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Transcriptomics of environmental acclimatization and survival in wild adult Pacific sockeye salmon (*Oncorhynchus nerka*) during spawning migration

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Abstract

Environmental shifts accompanying salmon spawning migrations from ocean feeding grounds to natal freshwater streams can be severe, with the underlying stress often cited as a cause of increased mortality. Here, a salmonid microarray was used to characterize changes in gene expression occurring between ocean and river habitats in gill and liver tissues of wild migrating sockeye salmon (*Oncorhynchus nerka* Walbaum) returning to spawn in the Fraser River, British Columbia, Canada. Expression profiles indicate that the transcriptome of migrating salmon is strongly affected by shifting abiotic and biotic conditions encountered along migration routes. Conspicuous shifts in gene expression associated with changing salinity, temperature, pathogen exposure and dissolved oxygen indicate that these environmental variables most strongly impact physiology during spawning migrations. Notably, transcriptional changes related to osmoregulation were largely preparatory and occurred well before salmon encountered freshwater. In the river environment, differential expression of genes linked with elevated temperatures indicated that thermal regimes within the Fraser River are approaching tolerance limits for adult salmon. To empirically correlate gene expression with survival, biopsy sampling of gill tissue and transcriptomic profiling were combined with telemetry. Many genes correlated with environmental variables were differentially expressed between premature mortalities and successful migrants. Parametric survival analyses demonstrated a broad-scale transcriptional regulator, cofactor required for Sp1 transcriptional activation (CRSP), to be significantly predictive of survival. As the environmental characteristics of salmon habitats continue to change, establishing how current environmental conditions influence salmon physiology under natural conditions is critical to conserving this ecologically and economically important fish species.

Keywords: environment, genomics, migration, physiology, salmon, stress

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Introduction

Anadromous fish undertake migrations from marine to freshwater environments for reproductive purposes. While salmon have employed this strategy for millions of years, spawning migrations are costly and represent a particularly challenging phase of life history (Cooke *et al.* 2006; Pon *et al.* 2009). Underlying the difficulty of an anadromous lifestyle are broad-scale changes in the environmental characteristics of marine and freshwater habitats encountered during the ocean to river transition. During this period, migrating fish must adjust to a suite of new abiotic and biotic conditions, including

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reversed osmotic gradients (Clarke & Hirano 1995; Makino *et al.* 2007), increases in temperature (Crossin *et al.* 2008; Macdonald *et al.* 2010) and the presence of freshwater pathogens (Marcogliese 2001). Upper-limit tolerances to these potential stressors reflect adaptations to environmental conditions most frequently encountered during evolutionary history (Lee *et al.* 2003; Farrell *et al.* 2008; Eliason *et al.* 2011). As a consequence, recent shifts in the environmental characteristics of either freshwater or saltwater habitats, such as those stemming from global climate change, are anticipated to exert substantial stress on anadromous fish populations (Crozier *et al.* 2008; Pörnter & Farrell 2008).

In the Fraser River, British Columbia, Canada, there are a number of ecologically and economically important sockeye salmon stocks that exhibit an anadromous lifestyle. However, environmental conditions within the Fraser River have been falling increasingly outside historic ranges to which these salmon populations are adapted and are hypothesized to be driving unprecedented rates of premature mortality during spawning migrations (Cooke *et al.* 2004; Patterson *et al.* 2007). While historical levels of premature mortality in Fraser River sockeye varied between 10% and 20% during spawning migrations, average premature mortality rates exceed 50% over the last decade for several populations (Cooke *et al.* 2004). Strikingly, in years with atypical environmental conditions, prespawning mortality rates exceed 90% for some sockeye populations (Cooke *et al.* 2004). For example, in the summer of 2004, sockeye salmon suffered enhanced prespawning mortality when Fraser River water temperatures exceeded levels previously recorded in a 60-year time series for several consecutive days (Brander 2007). Such severe rises in premature mortality rates suggest that the impact of the environment is exceeding the capacity to acclimatize in adult salmon (Farrell *et al.* 2008; Hague *et al.* 2010). As a corollary, shifting environmental conditions may play a major role in dictating the population dynamics of Fraser River salmon and underscore the need to assess the potential effects of current and future environmental conditions on these important salmon populations.

Despite a large body of research evaluating salmonid migration and the fact that shifting environmental conditions are an often cited cause of recent sockeye salmon population declines (Macdonald 2000; Holt & Peterman 2006; Haeseker *et al.* 2008), mechanistic knowledge of the effects of the environment on the physiology and survival of wild migrating salmon is limited. Here, transcriptomics (quantifying changes in the cellular mRNA pool) and telemetry were used to better define the relationship between the environment, physiology and survival in wild sockeye salmon returning to spawn in the Fraser River. We hypothesized that migrating sockeye would respond to potential environmental stressors encountered along migration routes with adaptive shifts in gene expression and that transcriptional responses to the environment would differ between successful migrants and premature mortalities. High-throughput gene expression profiling has emerged as an insightful tool for investigating responses to environmental change (Gracey & Cossins 2003). Coordinated manipulation of gene expression represents one of the most rapid and versatile reactions available to organisms experiencing environmental perturbations and has been shown to facilitate responses to a multitude of environmental stressors in fish, including heat stress (Buckley *et al.* 2006), osmotic stress (Kalujnaia *et al.* 2007) and pathogen infection (Rise *et al.* 2004).

