance in fish species can arise from duplication and diversification of NKAα1a (Dalziel et al., 2014), we investigated relationship of several Sacramento splittail NKAα1 sequences to each other, as well as to other teleost species. Sacramento splittail sequences that exhibited similar expression patterns from the Jeffries et al. (2019) study, shown in Figure 6B, in some cases had greater homology, grouped by mRNA abundance. *Pogonichthys macrolepidotus atp1a1a* (a sequence further investigated via qPCR in this study), grouped most closely with sequences 3 and 4 (also predicted to be *nk1a1a*). *Pogonichthys macrolepidotus atp1a1b* (investigated via qPCR in this study), grouped most closely to sequence 11 (also predicted to be *nk1a1b*).These sequences 11, 12, 15, and 17 were predicted by their expression patterns to be *nka1b* paralogs, considering they were upregulated in both populations at day 7 at 14 PSU exposure in a previous study (Figure 5B) (Jeffries et al., 2019). Particularly, the sequences that were chosen for qPCR analysis at all exposures and time points (sequences 10 and 12, named *nka1a* and *nka1b*, respectively) are separated by one branch (Figure 6A). Although not as distant as expected, they were compared to NKAα1 sequences from other teleost species, in order to determine if the two sequences of interest aligned with different known isoforms and paralogs.

In the phylogenetic analysis comparing the proposed Sacramento splittail *nka1a* and *nka1b* sequences to NKAα of other teleost species, the Sacramento splittail NKAα grouped by proposed paralog (Figure 7). Particularly, the Sacramento splittail sequence *Pogonichthys macrolepidotus atp1a1a* is separated from those sequences predicted to be *nka1a* (sequences 3 & 4, and 8 & 9) by 3 branches. Outside of the other Sacramento splittail sequences, the *Pogonichthys macrolepidotus atp1a1a* sequence is closest in relation to *Danio rerio atp1a1.2* and *Danio rerio atp1a1.5*. In this same group were *Sinocyclocheilus grahmi atp1a1* and *Carassius auratus atp1a1*. All sequences in this group are from teleosts in the *Cypriniformes* order.

The *Pogonichthys macrolepidotus atp1a1b* sequence is located in a group separated by three branches from the *Pogonichthys macrolepidotus atp1a1a*. This sequence was most closely related to sequence 11, which is predicted to be *nka1b*. The group also contained sequences 17 and 16, which are also predicted to be *nka1b*. Outside of Sacramento splittail sequences, the closest sequences were *Carassius auratus atp1a1*, *Sinoclocheilus rhinocerous atp1a1*, and *Danio rerio atp1a1b* (all included in the order *Cypriniformes*). Also included in this group is *Astyanax mexicanus atp1a1* and *Pygocentrus natterei atp1a1* (both are included in the order *Characiformes*).

Interestingly, the Sacramento splittail predicted *nka1a* and *nka1b* are closer to different *Danio rerio* NKAα's. Although *Danio rerio* is a freshwater fish that cannot tolerate salt water, they do appear to have the ability to perform isoform switching in response to low-ion environment. Esbaugh et al. (2019) found *atp1a1a.2* to be the most abundant in *D. rerio* gill (10-fold more abundant than other subunits) when in 15mM Na^+ or 1.5mM Na^+ conditions. However, once the *D. rerio* were moved below 0.01mM Na^+ (hypoosmotic conditions), atp1a1a.5 increased 100-fold. It is important to note that although most abundant in gill, *the D. rerio atp1a1a.2* is not found in the HR-type ionocytes (H^+-ATP-ase-rich ionocytes),

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where *atp1a1a.5* is located (Liao et al 2009); therefore it is hypothesized that in hypoosmotic conditions, *D. rerio* undergo isoform switching in the HR cell type from *atp1a1a.3* to *atp1a1a.5.*

Considering the groups of Sacramento splittail sequences are separated by 3 branches, this indicates the two groups are more similar by mRNA abundance group than by species alone. We suggest here that the Sacramento splittail contains at least two NKAα1 isoforms or paralogs in the gill. As described, it is uncertain whether these sequences are splice variants or variants of the same gene; therefore the existence of multiple paralogs or variants within the same mRNA abundance group is a possibility.

Also included in the analysis were sequences of NKAα isoforms known to respond to either fresh water or salt water (Figure 7). Neither Sacramento splittail group containing predicted *nka1a* nor *nka1b* grouped closer or further from either of the freshwater or saltwater inducible isoform group. This is informative of where these proposed paralogs fit more broadly, indicating closer relation to NKAα isoforms from teleosts in the same order (*Cypriniformes*), as the sequences in the freshwater or saltwater inducible groups are from teleosts in orders *Salmoniformes* or *Esociformes* (a freshwater sister clade to *Salmoniformes*).

