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Differential Gene Expression in Liver, Gill, and Olfactory Rosettes of Coho Salmon (*Oncorhynchus kisutch*) After Acclimation to Salinity

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Abstract

Most Pacific salmonids undergo smoltification and transition from freshwater to saltwater, making various adjustments in metabolism, catabolism, osmotic, and ion regulation. The molecular mechanisms underlying this transition are largely unknown. In the present study, we acclimated coho salmon (*Oncorhynchus kisutch*) to four different salinities and assessed gene expression through microarray analysis of gills, liver, and olfactory rosettes. Gills are involved in osmotic regulation, liver plays a role in energetics, and olfactory rosettes are involved in behavior. Between all salinity treatments, liver had the highest number of differentially expressed genes at 1616, gills had 1074, and olfactory rosettes had 924, using a 1.5-fold cutoff and a false discovery rate of 0.5. Higher responsiveness of liver to metabolic changes after salinity acclimation to provide energy for other osmoregulatory tissues such as the gills may explain the differences in number of differentially expressed genes. Differentially expressed genes were tissue- and salinity-dependent. There were no known genes differentially expressed that were common to all salinity treatments and all tissues. Gene ontology term analysis revealed biological processes, molecular functions, and cellular components that were significantly affected by salinity, a majority of which were tissue-dependent. For liver, oxygen binding and transport terms were highlighted. For gills, muscle, and cytoskeleton-related terms predominated and for olfactory rosettes, immune response-related genes were accentuated. Interaction networks were examined in combination with GO terms and determined similarities between tissues for potential osmosensors, signal transduction cascades, and transcription factors.

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Keywords

Salinity; Salmonids; Microarray; Gene expression

Introduction

Pacific salmonids are economically and ecologically critical species in the northwestern USA. Most of these species are anadromous, hatching in freshwater, migrating to the ocean for growth and maturation, and migrating back to their natal streams for reproduction (Quinn 2005). The process of transitioning from freshwater to saltwater, smoltification, involves various changes in color, shape, size, metabolism, catabolism, and osmotic and ion regulation (reviewed in Hoar 1988). However, there are still many aspects of Pacific salmonid smoltification that are currently unknown, particularly the regulation of smoltification at the molecular level.

Seear et al. (2010)) conducted a transcriptomic study of Atlantic salmon (*Salmo salar*) smoltification in brain, gill, and kidney tissues to try to elucidate some of the molecular mechanisms involved in smoltification regulation. These tissues were selected based upon their key roles in smoltification. The gills had the highest number of differentially expressed genes, and the differentially expressed genes in the three tissues were mostly involved in growth, metabolism, oxygen transport, or osmoregulation. Many of the genes that were upregulated complimented previous findings from other studies on salmonid smoltification physiology and biochemistry (D'cotta et al. 1996; Chakravarti et al. 1998; McCormick et al. 1989; Vanstone et al. 1964; Gallagher et al. 2008). Pacific salmon has also been the subject of a transcriptomics study of migration, although the focus was on the migration from the ocean to freshwater (Evans et al. 2011). Additionally, Gallagher et al. (2008) assessed differences in gene expression between smolts and adult coho salmon (*Oncorhynchus kisutch*) liver and found that differentially expressed genes were mainly involved in cellular processes related to protein biosynthesis and degradation, ion transport, transcription, cell structure, and cellular energetics. There is still a need to better understand the molecular responses of Pacific salmon transitioning from freshwater to saltwater.

In addition to salmon, transcriptomic studies of other euryhaline fish undergoing salinity acclimation have also been conducted. For example, Evans and Somero (2008) preformed a time course acclimation of goby, *Gillichthys mirabilis*, to hyper-and hypo-osmotic stress in gills. The authors examined changes in gene expression between 0, 1, 2, 4, and 12 h of acclimation and showed that cell signaling genes were among the most predominant groups of differentially expressed genes. Many of the genes they found to be differentially regulated had no previous reported role in osmotic stress adaptation, highlighting the value of the transcriptomic approach. Fiol et al. (2006)) also examined changes in gene expression after short-term acclimations. Tilapia (*Oreochromis mossambicus*) were transferred from freshwater to saltwater, and 20 novel genes involved in immediate hyperosmotic stress were identified after 4 h in gill epithelial cells. Network analysis revealed that more than half of the identified genes interact within a cellular stress response signaling network, with other important networks comprising stress response signal transduction, compatible organic

osmolyte accumulation, energy metabolism, lipid transport and cell membrane protection, actin-based cytoskeleton dynamics, and protein and messenger RNA (mRNA) stability. These and other novel genes may also be important in Pacific salmonid salinity acclimation, which could be determined through a transcriptomics approach.

The goal of the current study was to assess how salinity acclimation of coho salmon impacted gene expression in gills, liver, and olfactory rosettes. Gills were chosen because they play a vital role in osmoregulation. Liver was selected based on its role in energetics, and olfactory rosettes were chosen for their role in behavior and homing to natal streams. Establishing the underlying molecular responses of Pacific salmonids to changes in salinity will help in understanding the natural process of smoltification.

Methods

Organisms

Juvenile coho salmon (21.6±1.7-cm length and 100.7±28.7-g weight) were obtained from the Nimbus Hatchery (Gold River, CA), collected and maintained in freshwater. Organisms were maintained in a flow-through living-stream system at the University of California, Riverside, with dechlorinated carbon-filtered municipal water at 11–13 °C, on a 16-h light: 8-h dark cycle, and acclimated for a minimum of 2 months before experimental use. Organisms were fed with commercial fish feed (Silver Cup, Murray, UT) in excess. Water was replaced daily to avoid the food breaking down which can result in an overabundance of ammonia.

Hypersalinity Acclimation

Fish were transferred and sequentially acclimated to hypersaline water up to concentrations of <0.5- (control), 8- (low), 16- (medium), and 32-ppth (high) saline concentrations, with an increase of 4 ppth every 2 days (CrystalSea Marine Mix, Marine Enterprises International, Baltimore, MA) in 24-L tanks. The average full strength oceanic salinity that Pacific salmon typically encounter is about 32–33 ppth (Quinn 2005). All fish were left for 1 week at the specific salinity. All acclimations were performed concurrently. While the animals at 4 and 8 g/L stayed 1 week at that salinity, the rest of the tanks (regarding to 16 and 32 g/L) continued the acclimation steps. Previous studies in salmonids have indicated direct relationships with plasma osmolality, stress responses, and survival of individuals using this method (El-Alfy et al. 2002). Five animals and three tank replicates (total 15 animals) were used per experimental group. The density was approximately 20-g fish per liter. The water quality (ammonia, pH, PO4, DO, nitrate/nitrite) was measured by using the colorimetric API Freshwater Master Test Kit (Aquarium Pharmaceuticals, Chalfont, PA). The oxygen levels were monitored daily and were always near saturation levels (94 ±3 %). After acclimation to each salinity regime, animals were euthanized using tricaine methanesulfonate (MS-222) (Argent Chemical Laboratories, Redmond, WA) at 1 g/L buffered with phosphate buffer pH 7.6. Fish were then weighed and measured with subsequent removal of the liver, gills, and olfactory rosettes, which were frozen in liquid nitrogen and stored at −80 °C. Four biological replicates per tissue and condition were then randomly selected. All experiments were done in accordance with IACUC guidelines at The University of California, Riverside.