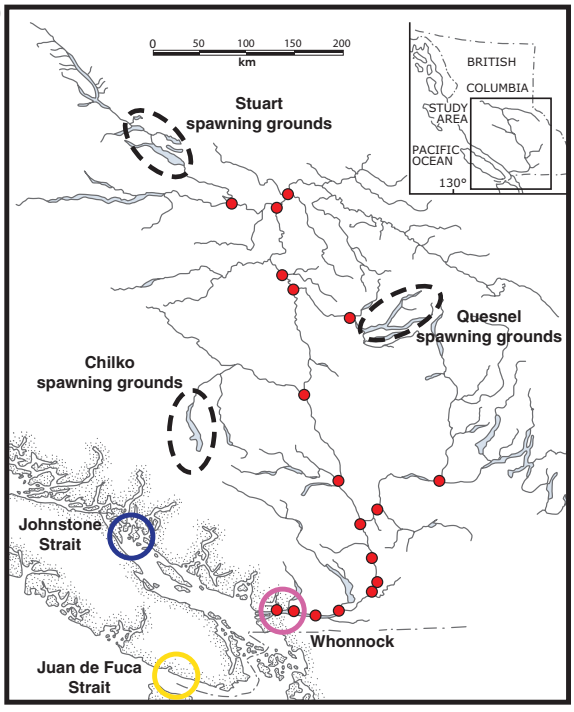
This study is intended to address several key issues surrounding salmon conservation. First, transcriptomic profiles were used to infer the relative importance of physiological responses to the environment. Second, we sought to isolate environmental variables most strongly affecting salmon physiology and most plausibly contributing to premature mortality. Third, expression patterns of genes tightly associated with specific environmental variables were used to resolve spatial and temporal patterns of environmentally regulated physiological change. Finally, by exploring how the transcriptome of fish upon entry into freshwater differs between successful migrants and premature mortalities, links between gene expression, survival and the environment can be empirically established.

Materials and methods

Lethal sampling of sockeye salmon

Lethal sampling was performed on wild migrating sockeye salmon at three sites: two within the marine environment and one within the Fraser River (Fig. 1A). Adult sockeye salmon return to the Fraser River using two distinct marine routes (Quinn *et al.* 1989). Fish taking the northern route pass through Johnstone Strait (JS) along the eastern side of Vancouver Island, while those taking the southern route follow the western coast of Vancouver Island through Juan De Fuca Strait (JDFS). Commercial fishing vessels and purse seine netting were used to capture wild fish within each route during the summer spawning migration of 2005. Fish were sampled within JS on August 22nd and within JDFS on August 23rd. Telemetry has shown that migration times from either sampling site to the Fraser River (approximately 300 km) are similar (Crossin *et al.* 2009). Nets were deployed and retracted in 20- to 30-min intervals and contained a maximum of six fish per interval. All fish were alive following capture and transferred on

(A)



(B)

Gill	JS (n = 14)	JDFS (n = 15)	W (n = 11)	Biopsy (n = 45)
Chilko	5	6	4	18 (10:8)
Quesnel	8	5	4	18 (10:8)
Stuart	1	4	3	9 (5:4)

Liver	JS (n = 15)	JDFS (n = 11)	W (n = 10)
Chilko	7	4	3
Quesnel	8	7	7
Stuart	0	0	0

Fig. 1 Map of study area (A). Lethal sampling sites (coloured circles: JS = blue, JDFS = gold, W = magenta), stock-specific spawning grounds (dashed circles) and the location of receiver stations used in telemetry (red dots; Crossin *et al.* 2009) are illustrated. The gill biopsy sampling site at Mission is contained within the W site. All samples were obtained from three major stocks of summer-run sockeye salmon (Chilko, Quesnel, Stuart). The proportions of fish from each stock are shown (B). In gill biopsy samples, bracketed numbers denote the ratio of survivors to premature mortalities for each stock.

deck using smaller nets in <5 min. Once on board, fish were killed and transferred to a chilled cooler for dissection. Gills (4 mm from the tips of 6–7 filaments from the first gill arch) and whole livers were dissected and frozen on dry ice within 15 min of fish being brought on board. Freshwater sampling within the Fraser River occurred on September 1st at Whonnock (W), approximately 65 km upstream from the river mouth, using commercial gill net boats. The river sampling site at W is fully freshwater with no estuarine influence and fish reaching W are committed to continuing upstream (Crossin *et al.* 2009). Gill nets were deployed and retracted in 20- to 25-min sets and sampling proceeded as described for marine sites. All tissue samples were stored in liquid nitrogen prior to being transferred to −80 °C freezers. Following tissue dissection, a fin clip from each fish was preserved in 95% ethanol for genetic stock identification (GSI) (Beacham *et al.* 2005). Any individuals that were injured or in poor condition (e.g. had visible wounds from predatory marine mammals or excessive scale loss) were excluded from the study. Only female fish were sampled in an attempt to limit overall variation in gene expression (Churchill 2002). Females were chosen over males because females appear to suffer higher rates of premature mortality during spawning migrations than males (e.g. Tierney & Farrell 2004; Crossin *et al.* 2008; Roscoe *et al.* 2010) and because conservation efforts rely on data from female fish. The decision to focus exclusively on female fish increases the probability of detecting changes in gene expression that may not be similar in males.