Gill remodeling

The genes chosen to investigate gill remodeling in this study carry out functions related to proliferation, cell restructuring, and cell signaling. Specifically, genes involved in Wingless type (Wnt) signaling process (*wnt4a*, and *fzd5*) and Mitogen-activated protein kinases (MAPK) cell signaling pathway (*m3k10*), are activated by osmotic stress in gill tissue (Angers and Moon, 2009; Lai et al., 2009; Kültz and Avila, 1999), and thus were predicted to be upregulated in this study. Involved in ERK and TGFβ signaling, and important for cell signaling and cartilage formation (Wharton and Derynck, 2009), *bmp2* was also predicted to be upregulated in this study. Additionally, a gene involved in extracellular matrix degradation and remodeling process (Pedersen et al., 2015), Collegenase-3 (*mmp13*), was also predicted to be upregulated in response to salinity exposure. In the SP population, gill remodeling genes were significantly upregulated at day 1 of exposure, but recovered to control levels at days 3 and 7 of exposure (Figures 3, 4). At day 1 of 11 PSU exposure, *wnt4a*, *bmp2*, *fzd5* and *m3k10* were upregulated in comparison to day 0. At day 7 and 14 at 11 PSU exposure, *mmp13* was found to be significantly increased in the SP population in comparison to the CV population (Figure 4). In the 14 PSU exposure in the SP population, although *fkbp9* was increased on day 1 along with non-significant increases in *m3k10* and *mmp13*, a decrease in *m3k10* and *mmp13* was seen on day 3. In the SP population, the pattern of initial upregulation and recovery of genes to control levels at days 3 and 7 of exposure at 11 PSU may be due to their ability to readily activate the gill remodeling process, and once the necessary protein levels are functionally active there may no longer be a need for further transcriptional activity. Because 14 PSU is near the upper limits of this organism’s salinity tolerance, this pattern may signify a weaker activation of the gill remodeling process, or potentially that the fish are diverting energy to processes outside the gill, physiological parameters that were not evaluated as part of this study.

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The CV population seemingly have the ability to moderately upregulate some gill remodeling genes, but these are mostly downregulated at day 1 and are unable to recover to pre-salinity-stress conditions (0.4 PSU), with significant downregulation by 14 days of exposure to 11 PSU or 14 PSU (Figures 3, 4). These findings suggest that the SP population may have the ability to initially handle and recover from a mild osmotic challenge at 11 PSU; whereas the CV population does not upregulate the transcripts necessary to perform the physiological function of gill remodeling.

General Stress Response

A general stress response can be defined as a reaction to the threat of macromolecular damage, and the pathways involved are often evolutionarily conserved and present in a vast array of cell types (Kültz, 2003). A salinity challenge, especially when outside of, or close to, an organism's range of tolerance can therefore be considered an environmental stressor. Thus, exploring some conserved general stress responses in gill tissue during a salinity challenge can provide further insight into the physiological processes occurring at the gill. There were no significant differences in general stress response genes between the two populations of Sacramento splittail. In the SP population, there was an initial upregulation in general stress genes (*ch10*, *egr1*, and *fkbp9*) at days 1 and 3, which were then downregulated days 7 and 14 (Figures 3, 5). Upregulated on day 1 at 11 and 14 PSU exposure, *egr1* is an immediate early response gene and zinc finger transcription factor, known to function in response to NaCl and urea, and signal for apoptosis and cell proliferation (Cai et al., 2004). Heat shock protein 10kDA (*ch10*), upregulated on day 1 at 11 PSU exposure, is a chaperone protein localized in the mitochondrial matrix, and functions in cell signaling and transduction, especially under environmental stress (Mohindra et al., 2015). Upregulated on days 1 and 3 at 11 PSU exposure, *fkpb9* contains regions identified with localization to the endoplasmic reticulum, where it is hypothesized to act as a molecular chaperone during cell stress (Patterson et al., 2002).

In the CV population, a similar trend of upregulation on day 1 is apparent in the 11 PSU exposure, and is significant with *egr1*; however, in the 14 PSU exposure this trend is not as apparent (Figures 3, 5). The mRNA abundance patterns of the general stress genes appearing similar between populations is not unexpected, as these chaperones and early response genes functions are generally conserved and they are typically upregulated under environmental stresses, including salinity, hypoxia, thermal stresses (Feder and Hofmann, 1999; Kültz, 2005; Morimoto, 1998).

CONCLUSIONS

Examining how fish populations respond to salinity challenges is critical, considering the increasing rates of saltwater intrusion events in coastal systems. Here we found that differences exist in patterns of mRNA abundance between the SP and CV populations of Sacramento splittail, the results of which suggest that the SP population has the ability to better cope with osmoregulatory challenges. Transcriptomic responses have been suggested to be a particularly useful approach for identifying organismal responses to environmental stressors in fishes of conservation importance (Connon et al., 2018). The Sacramento

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splittail is a species of special concern, considered as a single population under the United States Fish and Wildlife Service. The physiological differences between populations determined in this and prior studies can inform conservation efforts of the species, such as providing insight to salinity tolerance, and potential outcomes of predicted salinity exposures of the populations. Specifically, conservation managers may consider directing resources to favor the preservation of the SP population, considering the SP population is known to have a smaller population size in comparison to the CV population, but is potentially an important source of genetic variation required for adaptation to salinity tolerance in the species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlight:

- Osmoregulatory transcripts are differentially expressed in two populations of a semi-anadromous fish.
- Possible Na^+/K^+-ATPase paralogs are identified in a non-model teleost species.