RNA Extraction

Total RNA was isolated from salmon tissue using the miRNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the vendor's defined method and stored at minus 70 °C. The quantity (ng/μL) of RNA was determined measuring the OD_{260} with a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and the RNA purity was assessed measuring $OD_{260/280}$ and $OD_{260/230}$ ratios. RNA integrity was characterized using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only total RNA samples with an RNA integrity ratio (RIN) score of >7.0 and $OD_{260/280}$ as well as $OD_{260/230}$ ratios of 1.8–2.1 were used for microarray-based analysis.

RNA Sample Processing for Microarray Analysis

The RNA samples were labeled and prepared for hybridization onto Agilent 4×44K salmon arrays (Cat.# G2519F-020938; Agilent Technologies, Inc. Santa Clara, CA) using the manufacturer's established protocols. Briefly, 100 ng of total RNA was converted into fluorescently labeled Cy3 complimentary RNA (cRNA) using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescent targets were purified to remove unincorporated nucleotides using RNeasy (Qiagen). Absorbance (OD) at 260 nm was used to quantify the cRNA concentrations, and absorbance at 550 nm was used to measure the efficiency of Cy3 dye incorporation. An incorporation efficiency of 9 pmol/μg or greater was deemed necessary before proceeding with hybridization. One micrograms of fragmented cRNA for each sample was hybridized to the array in accordance with single color Agilent hybridization protocols. The hybridization conditions were such that the hybridization was carried out under high salt conditions to facilitate specific probe and target interactions. Hybridization and washing of these arrays were accomplished using HS 400 Pro hybridization and wash stations (Tecan Systems, Inc., San Jose, CA) and scanned using an Agilent DNA Microarray Scanner (Agilent Technologies, Inc. Santa Clara, CA) using previously established methods.

Microarray Data Analysis

Expression level data from the Agilent Microarray Scanner and Feature Extraction Software were also normalized using a multiple-loess algorithm (Sasik et al. 2004). Probes whose expression level exceeds a threshold value in at least one biological replicate sample were called detected. The threshold value was found by inspection from the distribution plots of (log) expression levels in a similar manner as described by Baker et al. (2013). Detected probes are sorted according to their q-value, which is the smallest false discovery rate (FDR) at which the gene is called significant. FDR is the expected fraction of false positive tests among significant tests (Benjamini and Hochberg 1995). We evaluate FDR using significance analysis of microarrays (SAM) and its implementation in the official statistical package *samr* (Tusher et al. 2001). In order to not be unduly impressed by accidentally small variances, we set the percentile of standard deviation values used for the exchangeability factor in the test statistic to 75.

Each gene ontology term or a pathway was treated simply as a set of genes. The probe list, sorted by *q*-value in ascending order, is translated into Entrez gene ID's and parsed so that whenever several different probes represent the same gene, only the highest-ranking probe is

kept for further analysis. The sorted list of genes is subjected to a nonparametric variant of the gene set enrichment analysis (GSEA) (Subramanian et al. 2005), in which the p value of a gene set of size *n* is defined as follows: Let us denote the *k*th highest rank in gene set as r_k and define p_k as the probability that out of n randomly chosen ranks (without replacement), the kth highest is not smaller than r_k. The *p* value of the gene set is defined as $\min_k [P_k]$. It is designed to detect overrepresented gene sets at the top of the list. Unlike the Kolmogorov-Smirnov statistic used in GSEA, it will not detect underrepresented or other, pathologically distributed, gene sets. Finding the *p* value of a gene set of size n requires calculation of n rank-order values p_k; however, there is no need to adjust the *p* values for multiple testing, as the rank-order tests are highly statistically dependent. A Bonferroni adjustment of gene *set p* values for the number of gene *sets* tested was performed, even though there are often several gene sets with overlapping gene content (and therefore are statistically dependent), which is partly due to the design of the gene ontology database and partly because genes tend to be involved in multiple processes. We report only gene sets with adjusted *p* values ≤0.01. Array data had been deposited in NCBI's Gene Expression Omnibus (GEO) (accession number GSE67461).

qPCR Validation

Primers were designed using Primer3 software for CCAAT/enhancer binding protein β (*cebpb*), calpain 1 (*capn1*), proto-oncogene, serine/threonine kinase (*pim1*), aldolase B, fructose-bisphosphate (*aldob*), and complement component 3 (*c3*) (Rozen and Skaletsky 1999). *Cebpb* was selected based on its potential function as a transcription factor in response to salinity acclimation. *C3* was chosen for its role in immune response and differential regulation in two tissues. *Capn1*, *pim1*, and *aldob* were chosen as representatives for the different tissues based on similar patterns of differential regulation (all up or all downregulated) in all three salinity treatments. β-Actin was used as the housekeeping gene. Primer sequences are listed in supplemental Table 1. Primers were optimized based on annealing temperature, template concentration, and primer concentration. After optimization, PCR products were run on DNA electrophoresis gels to confirm the size of the amplicon. Quantitative PCR (qPCR) was run for each gene comparing freshwater acclimated fish with fish acclimated to the high salinity (32 ppth; *n*=6–8) using the iScript One-step RT-PCR kit with SYBR Green from Bio-Rad. Two hundred fifty nanomoles of each primer was added to 25-μL PCR reactions containing SYBR Green RT-PCR Reaction Mix and 100 ng of RNA sample. Thermocycling parameters were as follows: 10 min at 50 °C, 5 min at 95 °C, 40 cycles of 10 s at 95 °C, and 30 s at 57 °C. At the end of each cycle, fluorescence data was collected. A melting curve analysis was run between 60 and 95 °C following the amplification reaction. The C(t) was selected to be in the linear phase of amplification. The reactions were done in an iCycler-MyIQ Single Color Real-Time PCR Detection System (Bio-Rad), and data analysis was done using IG5 (Bio-Rad). The comparative quantification method that employs the formula $2^{-\Delta\Delta CT}$ was used to derive relative gene copy numbers.

Results

Using a 1.5-fold cutoff at $q<0.5$, we observed 119, 101, and 81 differentially expressed genes in all three salinity treatments (low, medium, and high) as compared to the freshwater controls in liver, olfactory rosettes, and gills respectively (Fig. 1). Among the salinity treatments, liver had the highest number of differentially expressed genes (1616 differentially expressed genes). Gills had 1074 differentially expressed genes, and olfactory rosettes had 924. The high salinity treatment had the highest number of differentially expressed genes for liver with 556, whereas in the gills and olfactory rosettes, the medium salinity treatment caused the highest number of differentially expressed genes with 440 and 361, respectively. The number of differentially expressed genes in each individual salinity treatment was greater than the shared genes between treatments for all salinities and all tissues.

There were no known differentially expressed genes that were common and inclusive to all salinity treatments and all three tissues. Five genes were differentially expressed in all salinity treatments and shared between two tissues (Table 1). Sodium/potassium-transporting ATPase subunit alpha-1 precursor (*atp1a1*) was differentially expressed in gills and olfactory rosettes, although this gene was upregulated in gills and downregulated in olfactory rosettes. Sodium-coupled neutral amino acid transporter 2 (*snat2*) was upregulated in both gills and liver. Gastrulation-specific protein G12 (*g12*), complement C1q-like protein 2 precursor (*c1ql2*), and complement C3-1 (*c3*) were all differentially expressed in both liver and olfactory rosettes relative to freshwater controls. *G12* was downregulated in liver and upregulated in olfactory rosettes, while *c1ql2* was upregulated in liver and downregulated in olfactory rosettes. *C3* was downregulated in both liver and olfactory rosettes relative to freshwater controls.