Biopsy sampling and telemetry

Nonlethal biopsy sampling of gill tissue and transcriptomic profiling were combined with telemetry to determine whether differences in gene expression at the time of sampling influenced survival during subsequent upstream migration. Biopsy sampling was performed on wild migrating sockeye salmon intercepted within the Fraser River at Mission (15 km upstream from the W sampling site) between 3 August and 31 August 2005. A detailed description of the sampling and radiotagging procedure used in this study is available elsewhere (Cooke *et al.* 2006, 2008; Crossin *et al.* 2009). Previous studies have demonstrated that biopsy sampling and radiotransmitter implantation have a negligible effect on the subsequent survival and migratory behaviour of sockeye salmon (Cooke *et al.* 2005). Gill biopsies for microarray analysis (4 mm from the tips of 2–3 filaments from the first gill arch) were frozen on dry ice and stored in liquid nitrogen prior to being transferred to −80 °C freezers until further processing. Fin clips for GSI were placed in 95% ethanol.

The progress of radio-tagged fish through the Fraser River was monitored using 18 fixed receiver stations set at strategic locations throughout the watershed (Fig. 1A). Ninety-eight per cent of fish implanted with radiotransmitters in 2005 were detected at at least one monitoring station (Roubichaud & English 2005). Data downloaded from receiver sites were processed using the custom database software Telemetry Manager (LGL Limited). GSI was used to determine the stock-specific spawning grounds for each fish (Beacham *et al.* 2005) giving an intended end-point of migration for each of the three stocks investigated in this study, namely Chilko, Quesnel and Stuart. Fish were classified as probable

survivors if they were detected at the receiver station closest to their intended spawning ground. Those fish that did not reach this receiver station were assumed mortalities. Tagged fish caught through in-river fisheries were removed from analyses; however, some fish classified as mortalities could represent unreported fisheries losses. The actual spawning success of fish classified as survivors was not assessed.

RNA extraction, amplification and labelling

Total RNA from lethally sampled gill and liver tissues and biopsy-sampled gill tissue was extracted, amplified and labelled as described in the study by Miller *et al.* (2009). Approximately 10 mg of frozen tissue was homogenized with stainless steel beads in TRI reagent (Ambion Inc.) on a MM301 tissue homogenizer (Retsch Inc.). Aliquots of homogenate (100 μL) were pipetted into 96-well plates, and extractions were carried out using Magmax™-96 for Microarrays Kits (Ambion Inc.) with a Biomek NXP automated liquid-handling instrument (Beckman-Coulter). RNA yield and purity were assessed by measuring A_{260} and A_{260}/A_{280} ratio, respectively, with a NanoDrop spectrophotometer (NanoDrop Technologies). Total RNA (500 ng to 5 μg) was amplified using a MessageAmp™II-96 kit (Ambion Inc.) according to manufacturer's instructions. Amplification steps were performed in batches in 96-well plates with each batch containing all samples from either lethally sampled gill RNA, lethally sampled liver RNA or biopsy-sampled gill RNA. A separate amplification was performed for the reference RNA sample, compromised of pooled gill and liver RNA extracted from all lethally sampled fish. Amplified RNA (5 μg) were reverse-transcribed into cDNA and labelled with Alexa dyes using a SuperScript Plus Indirect Labeling Kit (Invitrogen) according to manufacturer's instructions. Experimental samples were fluorescently tagged with Alexa 555 (red) and the reference labelled with Alexa 647 (green).

Gene expression profiling with cDNA microarrays

The 16 006 feature microarray was produced by the Genomics Research on Atlantic Salmon Project (GRASP http://www.uvic.ca/cbr/grasp; von Schalburg *et al.* 2005). Gene expression levels were determined by comparing the amount of RNA transcript in the experimental samples relative to a common reference sample. A total of 121 microarrays were used to generate the data set, corresponding to individual hybridizations of both gill and liver samples collected from JS (gill $n = 14$; liver $n = 15$), JDFS (gill $n = 15$; liver $n = 11$), W (gill $n = 11$; liver $n = 10$) and biopsy-sampled gill tissue collected at Mission ($n = 45$) (Fig. 1B). Gill and liver were chosen because these tissues exhibit dynamic changes in gene expression in response to changing environmental conditions in fish, including stresses probably incurred by salmon during spawning migrations (Douglas 2006). GSI indicated that these samples were obtained from three major salmon stocks: Chilko, Quesnel and Stuart. The number of fish sampled from each stock at each site is reported in Fig. 1B.

Slide hybridizations were performed as described in the study by Miller *et al.* (2009). A ScanArray Express microarray scanner (Perkin Elmer) was used to capture fluorescent images, adjusting the PMT gain for optimized visualization of each slide. Resulting fluorescent images were quantified using ImaGene software (BioDiscovery Inc.). Spots with poor quality (<2 standard deviations from background) were flagged as missing. Raw microarray intensity data were then normalized within the BioArray Software Environment (Vallon-Christersson *et al.* 2009) using the print-tip locally weighted linear regression (LOESS) method. All data were log-transformed (base 2), and an intensity ratio was computed by taking the difference in normalized log-transformed intensities between the sample and reference. Features flagged as missing in <50% of the samples had intensity ratios imputed with the K-nearest neighbours method. Features with missing values in >50% of the samples were removed. This gave gene sets of 14 420 features for gill, 14 962 features for liver and 14 411 features for radio-tagged fish to be used for statistical analyses. Thus, a high proportion of the transcriptome was considered expressed and generally exceeds the proportion of expressed genes reported in other microarray studies. Extensive quality control, including the use of the arrayQualityMetrics package for R, refutes the possibility of technical artefacts driving this trend. Alternatively, the large proportion of expressed genes may relate to the fact that expression above background needs only to be detected in 50% +1 of all individuals to be considered expressed and the extreme breadth of physiological change associated with salmon migration (e.g. osmoregulation, sexual maturation, prolonged swimming, starvation and senescence).