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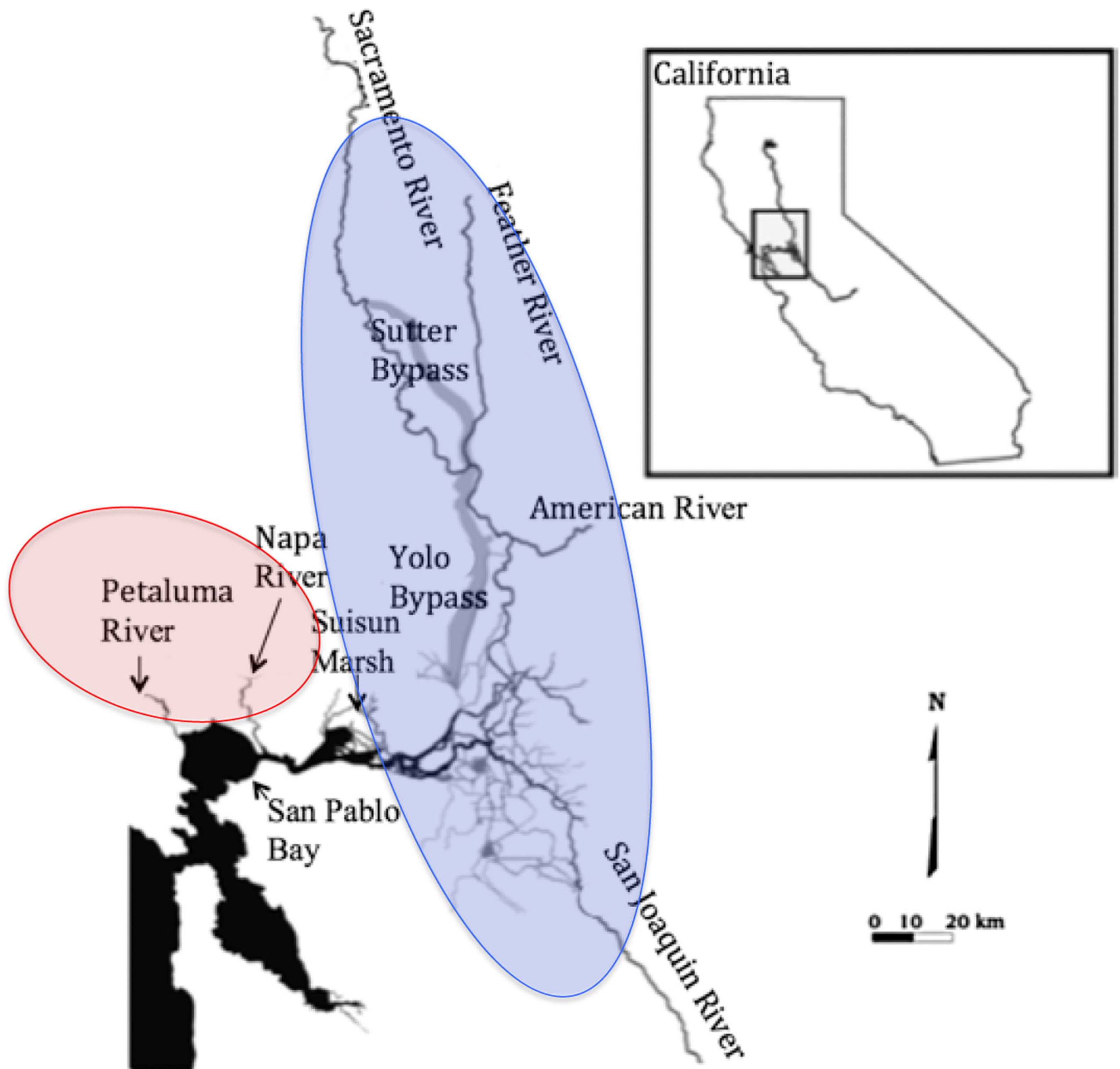

Figure 1. Map of SFE.

Early rearing habitats for both the Central Valley (blue) population and San Pablo (red) population of Sacramento splittail (*Pogonichthys macrolepidotus)*, modified from Verhille et al. (2016). It should be noted that the areas depicted do not fully reflect the extent of distributions of all life stages of the Sacramento splittail, but are representative of the areas in which they were caught for this study. Fish from the SP population were caught in the Napa River within 2 km up or down stream of the city of Napa. Considering the spawning habitat of the CV population is more extensive that that of the SP population, fish from the CV population were caught across 24 sites throughout the Suisun Marsh, Sacramento River,

and American River. The salinities at which the fish were caught ranged from 12.8 – 13.2 PSU for the SP population, and 0.1 – 5.5 PSU for the CV population.

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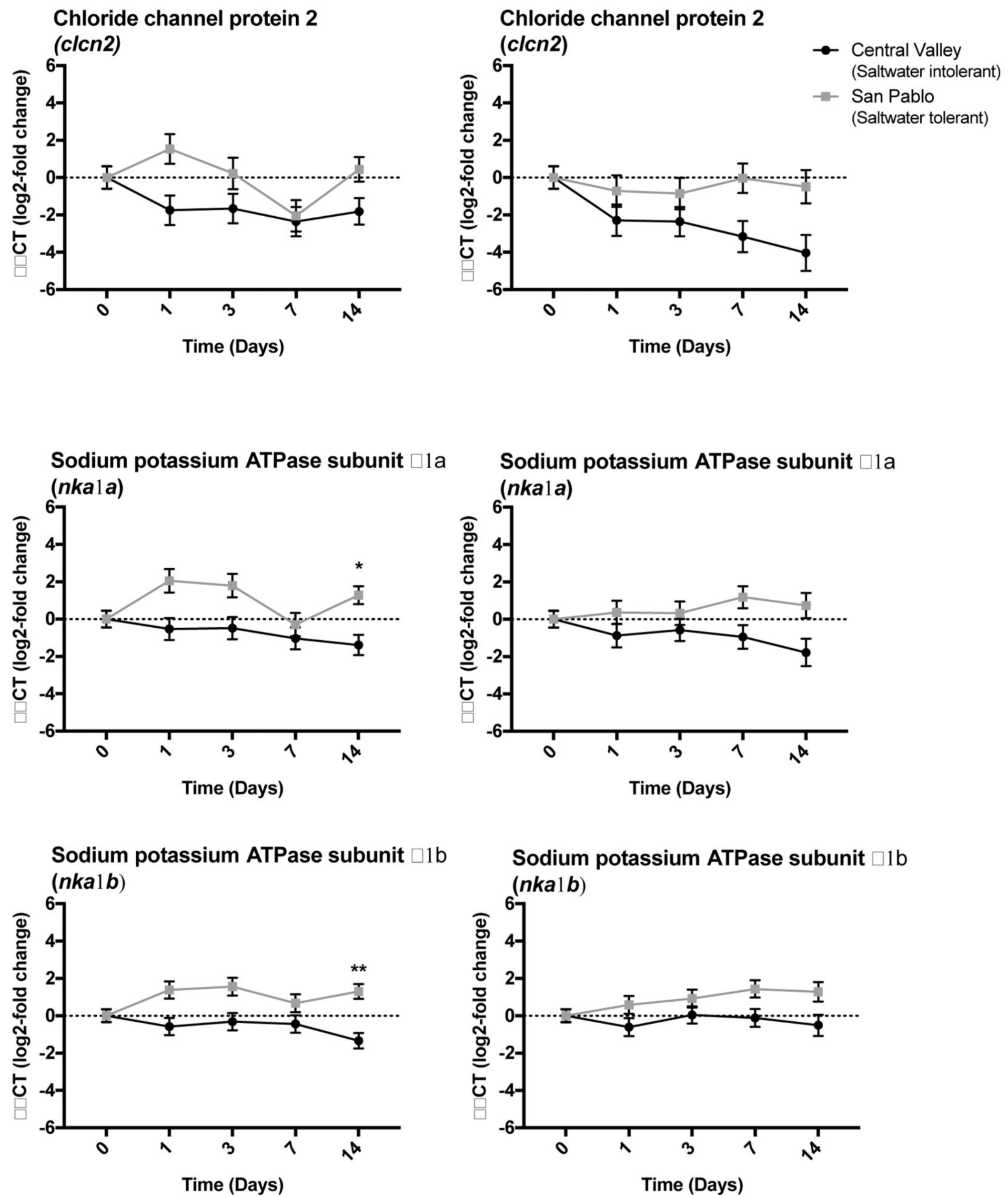