Gene ontology (GO) term analysis revealed that most significant terms were tissue specific. For liver, 13 GO terms were significant: 3 biological processes, 5 molecular functions, and 5 cellular components (Table 2; Bonferroni $p< 0.05$). For gills, 17 GO terms were significant: 6 biological processes, 4 molecular functions, and 7 cellular components (Table 3; Bonferroni $p<0.05$). For olfactory rosettes, 26 GO terms were significant: 14 biological processes, 10 molecular functions, and 2 cellular components (Table 4; Bonferroni $p<0.05$). No GO terms were significant for all three tissues.

Liver did not share any significant terms with either gills or olfactory rosettes. Gills and olfactory rosettes shared three significant terms: the biological process of the classical pathway of complement activation, the molecular function of serine-type endopeptidase inhibitor activity, and the cellular component for extracellular space.

Heatmaps were created for select significant terms for each tissue depicting changes in gene expression levels between each salinity treatment and control (Fig. 2). For liver, hemoglobin complex and oxygen binding terms were significant. The heatmap shows that some associated genes were upregulated after salinity acclimation; however, the majority was downregulated. Additionally, a majority of the genes were consistent in direction of change (either up or down) for all salinity treatments compared to control (Fig. 2a).

For gills, terms for complement activation, glycolysis, structural constituent of muscle, and collagen were significant. Associated genes for complement activation were mixed between upregulation and downregulation; however, a majority of the genes were consistent in direction of change for all salinity treatments compared to control (Fig. 2b). For glycolysis, associated genes had a more complex response to salinity acclimation, with many genes switching in direction of regulation (either up or down) based on specific salinity treatments (Fig. 2c). A majority of genes associated with structural constituent of muscle showed a salinity treatment pattern in that most were upregulated at low salinity but downregulated at medium and high salinity (Fig. 2d). For collagen, associated genes were mixed between upregulation and downregulation; however, a majority of the genes were consistent in direction of change for all salinity treatments compared to control (Fig. 2e).

For olfactory rosettes, complement activation (classical and alternative pathway) and antigen binding terms were significant. Almost all genes associated with complement activation were downregulated in all salinity treatments compared to control (Fig. 2f). For antigen binding, associated genes had a more complex response to salinity acclimation, with some genes switching in direction of regulation based on specific salinity treatments (Fig. 2g).

The MetaCore™ knowledge base was used to create interaction networks for each tissue comparing freshwater acclimated fish and fish acclimated to the high salinity to depict how differentially expressed genes interact (Bugrim et al. 2004). Different fold changes were used for each tissue to create networks that gave an overall picture of the main changes occurring, without being too convoluted. In order to generate meaningful networks, the number of differentially expressed genes was inputted empirically from a list reflecting fold changes between 1.25- and 1.75-fold in each tissue. Representative networks are included in the supplemental materials. As liver had the highest number of differentially expressed genes, a stricter cutoff of 1.75-fold change was used. For gills, only genes with 1.5-fold change or greater were included. For olfactory rosettes, which had the least genes differentially expressed, a 1.25-fold change was used.

For the liver, one main network with four major nodes was revealed, as well as seven pairs of interacting genes and one small network of three related genes (Supplemental Fig. 1). The four nodes on the major network were complement component 3 (c3), CCAAT/enhancer binding protein δ (*cebpd*), CCAAT/enhancer binding protein α (*cebpa*), and jun proto-oncogene (*jun*). The total number of genes included in the liver network was 37. Confirmation of hepatic *c3* downregulation was achieved by qPCR analyses in animals from the high salinity treatments (Fig. 3). To confirm upregulation of hepatic transcripts, qPCR was performed on *capn1* in the liver where similar increases of mRNA were consistent with those of the array. The interaction network for gills, which had a lower fold-cutoff, was more complex than observed for liver (Supplemental Fig. 2). For example, there was one major network with multiple nodes identified in the gills, as well as three separate interactions with two genes each. Sixty-two genes were included in the gills network. For gills, both *cebpb* and *pim1* were confirmed to be upregulated through qPCR (Fig. 3). The olfactory rosettes network, which had the least strict fold-cutoff, was the most complex of the three tissues studied (Supplemental Fig. 3). Specifically, the olfactory rosettes network contained one main network with multiple nodes and short networks with four genes or less.

A total of 108 genes were included in the network. In olfactory rosettes, transcripts of *aldob* and *c3* were both downregulated and consistent with array data in the high salinity treatment (Fig. 3).

A systems view network of interacting genes and gene products identified similarities and differences in how each tissue responded to the high salinity acclimation as compared to control. For example, CCAAT/enhancer binding protein α (*cebpa*) was upregulated and a main node in the networks for all three tissues. The interaction this gene had with other differentially regulated genes was similar between tissues. Another similarity between the three networks was upregulation of growth arrest and DNA-damage-inducible genes (*gadd45*). For liver, *gadd45g* was upregulated. For gills, *gadd45b* was upregulated, and for olfactory rosettes, both *gadd45b* and *gadd45a* were upregulated. Insulin-like growth factor-binding protein (*igfbp*) genes were also present in each network; however, the specific gene and direction of change differed by tissue. *igfbp1* mRNA levels were upregulated in liver and downregulated in olfactory rosettes compared to freshwater controls. For gills, *igfbp5* mRNA levels were downregulated compared to freshwater controls. A few genes were involved in networks for two of the three tissues. Jun proto-oncogenes (*jun*) were also observed in both the liver and gills networks, with *jun* being upregulated in liver and *junb* being upregulated in gills. *Jun* genes also shared a connection with *cebpa* in both networks.

Although there was some overlap in genes between the different networks, there were many salinity-modulated genes that were specific to each tissue. For liver, the four main nodes were shared with other tissues, although some of the genes branching from *cebpa* were not shared, including genes involved in metabolic processes (e.g., cytochrome P450 3A, which for salmonids would likely be *cyp3a27*) (Lee et al. 1998). None of the smaller two and three gene interactions separate from the main network were shared between tissues but included genes involved in blood coagulation, such as coagulation factor XIII, B polypeptide (*f13b*), and protein folding, such as heat shock 70 kDa protein 8 (*hspa8*).

For the gills, some of the major nodes in the network were not shared with other tissues. Avian myelocytomatosis viral oncogene homolog (*myc*) was upregulated and linked to 19 different genes. Transforming growth factor, β1 (*tgfb1*), which was linked to 20 genes, not shared, and downregulated on high salinity acclimation. Another major node that was specific for gills was insulin (*ins*), which was upregulated by high salinity and connected to 19 genes. The gills network also included various genes that impact the cytoskeleton and cell growth, such as collagen, type I, alpha 1 (*col1a1*), which was downregulated, and actin gamma 1 (*actg1*), which was upregulated. Many genes involved in immune response were also included in the network, such as lectin galactoside-binding soluble 3 (*lgals3*), which was downregulated and chemokine (C-C motif) ligand 7 (*ccl7*), which was upregulated by high salinity.

Unshared nodes in the olfactory network included signal transducer and activator of transcription 1 (*stat1*), which was upregulated and linked to 13 genes. Caspase 8 (*casp8*) was also upregulated in high salinity and interacted with eight genes. Ubiquilin 4 (*ubqln4*) was downregulated and connected to nine genes. Genes involved in immune response were also present in the olfactory network, such as major histocompatibility complex, class I-

related (*mr1*), and SLC46A2 solute carrier family 46, member 2 (*tscot*), which were both downregulated. Genes involved in growth and impacts of the cytoskeleton were also part of the network and included talin 1 (*tln1*), which was upregulated by high salinity acclimation, and plasminogen activator, tissue (*plat*), which was downregulated.