Statistical analyses of microarray data

For lethally sampled gill and liver tissues, we were interested in features that showed a significant change in expression between each sampling site. Small sample sizes for each stock at certain sampling sites prevented a stock-by-stock analysis (Fig. 1B). Alternatively, statistical tests were performed independent of stock using a one-way analysis of variance (ANOVA) that modelled sampling location as a fixed effect and log-transformed

intensities for each feature as the response variable. The *q*-value method was used to correct for multiple hypothesis testing (Storey 2002), and Tukey's post hoc tests were used to identify features that differed in expression between each sampling site (i.e. JS vs. JDFS, JS vs. W and JDFS vs. W). Few genes had *q*-values < 0.05, which we predict to be the result sampling fish in their natural habitat without laboratory acclimation and across genetically distinct stocks. Consequently, a less stringent significance threshold was chosen, and features with Tukey's *P*-values < 0.01 and *q*-values < 0.1 were considered significant. Statistical analyses were performed independently for gill and liver tissue as expression levels of specific genes were not compared.

For biopsy-sampled and radio-tagged fish, we were interested in significant differences in gene expression between successful migrants and premature mortalities. We therefore used a *t*-test to determine those transcripts that differed significantly between mortalities and survivors, correcting for multiple hypothesis testing using the *q*-value method. Because GSI indicated that mortality was not skewed towards a particular stock (Fig. 1B), we performed the test independent of stock as a means of identifying genes potentially affecting survival in all three populations sampled. No genes were significant at a *q*-value threshold of $P = 0.1$ (Fig. S9). Alternatively, features with *P*-values < 0.01 were further analysed using principal components and parametric survival analyses (an extension of generalized linear models). Parametric survival analysis is appropriate for time to event data that do not have a constant variance (Harrell 2001; Crawley 2007). Two variables were used as objects in the survival analysis: the number of days survived following tagging, calculated as the number of days between tagging and last receiver detection, and distance travelled, calculated as the approximate distance between the tagging site at Mission and the receiver site fish were last detected. All statistical tests were performed in R (R Development Core Team 2009).

Functional annotation of significantly differentially expressed genes

Each significantly differentially expressed gene was assigned to a broad-scale functional category and given a putative function to provide a cursory view of the types of genes transcriptionally affected by migration. Multiple significant features that annotated as the same gene and were expressed in a similar direction were considered a single gene. Features that annotated alike but diverged in expression pattern were considered distinct and the result of residual tetraploidy within the salmon genome, alternative splicing or a false-positive statistical test. This occurred 28 times among all 1244 features significantly differentially expressed. Functional annotations were performed manually for each gene based on entries contained in the Uniprot Database. Hypothesized functions were based on descriptions included under the 'Function' heading within the 'General Annotations (Comments)' section. Functional categories were based on the 'Biological Process' heading within the 'Gene Ontology' section. Each significant gene was cross-referenced with information from the primary literature to accurately classify those with multiple ontologies or fish-specific functions not mentioned in database entries. Integrating information across these multiple sources provided the most accurate means of functional annotation for the nonmodel and nonmammalian species used in this study (Larsen *et al.* 2007; Evans & Somero 2008). Fold-change was calculated by averaging intensity ratios across individuals within a given sampling site for each gene, then calculating the difference in averaged ratios between sampling sites and converting this value (x) to fold-change [if $x > 0$, then fold-change = $2^{(x)}$; if $x < 0$, then fold-change = $(-1)2^{-(x)}$].

Gene set enrichment analysis (GSEA)

GSEA was performed using the publicly available program Database for Annotation, Visualization and Integrated Discovery (DAVID; Huang *et al.* 2009). Gene identifiers were extracted from the UniProt database and assigned to annotated features on the array. DAVID analyses use this annotation to agglomerate species-specific gene information from a variety of public genomic databases and identify ontologies significantly enriched within lists of differentially expressed genes relative to all features present on the entire array (EASE $P < 0.05$ and reported as a fold-enrichment value).

Quantitative real-time polymerase chain reaction (qPCR)

The expression patterns of four genes significantly differentially expressed between marine and freshwater sampling sites were also investigated using qPCR. Primers were designed to amplify heat shock protein 47 (Hsp47; forward 5′-TCCACTTTCCACCCTGCAAAG-3′ and reverse 5′-AGTTTGGTTGGCAAATGGCATAG-3′), cold-inducible RNA-binding protein (CIRP; forward 5′-AAGCTGTGATTGTGCTCTAAAGAC-3′ and reverse 5′-TCCCACTTAGCTTCCATCCTTG-3′), haemoglobin alpha (forward 5′-GGTTCCGCTCCAGTGAAGAAG-3′ and reverse 5′-CGGCAACGACCACAATCAGG-3′) and haemoglobin beta (forward 5′-GTCGTTGTGTCCGCTCT