Figure 2. Ion channel mRNA abundance.

Central Valley (CV) and San Pablo (SP) populations of Sacramento splittail (*Pogonichthys macrolepidotus)* are shown on each graph, separated by salinity exposures; 11 PSU **(A)** and 14 PSU**(B)** to highlight population differences in expression. * $p < 0.05$, ** $p < 0.01$, in Tukey HSD test.

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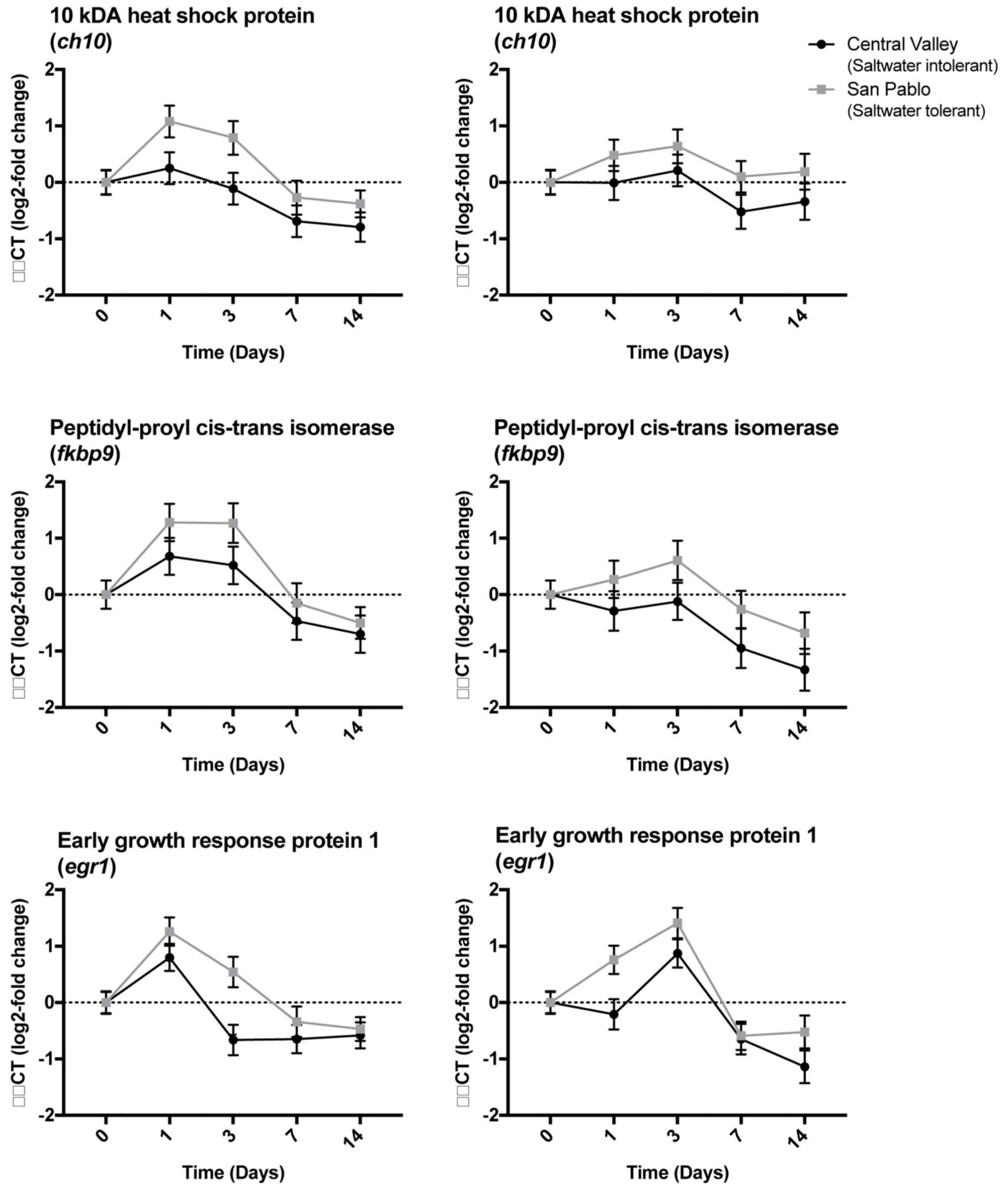

Figure 3. General Stress mRNA abundance.