Discussion

An interesting finding from our study was that gene expression induced by salinity was highly tissue-dependent, as we did not observe changes in gene expression that were common to salinity and all three tissues analyzed. Similarly, Seear et al. (2010) reported that only one gene upregulated more than 2-fold in the three tissues in their study (gills, brain, and kidney) in Atlantic salmon after smoltification. It has previously been proposed that the different cell types within an organism may be able to detect and respond in a unique fashion to different ranges of osmolality (reviewed in Fiol and Kültz 2007). As some of the tissues of euryhaline fish, such as gills and olfactory rosettes, are exposed directly to the aquatic environment, response to changes in salinity would be different compared to most of the other tissues, such as the liver which are in a more homeostatic environment.

Despite not being in direct contact with the external environment, we found it interesting that the liver had the highest number of differentially expressed genes following salinity treatments compared to the gills and the olfactory rosettes. In previous studies, salinity acclimation caused tissue-specific reorganization of energy metabolism in sea bream (*Sparus aurata*) liver, brain, kidney, and gills (Sangiao-Alvarellos et al. 2003). Interestingly, the nonosmoregulating organs, liver and brain, were more metabolically responsive to changes in salinity than the gills and kidney, which have established roles as osmoregulatory organs. Salinity acclimation may cause an increase in liver metabolism as liver is the main region for glycogen and glucose turnover in fish and could provide energy to fuel other metabolic and osmoregulatory tissues like gills (Sangiao-Alvarellos et al. 2003). Support for this hypothesis comes from a previous report showing enhanced liver metabolism in rainbow trout after salinity acclimation (Soengas et al. 1995). This difference in metabolism may impact the number of differentially expressed genes observed for each tissue in the current study.

Differential gene expression was also salinity-dependent as the number of treatment-specific genes was greater than the shared genes between treatments for all salinities and all tissues. Also, there was no discernable salinity concentration-dependent pattern in the number of differentially expressed genes. In liver, the high salinity caused the greatest number of differentially expressed genes, followed by the low concentration, with the medium concentration causing the least number of differentially expressed genes. The medium salinity concentration caused the highest number of differentially expressed genes in gills and olfactory rosettes. However, for gills, the low concentration resulted in a greater number of differentially expressed genes than the high concentration, while for olfactory rosettes, the high concentration caused a greater number of differentially expressed genes than the low salinity treatment. Evans and Somero (2008) analyzed gene expression of goby gills at different time points after acclimation to hyper- and hypo-osmotic stress and found that

many genes were only significantly altered at specific time points. Similarly, salinity only alters certain genes at specific concentrations.

Five known genes were differentially expressed in all salinity treatments and shared between two tissues (Table 1). Sodium/potassium-transporting ATPase subunit alpha-1 precursor (*atp1a1*) mRNA was upregulated in gills and downregulated in olfactory rosettes. Sodium/potassium ATPases are membrane proteins which establish and maintain electrochemical gradients that are necessary for osmoregulation, as well as for sodium-coupled transport of a variety of organic and inorganic molecules, and for electrical excitability of nerve and muscle. Sodium/potassium ATPases consist of two subunits: alpha, the larger catalytic subunit and beta, the smaller glycoprotein subunit. *Atp1a1* encodes an alpha 1 subunit; the alpha subunit is encoded by multiple genes (reviewed in Kaplan 2002). Upregulation of *atp1a1* in the gill is consistent with previous studies, as an increase in activity of gill sodium/potassium ATPases has been used in previous studies as a molecular indicator of smoltification (D'cotta et al. 1996; Yada et al. 2008; Riar et al. 2013). Evan and Somero (2008) reported a 1.6-fold increase in the alpha subunit of Na^+/K^+ ATPase mRNA during 12 h of hyperosmotic stress of *Gillichthys mirabilis*. Additionally, Seear et al. (2010) found that Na^+/K^+ ATPase alpha subunit isoform 1b was upregulated 2.1-fold in gill tissue of Atlantic salmon smolts. The difference in expression of *atp1a1* in gills and olfactory rosettes may be due to tissue-specific distributions of alpha isoforms. Richards et al. (2003) demonstrated the expression of alpha 1c and 3 in several tissues of rainbow trout (*Oncorhynchus mykiss*); however, olfactory rosettes were not included in the study. Other isoforms (alpha 1a, 1b, and 2) had tissue-specific distributions that were altered after transfer to seawater, suggesting that the different isoforms play different roles in salinity acclimation (Richards et al. 2003). It is possible that the gills and olfactory rosettes have different distributions of subunit isoforms with unique roles in salinity acclimation.

Sodium-coupled neutral amino acid transporter 2 (*snat2*) mRNA was upregulated in both gills and liver. SNAT2 is member of System A which transports small, non-branched amino acids (Jones et al. 2006) and couples the transport of amino acids against the inward movement of sodium down its electrochemical gradient (Baird et al. 2009). In humans, SNAT2 has ubiquitous tissue distribution (Glover et al. 2005) and is involved in the adaptive regulation of System A to nutritional challenge (Franchi-Gazzola et al. 1999). Previous studies in a human placental cell line showed that cor-tisol upregulates SNAT2 expression, which may ensure sufficient amino acid supply to the developing fetus (Jones et al. 2006). Salinity acclimation increases cortisol in euryhaline fish (Assem and Hanke 1981; Borski et al. 1991; McLean et al. 1997), which could result in the upregulation of *snat2*. SNAT2 has also been shown in mammals to be involved in cell volume control after hypertonic exposure as amino acids make up a large faction of organic osmolytes within the cytosol. Neutral amino acids may act as compatible osmolytes in hypertonically stressed cells, and SNAT2 can help regulate cell volume through transport (reviewed in Franchi-Gazzola et al. 2006).

Expression of gastrulation-specific protein G12 (*g12*) mRNA was downregulated in liver and upregulated in olfactory rosettes in salinity acclimated salmon. Although *g12* was expressed in the enveloping layer of zebrafish during different developmental stages, little is

known about its function (Conway 1995). This gene shares identity to mammalian genes, rat spot 14 and human MID1 interacting protein 1 (MID1). Rat spot 14 is purported to be involved in lipogenesis (Grillasca et al. 1997), whereas MID1 may function in maintaining microtubule dynamics (Schweiger et al. 1999). Evans and Somero (2008) found that MID1 interacting protein mRNA was downregulated in hypersaline acclimated goby gills. Other environmental factors have also altered *g12* expression in fish. Castilho et al. (2009) found that a *g12-like* gene was differentially expressed in tissues of bluefin tuna (*Thunnus orientalis*) after thermal acclimation in the ventricle and white muscle.

Complement C1q-like protein 2 precursor (*c1ql2*) mRNA was upregulated in liver and downregulated in olfactory rosettes. C1ql2 belongs to the C1q family which is involved in immunological processes. The C1q family plays a role in the classical complement pathway, impacting innate and acquired immunity (reviewed in Ghai et al. 2007). Seear et al. (2010) found that *C1q*-like protein mRNA was upregulated 3.38-fold in kidney tissue of Atlantic salmon smolts after smoltification. The final shared gene, complement C3-1 (*c3*), is also involved in the complement pathway and was downregulated in both liver and olfactory rosettes. C3 is involved in immunological processes and helps the host defend against infection. C3 has been suggested to be the most versatile and multifunctional component of the complement system (Lambris 1990). Hardiman et al. (1994) found through Northern blot analysis that *c3* was induced modestly during smoltification in Atlantic salmon liver. The difference from the current study may be from species difference, possible paralog differences, or from enhanced specificity from the techniques used in the current study compared to Northern blot.