TGG-3′ and reverse 5′-CAGCCTAACAGACTGGAGT TGG 3′). Primers were designed using AlleleID software version 4.0 (Premier Biosoft), and careful consideration was given to the tetraploidy of the salmon genome to ensure that assays detected only the gene copy of interest. cDNAs were synthesized from 1 μg total RNA from either gill or liver tissue using a two-step reverse transcription–PCR with SuperScript II First-Strand Synthesis SuperMix (Invitrogen) and Power SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's instructions. qPCR assays were performed on an ABI 7900HT PCR system (Applied Biosystems) in 384-well plates containing 1× Power SYBR Green PCR Master Mix, 300 nM forward and reverse primers and diluted template cDNA (1:5). Cycling conditions were 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. All samples were run in duplicate and nontemplate controls were included. The relative quantification $2^{-\Delta\Delta CT}$method described in Livak & Schmittgen (2001) was used to determine expression changes relative to the housekeeping gene acidic ribosomal phosphoprotein (forward 5′-GGAAAAGGGCGA GATGTTCAAC-3′ and reverse 5′GAAATCCTGAGCG ATGTGATGC-3′). Statistical significance was determined by one-way ANOVA using GraphPad Instat software (GraphPad Software).

Results

Transcriptional responses to the marine environment

We predicted that differences in gene expression between fish sampled in JS and those sampled in JDFS would be minimal, assuming that these two migration routes represented similar habitats. Contrary to this expectation, transcriptional differentiation between the two marine sites exceeded that occurring between either ocean site and freshwater in gill (Fig. 2A and Fig. S1). The vast majority of this transcriptional change represents shifts occurring only in the marine environment. Three-hundred and seventy genes were significantly differentially expressed between JS and JDFS in gill, of which 300 (82%) were exclusive to marine sampling sites. Because salmon were sampled approximately equidistant from the river and migration times from each marine sampling site to the river are similar, we hypothesized that these transcriptional differences are the result of divergent environmental conditions between JS and JDFS.

Functional annotation and GSEA performed on all genes significantly differentially expressed between JS and JDFS in gill suggest that temperature, salinity and pathogen exposure represent plausible causes for the extensive transcriptional change observed in the marine environment. Previous transcriptomic studies have demonstrated that changes in these variables drive broad-scale changes in gene expression, which may explain the large number and functional diversity of genes significantly differentially expressed (Rise *et al.* 2004; Buckley *et al.* 2006; Kalujnaia *et al.* 2007). Potential differences in thermal regimes were reflected by a significant enrichment for the 'protein folding' (2.3-fold) ontology (Fig. S6C) and a total of ten molecular chaperones significantly differentially expressed between JS and JDFS in gill (Fig. 3A). Included among these genes were three members of the Hsp70 family, whose

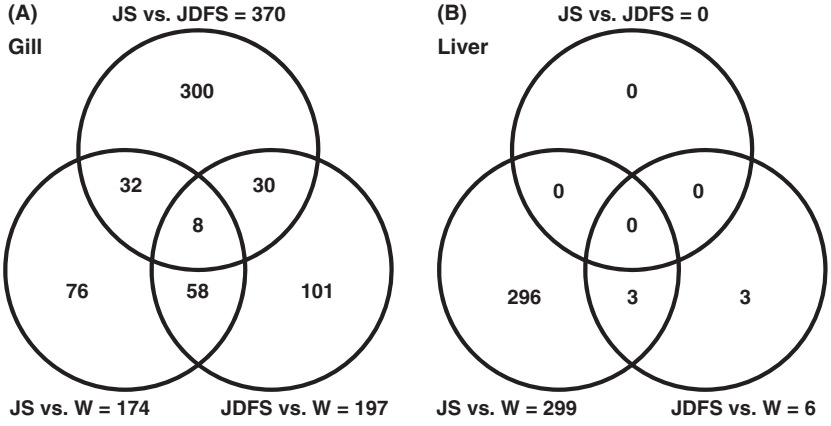


Fig. 2 Venn diagrams showing the relationship between significant genes for each sampling location and tissue. The number of probable unigenes, excluding genes that could not be annotated, is shown for each pairwise comparison of sampling location [Juan De Fuca Strait (JDFS), Johnstone Strait (JS) and Whonnock (W)] for gill (A) and liver tissue (B). Multiple significant features that annotated as the same gene and were expressed in a similar direction were considered a single gene. Features that annotated alike but diverged in expression pattern were considered distinct genes. Venn diagrams were created using GeneVenn (Pirooznia *et al.* 2007).

(A) Temperature change

Name	Fold change
Heat shock protein 70a	1.64
Heat shock protein_Hsp70 (*n* = 2)	1.58
Small glutamine-rich protein with tetratricopeptide repeats 2	1.51
Gamma-B-crystallin	1.50
Heat shock protein 60	1.44
Peptidyl-prolyl cis-trans isomerase (*n* = 2)	1.33
T-complex protein 1 subunit epsilon	1.29
Tetratricopeptide repeat protein 2 (*n* = 2)	−1.24
DnaJ homolog subfamily C member 1	−1.35
Heat shock 70 kDa protein 88E	−1.92