Central Valley (CV) and San Pablo (SP) populations of Sacramento splittail (*Pogonichthys macrolepidotus)* are shown on each graph, separated by salinity exposures; 11 PSU **(A)** and 14 PSU (**B**), to highlight population differences in expression. * $p < 0.05$, ** $p < 0.01$, in Tukey HSD test.

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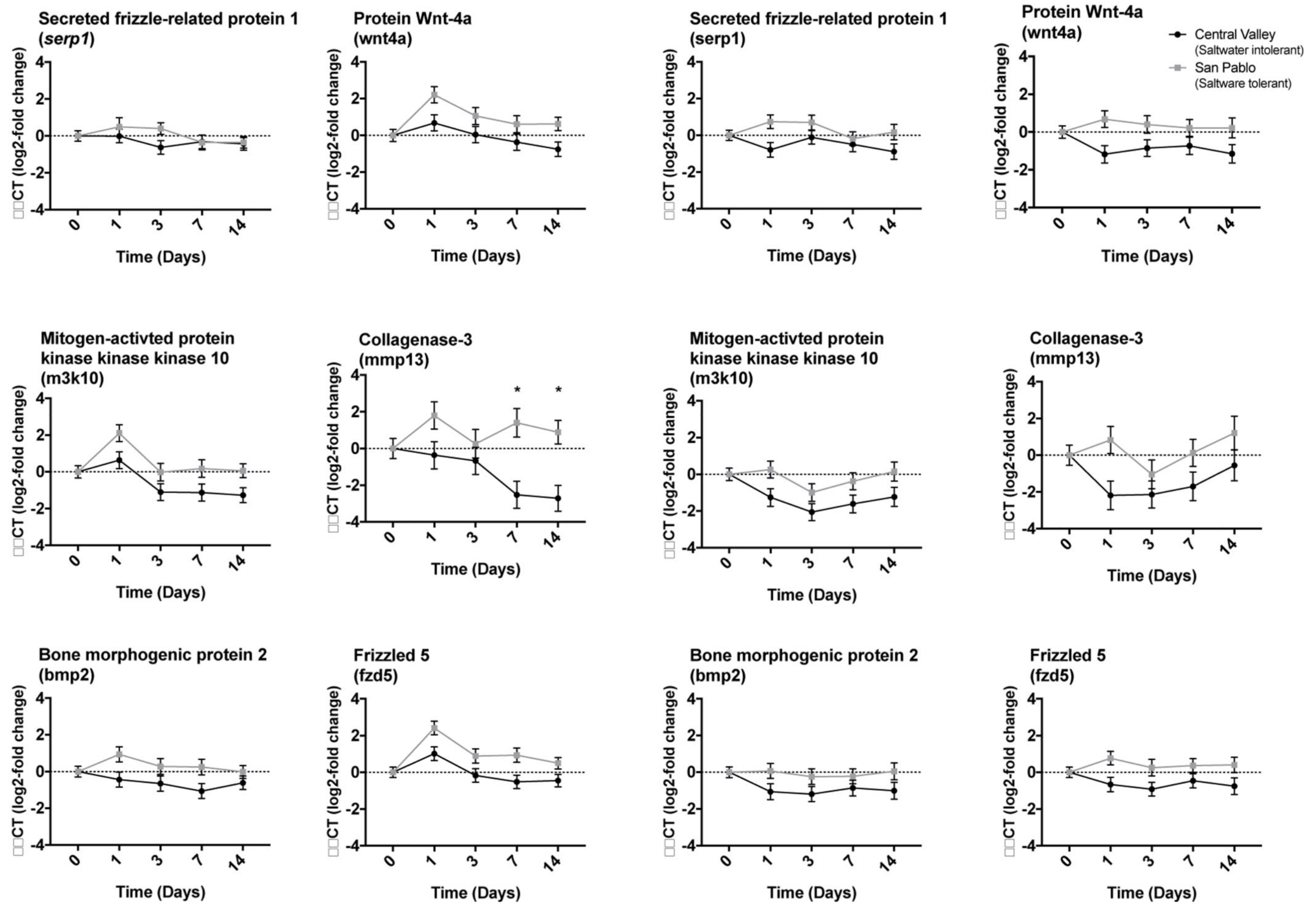

Figure 4. Gill remodeling mRNA abundance.

Central Valley (CV) and San Pablo (SP) populations of Sacramento splittail (*Pogonichthys macrolepidotus)* are shown on each graph, separated by salinity exposures; 11PSU **(A)** and 14 PSU **(B)**, to highlight population differences in expression. * $p < 0.05$, ** $p < 0.01$, in Tukey HSD test.