Salinity has been shown to exhibit complex effects on the immune system, and the results of our study revealed that many differentially regulated transcripts in all three tissues examined were associated with maintenance of immunity. Birrer et al. (2012) found that salinity changes caused an increase in activity and proliferation of immune cells in the broad-nosed pipefish (*Syngnathus typhle*), but gene expression of certain immune-related components was downregulated after infection of Vibrio in lower salinities. The authors suggested that energy needed for osmoregulation may result in less resources available to combat infection (Birrer et al. 2012). The stress response, as would occur during salinity acclimation, is thought to cause immunosuppression through release of cortisol and activation of corticosteroid receptors; however, this hypothesis may be tissue specific. For some species of euryhaline fish, salinity acclimation has enhanced some immune functions (reviewed in Yada and Nakanishi 2002). Yada et al. (2007) found that acute stress of rainbow trout decreases the number of antibody producing leucocytes and circulating level of immunoglobulin, but in gills, expression of glucocorticoid receptor 1 and 2 was unaltered. In contrast, saltwater acclimation in steelhead trout increased mRNA of corticosteroid receptor genes in gill and body kidney, while head kidney and spleen were unaltered, and leucocytes and peripheral blood showed decreases (Yada et al. 2008).

In evaluating the complement component of immune function, gill and olfactory rosettes shared the biological process GO term of classical pathway of complement activation; however, this term was not significant for liver. In the gills of Atlantic salmon, Seear et al. (2010) found downregulation of *CC chemokine SCYA112*. Chemokines play a role in innate

immune response and are involved in responding to sites of injury or infection (Moser and Loetscher 2001). Evans et al. (2011) also observed significant enrichment of ontologies associated with immune response, including the complement system, in gills from salmon from different migrating routes in the marine environment.

For olfactory rosettes, six of the top ten GO terms were related to immune response. Network analysis identified downregulated immune-related genes in all three tissues. Although immune response in liver may not be one of most significant effects, as it did not appear in the GO terms, it is still affected, as seen by the interaction network. In the liver network comparing high salinity treatment to control, *c3* was also adjoined to four other genes involved in immune response: complement component 5 (*c5*), complement component 7 (*c7*), clusterin (*clu*), and complement component 8, beta polypeptide (*c8b*); all of which were downregulated. The current study is in agreement with previous studies mentioned above in that salinity acclimation can impact tissue immune response to differing extents, yet overall, the current study observed immunosuppression in response to salinity acclimation for olfactory rosettes, gills, and liver.

A systems level analysis of interacting genes and gene products also helped to identify the differential regulation of cell cycle-related genes, such as growth arrest and DNA-damage-inducible genes (*gadd45*) in the three tissues. Cell proliferation, resulting from hormones and growth factors, may play a role in osmoregulation (Evans and Somero 2008). The expression of *gadd45g* genes was upregulated in all three tissues in high salinity compared to control. *Gadd45* increases following stressful growth arrest conditions and treatment with DNA-damaging agents and mediates activation of the p38/JNK pathway via MTK1/MEKK4 kinase (reviewed in Johnson and Lapadat 2002). Evans and Somero (2008) found that *gadd45* was downregulated after hyposmotic stress, supporting the role of *gadd45* in salinity acclimation. DNA damage sensors have been suggested as possible osmosensors, recognizing osmotic stress and initiating signal transduction pathways (reviewed in Fiol and Kültz 2007). As *gadd45* genes were upregulated in all three tissues, it is possible that these genes are working as osmosensors.

Another gene identified by interaction networks that appears to be regulated by high salinity acclimation was insulin-like growth factor binding protein 1 (*igfbp1*). It was linked to CCAAT/enhancer binding protein α (*cebpa*), upregualted in liver, and downregulated in olfactory rosettes. *Igfbp5* mRNA was downregulated in gills but did not link to similar genes in the liver or olfactory rosettes. Evans and Somero (2008) saw an increase in expression of *igfbp* in goby gills after hypersaline acclimation. IGFBPs have been hypothesized to prevent proteolytic degradation of insulin-like growth factors (IGFs) by mediating the efflux of IGFs from the vascular space to the cell surface and thus modulating interactions between ligands and receptors (Wood et al. 2005). IGFs couple with prolactin and cortisol to aid in osmo-regulation (McCormick 2001). Application of exogenous IGF-1 increases salinity tolerance of certain euryhaline fish (Mancera and McCormick 1998).

Signal transduction pathways were also impacted by salinity. Knowledge on osmosensing and osmotic stress signal transduction is limited in euryhaline fish; however, mitogen-activated protein kinase (MAPK) signaling has been identified in gills (reviewed in Fiol and

Kültz 2007). In gills of coho salmon, network analysis showed that *mapk14* mRNA was upregulated and interacted with five genes in the current study in high salinity compared to control. MAPKs receive signals from osmosensors that are then integrated and amplified to activate downstream targets. MAPKs are important in regulating cell cycle, cell growth, differentiation, cell death, and pathogen defense systems (Treisman 1996). Kültz and Avila (2001) observed altered activity and phosphorylation of the three main euryhaline MAPKs in the gills of killifish (*Fundulus heteroclitus*) after osmotic stress leading to the conclusion that MAPKs are important parts of salinity acclimation osmosensory pathways. Similarly, mRNA of jun proto-oncogenes (*jun* and *junb*) was upregulated in liver and gills in high salinity as compared to control. JUN is activated by the JNK pathway, part of a MAPK cascade, which can be regulated by cellular and environmental stress. Marshall et al. (2005)) saw an upregulation of JNK in gills of killifish (*F. heteroclitus*) after hypersaline acclimation.

In olfactory rosettes, another kinase signaling pathway was impacted, as protein kinase N1 (*pkn1*) mRNA was upregulated in high salinity compared to control. PKN1 is part of the protein kinase C (PKC) superfamily and is activated by Rho family small G proteins. PKC is involved in osmotic stress signaling and promotes volume regulation after hypo-osmotic stress (Ollivier et al. 2006). During hyperosmotic stress, PKC has been shown to activate $Na^+/K^+/Cl^-$ co-transporter (Lionetto et al. 2002).

Fiol et al. (2006) found one of the main processes of novel immediate hyperosmotic stress response genes for tilapia 4 h after transfer from freshwater to saltwater was actin-based cytoskeleton dynamics. The current study also observed differential regulation of cytoskeletal-related genes, which is apparent in the GO terms and interaction networks for the gills. One of the significant cellular component GO terms in the current study was collagen. Collagen is involved in growth or reorganization. Seear et al. (2010) found that multiple collagen-related genes were upregulated in gills and brain. In the gill of coho salmon, actin, gamma 1 (*actg1*) mRNA was upregulated. ACTG1 is involved in cell motility and maintenance of the cytoskeleton. Evans and Somero (2008) observed differential regulation of a variety of cytoskeleton-related genes in goby gills after hyperosmotic stress. Additionally, a majority of the significant GO terms for gills were related to muscle organization and movement. Seear et al. (2010) also saw upregulation of a gene, myomesin 1, involved in muscle contraction. Evans and Somero (2008) observed upregulation of myosin light chain 1, which is involved in cell contractile events after hypo-osmotic stress in goby gills.

Transcription factors can also play a vital role in combating osmotic stress by responding to osmotic signal transduction through activating effector mechanisms (reviewed in Fiol and Kültz 2007). Interaction network analysis identified CCAAT/enhancer binding protein α (*cebpa*) as a main component that was upregulated in the networks for all three tissues. CEBPA is a transcription factor that can regulate the expression of genes involved in cell cycle regulation and body weight ho-meostasis (Lekstrom-Himes and Xanthopoulos 1998). *Cebp* mRNA was shown to be downregulated in gill of two types of mussel, *Mytilus galloprovincialis* and *Mytilus trossulus*, after transfer from saltwater to low salinity, supporting a role for *cebps* in saltwater acclimation.