(B) Osmoregulation

Name	Fold change
Growth hormone	1.64
Prolactin	1.60
Inducible nitric oxide synthase	1.57
Prolactin precursor	1.52
Sodium/potassium-transporting ATPase subunit alpha-3	1.42
Na-K-Cl cotransporter	1.33
Sodium borate cotransporter 1	−1.20
Na(+)/H(+) exchange regulatory cofactor NHE-RF3	−1.30
Potassium channel tetramerisation domain containing protein 13	−1.32
Ion transport regulator 3 precursor	−1.45
Solute carrier family 12 member 8	−1.45

(C) Pathogen exposure

Name	Fold change
Reverse transcriptase-like protein	1.71
Immunoglobulin light chain	1.61
MHC class I antigen (*n* = 2)	1.48
Reverse transcriptase (*n* = 3)	1.48
Enediyne polyketide synthase	1.47
Chondromodulin 2	1.47
Immunoglobulin H A	1.46
C-type lectin, serum lectin isoform 1 precursor (*n* = 2)	1.45
Barrier-to-autointegration factor	1.42
Immunoglobulin A (*n* = 2)	1.42
Nonclassical MHC class I antigen	1.40
Interferon-induced very large GTPase 1	1.40
MHC class I A region (*n* = 3)	1.38
Proepithelin	1.36
CD81 antigen	1.34
Antigen peptide transporter 2	1.32
Collectin 11	1.32
T cell receptor beta chain	1.31
Interferon-induced, hepatitis C-associated microtubular aggregate	1.30
AN1-type zinc finger protein 5	1.29
Complement factor P	1.26
Beta-2-microglobulin precursor (*n* = 3)	−1.20
Minor capsid protein 6	−1.30
Prothymosin alpha	−1.32
Lysozyme g	−1.33
Aspartyl aminopeptidase	−1.34
Interferon regulatory factor 1	−1.35
Complement factor_C3 convertase activator	−1.36
Invariant chain S25-7 mRNA, complete cds	−1.37
T-cell receptor T3 zeta chain	−1.39
MHC class II-associated invariant chain	−1.44
Leukotriene-B(4) omega-hydroxylase 2	−1.49
Lysosome-associated membrane glycoprotein 3 (*n* = 2)	−1.64
Beta-2 microglobulin	−1.65
HLA class II histocompatibility antigen gamma chain	−1.66
C-type lectin, serum lectin isoform 1 precursor	−1.73
MHC B-F histocompatibility F10 antigen	−1.97

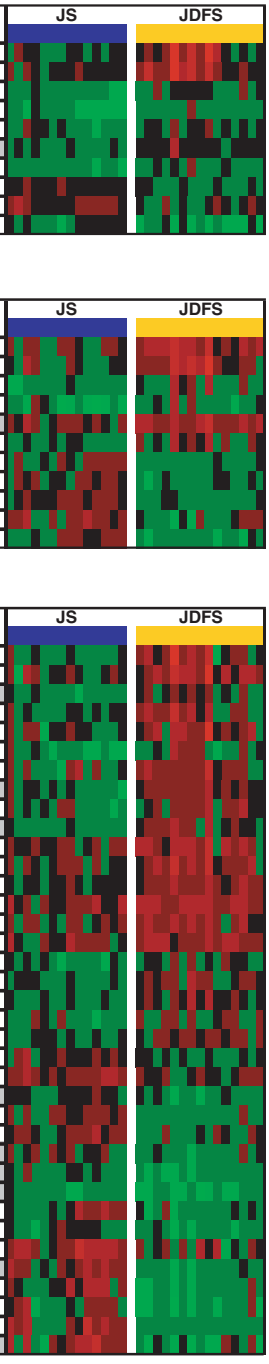


Fig. 3 Heatmaps of significantly differentially expressed genes relating to temperature change, osmoregulation and pathogen exposure between ocean sampling sites in gill. Each row represents a significantly differentially expressed gene, and each column, the corresponding expression level for individual fish sampled at Johnstone Strait (blue; JS) and Juan de Fuca Strait (gold; JDFS). Shaded rows indicate genes also differentially expressed between JS or JDFS and W. Functional classifications into either (A) temperature change, (B) osmoregulation or (C) pathogen exposure were based on information contained within the UniProt database and the primary literature. Genes are ordered by fold-change, which reflect changes that occurred in JDFS-sampled fish relative to those sampled in JS. The order of individual fish for each gene is random but consistent across each gene and heat map. In cases where multiple features annotated as the same gene and were expressed in a similar direction (number of features shown in brackets following the gene name), mean intensity values are shown. Features that annotated alike but diverged in expression pattern were considered distinct. Heatmaps were generated using a program written in Microsoft Excel (Microsoft Corp.).

expressions are tightly correlated with a well-characterized and highly conserved response to temperature change (Lindquist 1986; Basu *et al*. 2002). Significant enrichment of ontologies associated with salinity acclimatization, for example 'cellular cation homoeostasis' (3-fold), indicated that osmoregulatory processes also differed between migration routes (Fig. S6C). Ontologies relating to glucose metabolism and proteolysis were the most numerous in the GSEA and metabolic and proteolytic demands shift during the osmoregulatory remodelling of gills necessary to survive the saltwater to freshwater transition (Uchida *et al*. 1997; Tseng & Hwang 2008). Eleven genes belonging to the osmoregulation functional category were differentially expressed between ocean sampling sites in gill, including seven ion transporters (Fig. 3B). Significantly differentially expressed genes with extremely well-characterized roles in salinity acclimatization in fish included the ion

transport proteins Na^+, K^+ transporting-ATPase subunit α-3 and $Na^+/K^+/Cl^-$ cotransporter, as well as the osmoregulatory hormones growth hormone and prolactin. Differences in pathogen exposure between JS and JDFS were exemplified by the significant enrichment of ontologies associated with immune responses, such as 'regulation of macrophage activation' (10.2-fold) and 'regulation of interleukin-12 production' (12.3-fold) (Fig. S6C). A total of 37 genes annotating to the immune response functional category were differentially expressed between JS and JDFS in gill, including fundamental components of immune responses such as immunoglobulins and constituents of the major histocompatibility complex (Fig. 3C).