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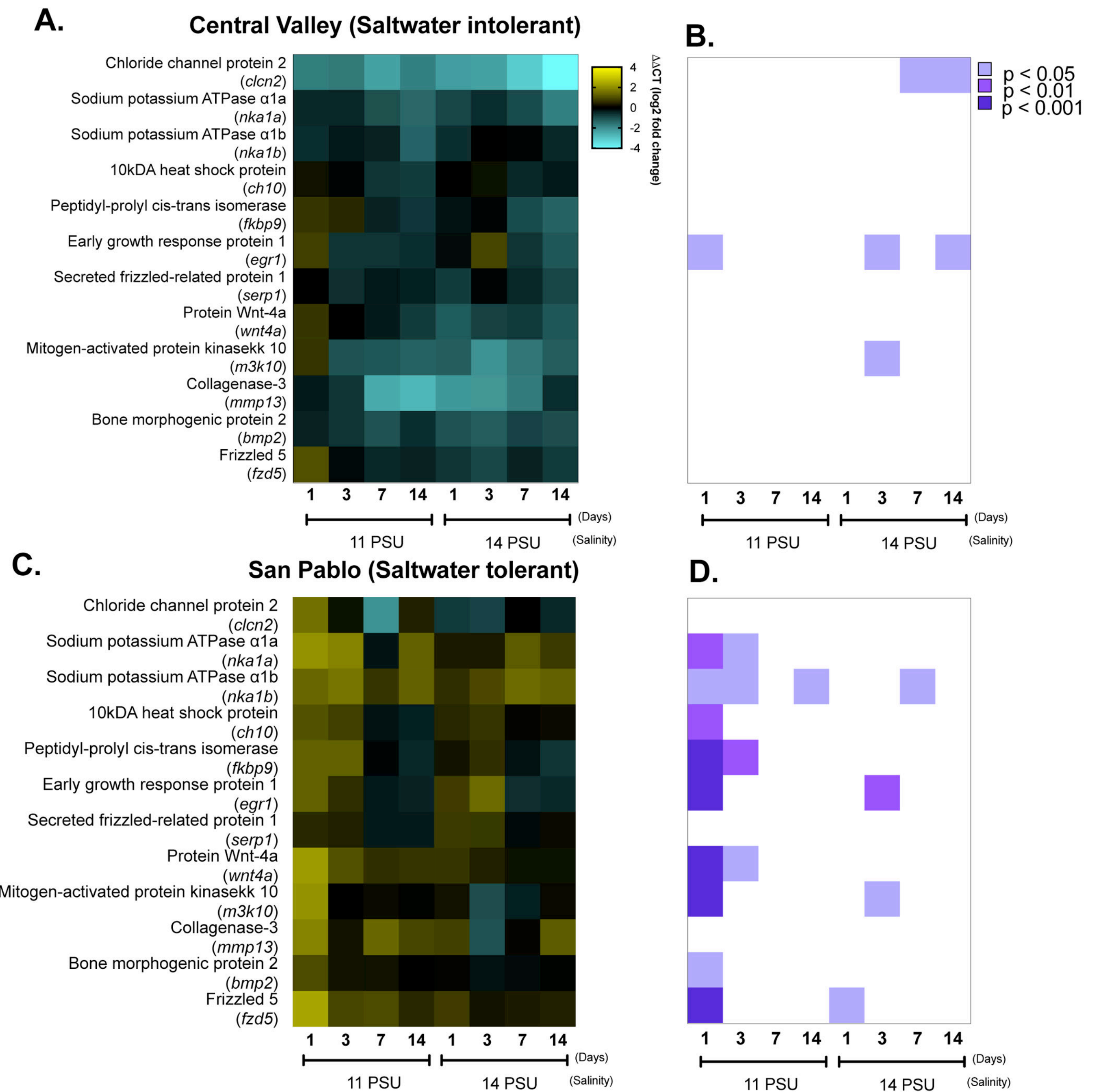

Figure 5. mRNA abundance of all twelve genes of interest over time.

(A) Expression of all twelve genes of interest in the Central Valley population of Sacramento splittail (*Pogonichthys macrolepidotus)* over time and salinity exposure. **(B)** Heatmap visualizing time points in specific salinity exposures for the Central Valley population that were statistically different from time 0 in the corresponding salinity exposure. (**C**) Expression of all 12 genes of interest in the San Pablo population over time and salinity exposure. (**D**) Heatmap visualizing time points in specific salinity exposure for the San Pablo population, which were statistically different from time 0 in the corresponding salinity

exposure. Light purple $p<0.05$, medium-purple $p<0.01$, dark purple $p<0.001$, in dunnetx test whereas time 0 is control and 1, 3, 7, and 14 are treatments for each salinity exposure.

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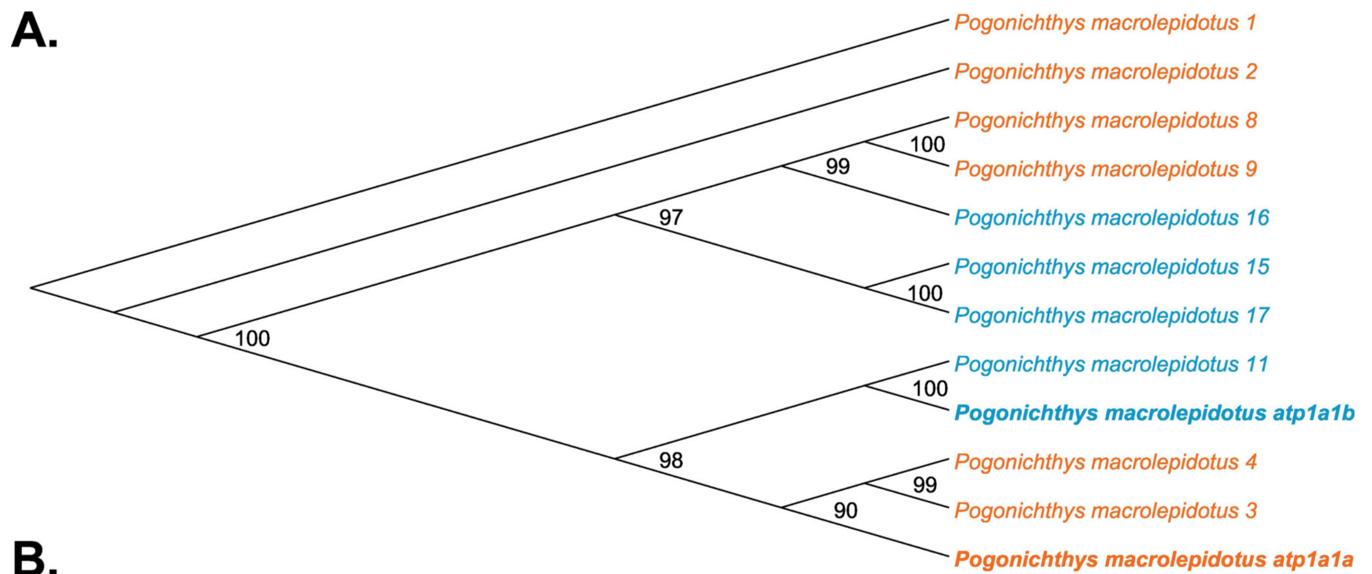