Although the four main nodes of the liver network were shared with other tissues, some of the genes branching from *cebpa* were not shared, including genes involved in metabolic processes, such as *cyp3a27*, which was downregulated. Downregulation of *cyp3a27* may impact metabolism of endogenous and exogenous compounds. None of the smaller two and three gene interactions separate from the main network were shared with gills or olfactory rosettes and included genes involved in blood coagulation, such as coagulation factor XIII, B polypeptide (*f13b*), and protein folding, such as heat shock 70 kDa protein 8 (*hspa8*). Salinity has previously been shown to impact blood characteristics, including coagulation, in redbelly tilapia (*Tilapia zilli*) (Farghaly et al. 1973). Protein unfolding can influence chaperones and trigger osmosensors to activate osmoregulatory signal transduc-tion networks (reviewed in Fiol and Kültz 2007). Other significant GO terms in the liver were related to oxygen binding and transport. One significant cellular component was the hemoglobin complex. During smoltification, the hemoglobin system of salmon becomes increasingly more complex, for example, increasing from three to ten hemoglobin forms from fry to juvenile and adult salmon (Vanstone et al. 1964; Giles and Vanstone 1976; Sullivan et al. 1985). Seear et al. (2010) found that alpha and beta hemoglobin genes were upregulated in gills and brain of smolts, but liver was not included in their study.

Glycolysis was one of the significant biological processes identified in the array for gills. Seear et al. (2010) found that two genes involved in glycolysis were upregulated in gills. Evans et al. (2011) who studied the migration of salmonids from saltwater to freshwater also found that ontologies relating to glucose metabolism were significant in gills of sockeye salmon (*Oncorhynchus nerka*) sampled in the marine environment from different migration routes. For the gills, some of the major nodes in the network were not shared with other tissues. Avian myelocytomatosis viral oncogene homolog (*myc*) mRNA was upregulated and connected to 19 different genes. MYC is involved in cell cycle progression, apoptosis, and cellular transformation and is a transcription factor. *Myc* mRNA was downregulated in gills of two types of mussel, *M. galloprovincialis* and *M. trossulus*, after transfer from saltwater to low salinity, suggesting a role for MYC in saltwater acclimation. Also, transforming growth factor, beta 1 (*tgfb1*), which was linked to 20 genes, was downregulated. TGFB1 is a cytokine that regulates proliferation, differentiation, adhesion, migration, and the MAPK cascade. Evans and Somero (2008) saw differential regulation of genes that inhibited cytokines after hypersaline acclimation in goby gills.

Olfactory-specific nodes included signal transducer and activator of transcription 1 (*stat1*), which was upregulated and connected to 13 genes. *Stat* genes are transcription activators that act in response to cytokines and growth factors. Caspase 8 (*casp8*) was also upregulated and connected to eight genes. CASP8 is involved in cell apoptosis. Cells may undergo apoptosis in response to salinity stress if they are no longer able to compensate, and the amount of damage is too large (Schwartz and Osborne 1993; Katsuhara 1997). Programed cell death allows for the elimination of malfunctioning and potentially harmful cells from the organism (reviewed in Kültz and Burg 1998).

In summary, the majority of differentially expressed genes were tissue- and salinity-dependent. Significant GO terms were also mostly tissue-dependent. For liver, terms involving oxygen binding and transport were significant, potentially impacting metabolism.

For gills, terms involving muscle and cytoskeleton were enriched, and for olfactory rosettes, immune response-related terms were highlighted. When interaction networks were examined in addition to GO terms, similarities between tissues were identified. For example, *gadd45* genes were upregulated in all three tissues and may function as potential osmosensors. Signal transduction cascades, which may integrate and amplify signals from osmosensors, were also differentially expressed in each tissue and deserve further study since the specific cascades also showed some tissue differences. *Cepba*, which is a transcription factor and may play a role in activating salinity acclimation effector mechanisms, was also upregulated in all three tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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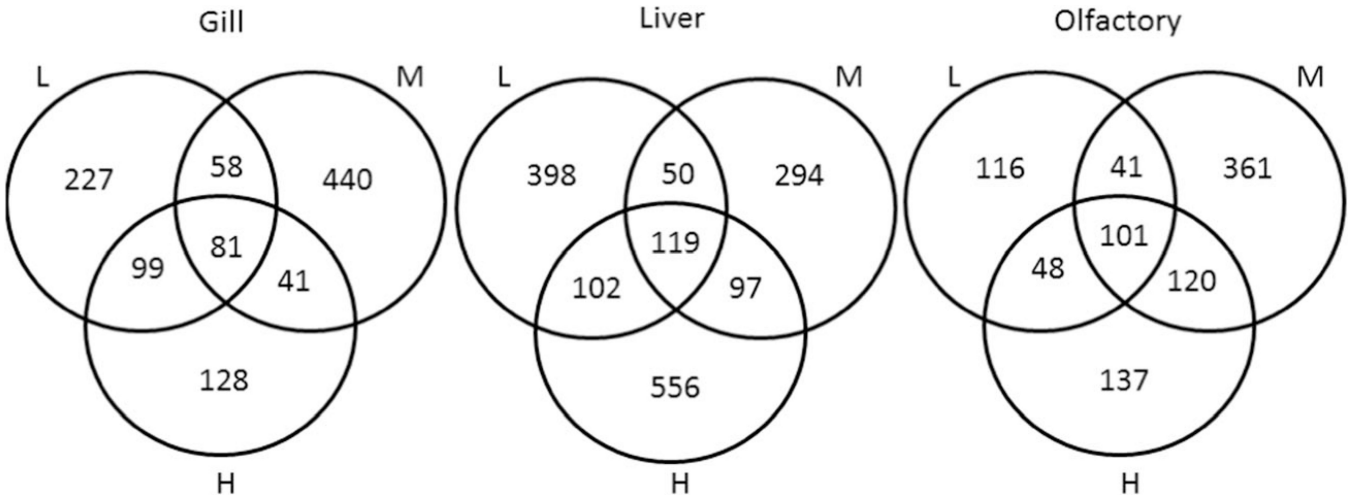
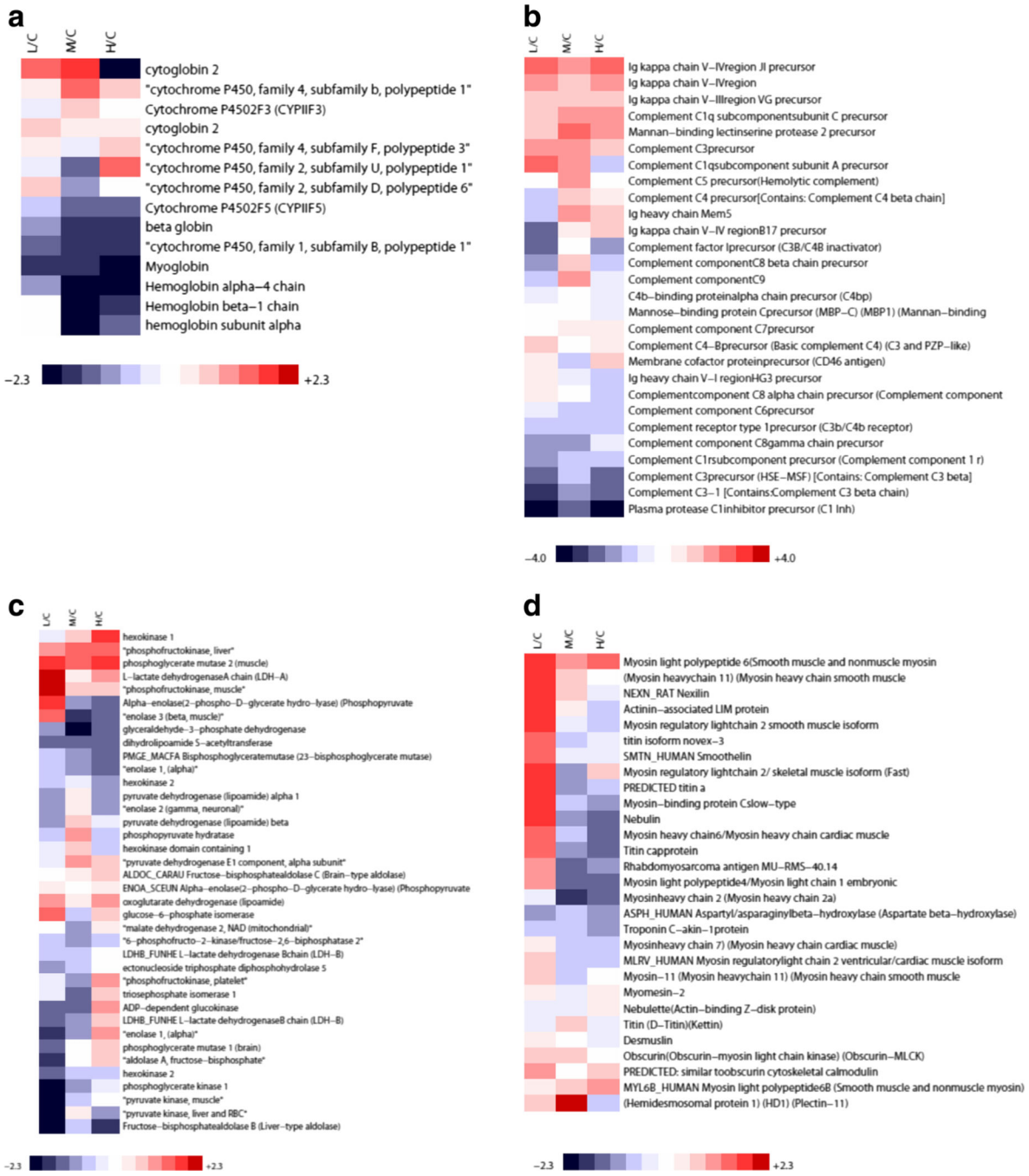