No significant differences in gene expression were detected between JS and JDFS in liver (Fig. 2B). While it is possible that the liver transcriptome is highly similar between migration routes, this would be in stark contrast to the extensive shifts in gene expression observed between marine sites in gill. An alternative explanation is that high interindividual variation within one or both of the marine sampling sites reduced the ability to detect significant differences in gene expression. We tested this possibility by generating a principal component loading plot that graphed the first principal component (PC1) against PC2 for each fish sampled at JS, JDFS and W in liver. This approach provided a method of estimating overall variation in expression between individual fish sampled at each site. The loading plot indicated higher variability in gene expression within JDFS relative to JS or W, which probably reduced the capacity to resolve significant differences in gene expression in pairwise comparisons with JDFS liver (data not shown). This trend is indicative of a lack of selective constraint on gene expression in liver.

Transcriptional responses to freshwater

Given major differences in the abiotic (e.g. salinity and temperature) and biotic (e.g. resident pathogens) characteristics of ocean and river habitats, we hypothesized that shifts in gene expression would occur as fish entered freshwater. Consistent with this prediction, 174 and 197 genes were significantly differentially expressed between JS and W and JDFS and W in gill, respectively (Fig. 2A, Figs S2 and S3). In liver, 299 genes were significantly differentially expressed between JS and W (Fig. 2B and Fig. S4). Only six genes were significant between JDFS and W (Fig. 2B and Fig. S5), a likely function of the higher interindividual variation in JDFS described previously. Gene expression patterns from gill provide clear evidence that the environment is a major factor driving physiological change between marine and freshwater habitats. Evidence for environmentally regulated gene expression in liver was limited.

Temperature. Putative responses to elevated temperature in freshwater were identified by GSEA and gene functional annotation. In gill tissue, GSEA conducted on all genes significantly differentially expressed between JS and W indicated a 6.5-fold enrichment for 'peptidyl-prolyl cis-trans isomerase activity', an enzyme involved in accelerating protein folding (Fig. S6A; Göthel & Marahiel 1999). In liver, GSEA indicated a 2.3-fold enrichment for the 'protein folding' ontology between JS and W (Fig. S7). Key molecular biomarkers of heat stress were significantly differentially expressed between ocean- and river-sampled fish in both tissues. Prominent were transcripts for molecular chaperones ('Protein Folding' functional category) involved in refolding denatured proteins and whose differential expression is strongly associated with exposure to elevated temperatures (Lindquist 1986; Basu *et al.* 2002). In gill, eight genes involved in protein folding were significantly differentially expressed between JS and W, six of which were upregulated in freshwater including Hsp70-5 and Hsp47. Between JDFS and W, five molecular chaperones were differentially expressed and three were upregulated in freshwater, including Hsp90β (Fig. 4A). Ten genes involved in protein folding were significantly differentially expressed between JS and W in liver and Hsp47 and Hsp90 were once again induced (Fig. S4). Members of the 90-, 70- and 40-kDa families of heat shock proteins are all induced in response to temperature stress in fish (Buckley *et al.* 2006).

Salinity. Movement from a marine to a freshwater habitat was anticipated to trigger considerable changes in the expressions of genes involved in osmoregulation. However, transcriptional change related to osmoregulation was minimal in freshwater with only five genes significantly differentially expressed in gill, the major osmoregulatory organ in fish (Fig. 4B). Despite dozens of features present on the array that annotate to ion transporters known to be osmotically regulated in fish, only Na^+, K^+ transporting-ATPase subunit α-3 was significantly differentially expressed in gill. Similarly, only two potassium channels were significantly differentially expressed in liver (Fig. S4). Significant differences in the expression of the freshwater-adapting hormone prolactin were conspicuously absent between marine and freshwater sampling sites. Evidence for osmoregulatory-based transcriptional change in freshwater came through the significant differential expression of CIRP, isotocin and vasoactive cardiac hormone. CIRP, whose expression is regulated by salinity in salmon (Pan *et al.*