Transcript name for phylogenetic tree	Predicted identity	CV 3 days	CV 7 days	SP 3 days	SP 7 days
Pogonichthys macrolepidotus 1	*nka1a*	0.75	0.74	0.84	0.72
Pogonichthys macrolepidotus 2	*nka1a*	0.77	0.73	0.85	0.71
Pogonichthys macrolepidotus 3	*nka1a*	0.74	0.74	0.73	0.66
Pogonichthys macrolepidotus 4	*nka1a*	0.68	0.68	0.69	0.56
Pogonichthys macrolepidotus 5	*nka1a*	0.79	0.79	0.76	0.74
Pogonichthys macrolepidotus 6	*nka1a*	0.63	0.64	0.67	0.49
Pogonichthys macrolepidotus 7	*nka1a*	0.47	0.37	0.51	0.27
Pogonichthys macrolepidotus 8	*nka1a*	0.67	0.58	0.72	0.52
Pogonichthys macrolepidotus 9	*nka1a*	0.45	0.32	0.55	0.23
Pogonichthys macrolepidotus atp1a1a	*nka1a*	0.72	0.70	0.79	0.64
Pogonichthys macrolepidotus 11	*nka1b*	1.29	1.35	1.19	1.26
Pogonichthys macrolepidotus atp1a1b	*nka1b*	1.27	1.30	1.18	1.18
Pogonichthys macrolepidotus 13	*nka1b*	1.34	1.37	1.23	1.29
Pogonichthys macrolepidotus 14	*nka1b*	1.93	1.87	1.35	1.09
Pogonichthys macrolepidotus 15	*nka1b*	1.18	1.07	1.20	0.72
Pogonichthys macrolepidotus 16	*nka1b*	1.18	1.12	1.17	0.93
Pogonichthys macrolepidotus 17	*nka1b*	1.13	1.07	1.11	0.89

Figure 6.

Phylogenetic tree of identified Sacramento splittail NKAα1 sequences. **(A)** Phylogenetic analysis of the seventeen transcript sequences, aligned and analyzed for maximal likelihood. Orange node labels are sequences of transcripts that were downregulated at 3 and 7 days in both populations, as shown in Figure 6B. Blue node labels are sequences of transcripts that were upregulated at 7 days, as shown in Figure 6B. Bolded node labels are sequences of transcripts that were analyzed for mRNA abundance at additional timepoints (Figures 2, 5). Bootstrap values resulting from 100 bootstrap replicates. **(B)** Representative table of transcripts that were returned as "NKA" from the Sacramento splittail reference transcriptome, renamed for phylogenetic tree visualization. Transcript abundance is shown as difference between exposure and freshwater control in Log2-fold change from the Jeffries et al. (2019) study. Those below 1 are downregulated (shaded in grey), and those above 1 are upregulated (not shaded) in response to 14 PSU exposure for 3 or 7 days. Transcript names in bold were used for qPCR analysis, named as predicted paralogs *nka1a (Pogonichthys macrolepidotus atp1a1a*) and *nka1b* (*Pogonichthys macrolepidotus atp1a1a*)