Fig. 1.

Venn diagrams for differentially expressed genes in gills, liver, and olfactory rosettes after acclimation to low (*L*), medium (*M*), and high (*H*) salinity as compared to freshwater controls. A 1.5-fold cutoff was used



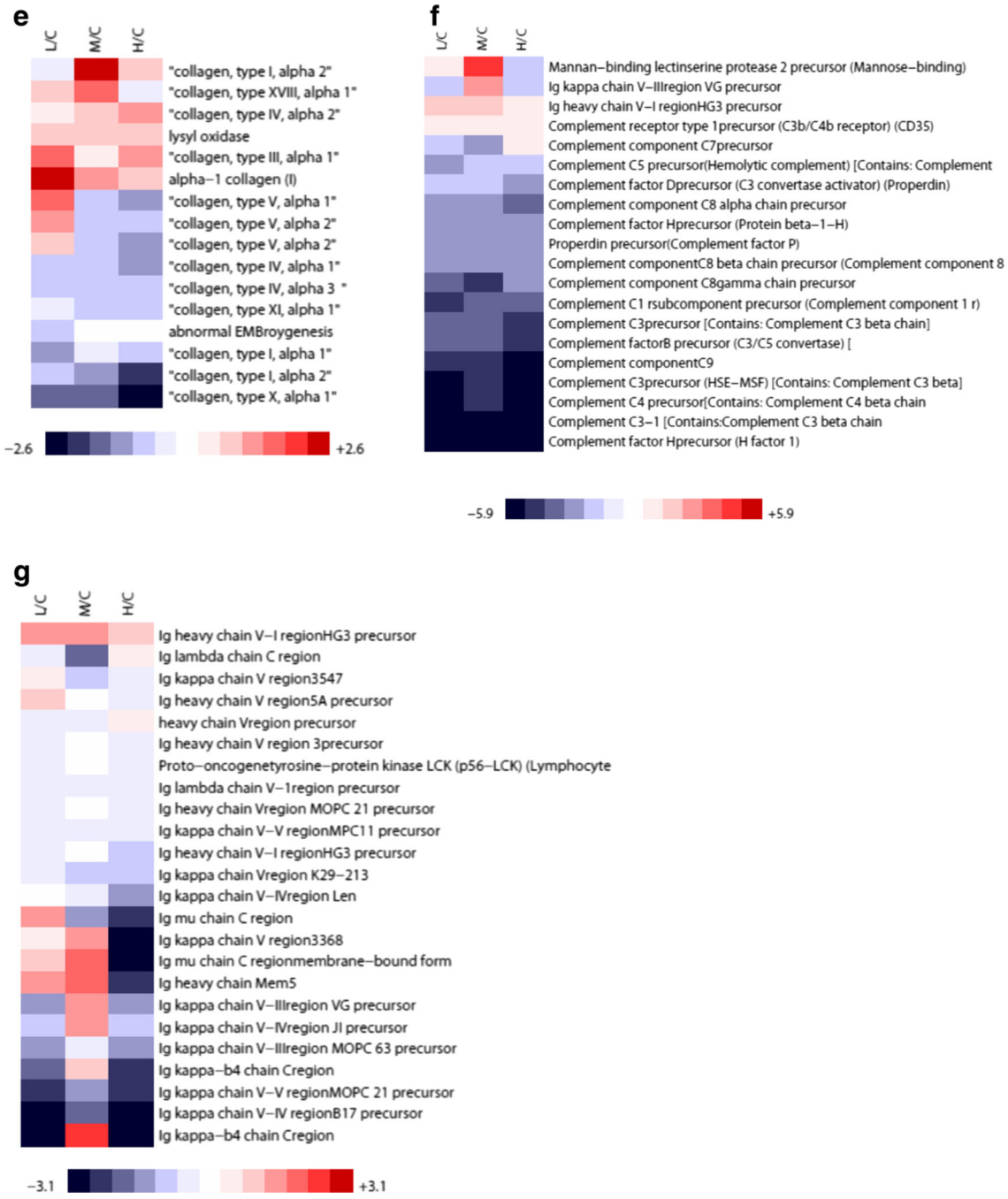


Fig. 2.