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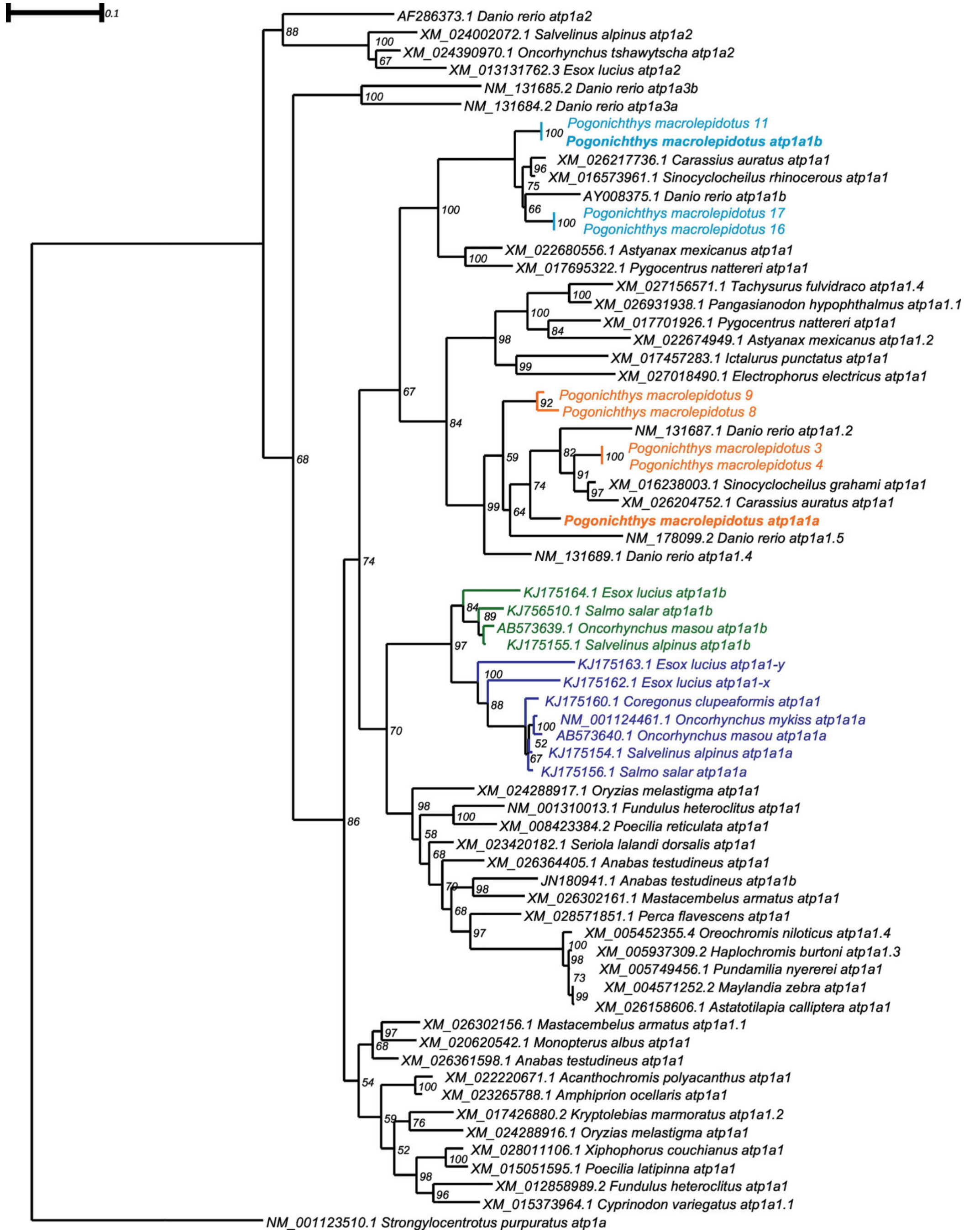

Figure 7.

Phylogenetic relationships of NKAα sequences across multiple species. This tree represents the phylogenetic analysis of the *Pogonichthys macrolepidotus* predicted paralog transcripts, in relation to other teleost NKAα, from both tBLASTX hits selected considering representation of taxa, with the best annotated genome, as well as manually selected sequences included for known gene expression patterns. Orange node labels are sequences of transcripts that were downregulated at 3 and 7 days in both populations, as shown in Figure 6B. Light blue node labels are sequences of transcripts that were upregulated at 7

days, as shown in Figure 6B. Bolded node labels are sequences of transcripts that were analyzed for mRNA abundance at additional timepoints (Figures 2, 5). Dark blue nodes are genes known to increase in transcript abundance in fresh water conditions. Green nodes are genes known to increase in transcript abundance in saltwater conditions. Bootstrap values resulting from 100 iterations. All branches not supported by bootstrap values of 50 or higher were collapsed.

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Table 1.

Genes selected for transcriptomic analysis. This table includes the genes selected in order of functional category including ion exchange, extracellular matrix (ECM) remodeling, and general stress.

Function	Gene Name	Gene code	Primer (5' -> 3')	Roche UPL #	%Efficiency
Reference	60s ribosomal protein L7	*rl7*	F: aacttcctctggccattcaaac R: cctcgacaaagtgggtggtct	55	97.7
Reference	40S ribosomal protein S9	*rs9*	F: tgggcttgaaagtggaggatt R: caaggccaagcttgaagacct	55	103.0
		Target Genes			
Ion Exchange	Chloride channel protein 2	*clcn2*	F: ttctggccagtttcagagacc R: ttgtggagctcagcggttt	82	100.1
Ion Exchange	Sodium potassium ATPase subunit α1a	*nka1a*	F: tgggtattgctggatctgacg R: tgaggcaaagttgtcgtcca	66	103.1
Ion Exchange	Sodium potassium ATPase subunit α1b	*nka1b*	F: ggaaacagcactagcggcttt R: gggtacatcctgagagccacat	37	101.7
ECM remodeling	Collagenase-3	*mmp13*	F: aagcaagtgtgaccctgaactg R:aaatgattgtctctccacggagtt	148	104.7
ECM remodeling	Bone morphogenic protein 2	*bmp2*	F: tctctcagcaacgctgcactac R: gagcacagctcttgtgccttg	5	95.6
ECM remodeling	Frizzled 5	*fzd5*	F: cctgctggctggctttgtt R: tggtgccgccctgtttaat	144	98.8
ECM remodeling	Secreted frizzled-related protein 1	*serp1*	F: tgtcacggtgtgggctacag R: cagccatagtctcgtgctcca	44	98.8
ECM remodeling	Protein Wnt-4a	*wnt4a*	F: cctgtgagaagctccgaggac R: cctccacgttacgcttgca	34	108.3
ECM remodeling	Mitogen-activated protein kinase kinase kinase 10	*m3k10*	F: gctcctgagctgggcagtaac R: gagcctattcccgatcctgag	25	108.9
General Stress	Early growth response protein 1	*egr1*	F: gaccagcagcttccagacg R:actgtagatgttgctgaccactgag	77	104.1
General Stress	10kDA heat shock protein	*ch10*	F: gaaattccttcccatgttcgac R: cacctcttgaaacggtctctgc	31	104.4
General Stress	Peptidyl-prolyl cis-trans isomerase	*fkbp9*	F: tgggaatgtgcgtaggagaaa R: tctctccatagccaaggaaagg	9	100.2