Heatmaps for select significant GO terms for differentially expressed genes in specific tissues comparing salinity treatments with freshwater control. *L/C* low salinity compared to control, *M/C* medium salinity compared to control, *H/C* high salinity compared to control. **a** Liver for GO terms hemoglobin complex and oxygen binding. **b** Gills for GO term complement pathway. **c** Gills for GO term glycolysis. **d** Gills for GO term structural constituent of muscle. **e** Gills for GO term collagen. **f** Olfactory rosettes for GO terms

complement activation—classical pathway, complement activation, complement activation—alternative pathway. **g** Olfactory rosettes for GO term antigen binding

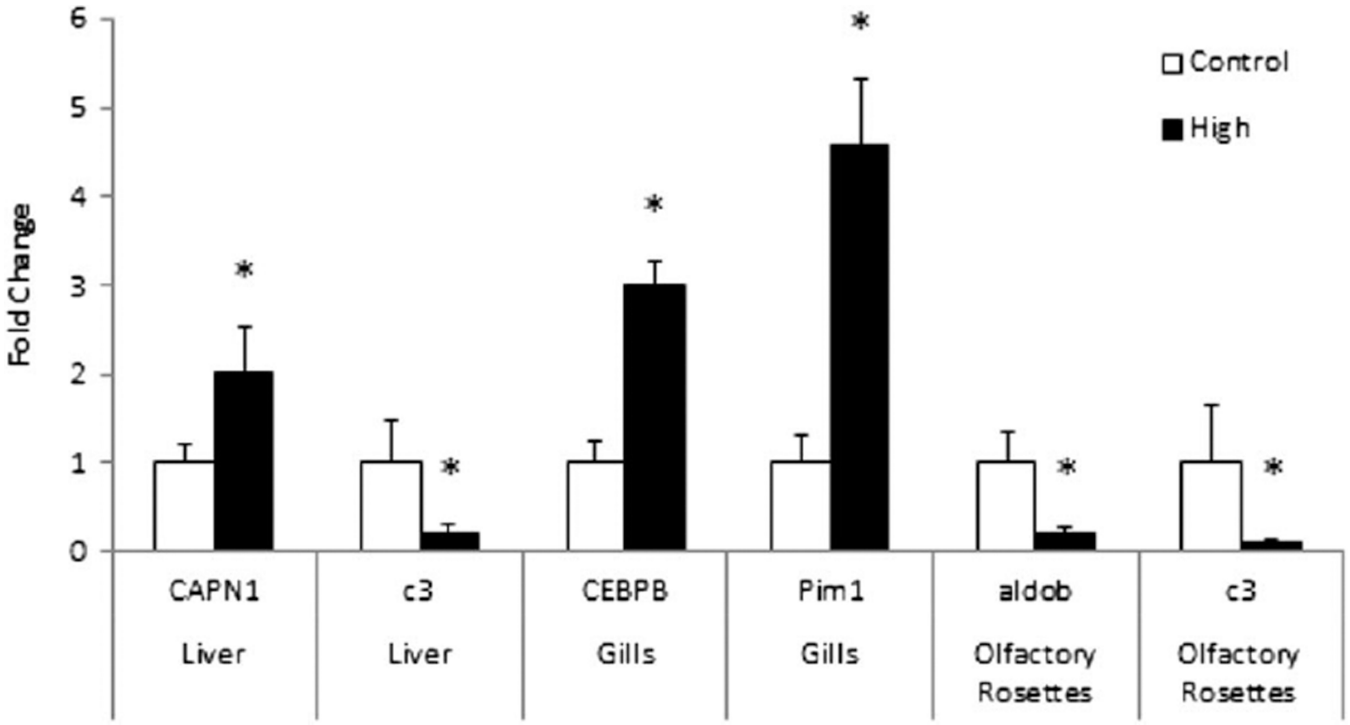


Fig. 3.

Relative gene expression for coho salmon tissues (liver, gills, and olfactory rosettes) acclimated to freshwater or high (32 ppth) salinity. Data are presented as fold change and mean±standard error (n=6–8). *Asterisk* indicates significant differences from freshwater acclimated fish (p<0.05, Student's t test). β-Actin was used as the housekeeping gene. *CAPN1* calpain 1, *c3* complement component 3, *CEBPB* CCAAT/ enhancer binding protein β, *Pim1* proto-oncogene, serine/threonine kinase, *aldob* aldolase B, fructose-bisphosphate

Table 1

Genes differentially expressed in all salinities in two tissues

Gene	Tissues		Expression	
Na/K-transporting ATPase subunit alpha-1 precursor	Gill	Olfactory	Up	Down
Na-coupled neutral amino acid transporter 2	Gill	Liver	Up	Up
Gastrulation-specific protein G12	Liver	Olfactory	Down	Up
Complement C1q-like protein 2 precursor	Liver	Olfactory	Up	Down
Complement C3-1	Liver	Olfactory	Down	Down

Table 2

Significant gene ontology terms for liver

GO term	GO term name	Bonferroni *p*
Biological process		
GO:0055114	Oxidation reduction	3.51E-05
GO:0042632	Cholesterol homeostasis	2.26E-02
GO:0042254	Ribosome biogenesis	3.85E-02
Molecular function		
GO:0005344	Oxygen transporter activity	5.75E-07
GO:0020037	Heme binding	6.68E-07
GO:0019825	Oxygen binding	1.72E-05
GO:0016491	Oxidoreductase activity	1.87E-03
GO:0009055	Electron carrier activity	3.34E-02
Cellular component		
GO:0005833	Hemoglobin complex	8.75E-06
GO:0005789	Endoplasmic reticulum membrane	4.57E-04
GO:0005783	Endoplasmic reticulum	2.43E-03
GO:0005788	Endoplasmic reticulum lumen	6.75E-03
GO:0005792	Microsome	1.23E-02

Table 3

Significant gene ontology terms for gills

GO term	GO term name	Bonferroni *p*
Biological process		
GO:0030049	Muscle filament sliding	1.32E-05
GO:0006096	Glycolysis	1.72E-04
GO:0045214	Sarcomere organization	1.91E-04
GO:0048739	Cardiac muscle fiber development	7.14E-03
GO:0055010	Ventricular cardiac muscle morphogenesis	2.21E-02
GO:0006958	Complement activation, classical pathway	4.22E-02
Molecular function		
GO:0008307	Structural constituent of muscle	8.03E-05
GO:0005509	Calcium ion binding	1.34E-04
GO:0003779	Actin binding	2.23E-03
GO:0004867	Serine-type endopeptidase inhibitor activity	2.46E-02
Cellular component		
GO:0005615	Extracellular space	3.75E-06
GO:0005861	Troponin complex	1.89E-03
GO:0005581	Collagen	3.30E-03
GO:0005743	Mitochondrial inner membrane	4.73E-03
GO:0005859	Muscle myosin complex	6.29E-03
GO:0030016	Myofibril	1.25E-02
GO:0030018	z disc	2.60E-02

Table 4

Significant gene ontology terms for olfactory rosettes

GO term	GO term name	Bonferroni *p*
Biological process		
GO:0006958	Complement activation, classical pathway	3.12E-10
GO:0006956	Complement activation	1.58E-05
GO:0006957	Complement activation, alternative pathway	4.36E-05
GO:0007596	Blood coagulation	5.04E-05
GO:0006879	Cellular iron ion homeostasis	1.87E-04
GO:0050766	Positive regulation of phagocytosis	4.92E-04
GO:0001798	Positive regulation of type IIa hypersensitivity	8.28E-04
GO:0006953	Acute-phase response	7.50E-03
GO:0007597	Blood coagulation, intrinsic pathway	1.56E-02
GO:0006642	Triglyceride mobilization	1.94E-02
GO:0016525	Negative regulation of angiogenesis	2.21E-02
GO:0006916	Anti-apoptosis	2.59E-02
GO:0006810	Transport	3.16E-02
GO:0008380	RNA splicing	4.47E-02
Molecular function		
GO:0003823	Antigen binding	8.97E-08
GO:0004867	Serine-type endopeptidase inhibitor activity	7.97E-06
GO:0030414	Peptidase inhibitor activity	1.10E-03
GO:0008289	Lipid binding	1.53E-03
GO:0032052	Bile acid binding	1.98E-03
GO:0002020	Protease binding	5.00E-03
GO:0004252	Serine-type endopeptidase activity	8.83E-03
GO:0043499	Eukaryotic cell surface binding	1.00E-02
GO:0005529	Sugar binding	3.23E-02
GO:0003705	RNA polymerase II transcription factor activity, enhancer binding	4.12E-02
Cellular component		
GO:0005615	Extracellular space	3.14E-15
GO:0000502	Proteasome complex	2.11E-02