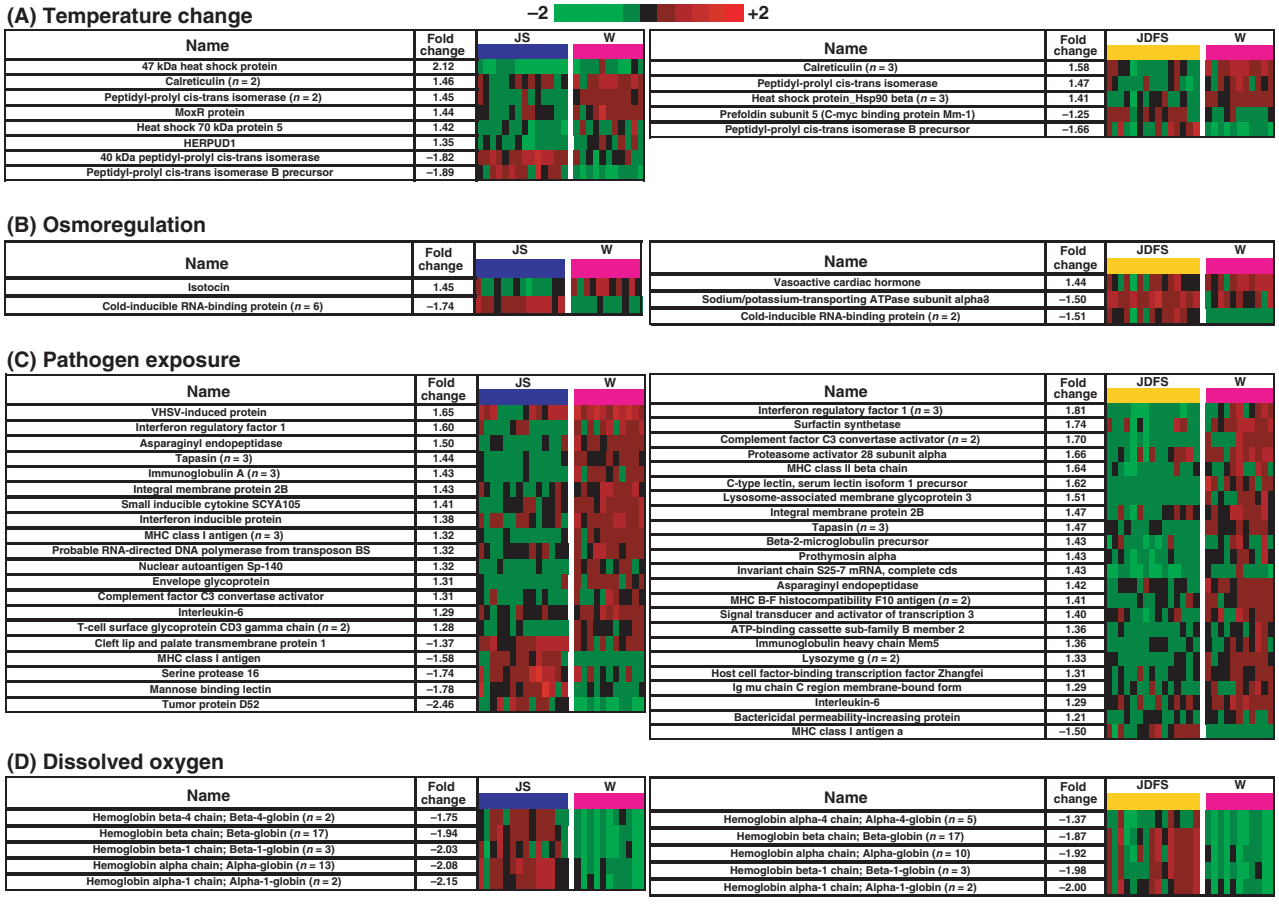


Fig. 4 Heatmaps of significantly differentially expressed genes relating to temperature change, osmoregulation, pathogen exposure and oxygen availability between ocean and freshwater sampling sites in gill. Expression patterns are displayed as described in Fig. 3 for ocean sampling sites within Juan de Fuca Strait (JDFS; gold) or Johnstone Strait (JS; blue) and the freshwater site at Whonnock (W; magenta). Fold-change values reflect changes that occurred in freshwater-sampled fish relative to those sampled in the marine environment. Functional classifications into either temperature change (A), osmoregulation (B) or pathogen exposure (C) or dissolved oxygen (D) were based on information contained within the UniProt database and the primary literature.

2004), was the only gene in the entire study to be significantly differentially expressed in freshwater in both gill and liver and between fish utilizing either migration route. Isotocin and vasoactive cardiac hormone (vasotocin) are hormones known to function during salinity acclimatization in fish (Balment *et al.* 1993; Takei & Hirose 2002; Chou *et al.* 2010).

Pathogen exposure. Functional annotation showed that transcripts associated with pathogen defence represented a dominant class of genes significantly differentially expressed in gill between ocean and freshwater sampling sites. The majority of significant genes were upregulated in freshwater (Fig. 4C). Genes highly correlated with both cellular immune responses, typical of exposure to viruses or intracellular parasites (e.g. MHC Class I antigens and interferon-related genes) and humoral immune responses, triggered by bacteria or extracellular parasites (MHC Class II antigens and immunoglobulins), were among those significantly upregulated in freshwater-sampled gill tissue relative to either ocean site. The prevalence of pathogen defence–related transcripts in gill between JS and W resulted in a 9.3-fold enrichment in 'antigen processing and presentation of the peptide antigen' in the GSEA (Fig. S6A). Multiple immune response–related ontologies were also significantly enriched between JDFS and W in gill, including 'immune response' (3.8-fold) and 'antigen processing and presentation' (11.2-fold) (Fig. S6B). A large number of pathogen and/or immune-related transcripts were significantly differentially expressed in liver between JS and W (Fig. S4). The trajectory of response was not as linear as observed in gill, and immune response–related genes were comprised of approximately equal portions of up- and down-regulated features.

Dissolved oxygen. GSEA indicated that altering the capacity for oxygen transport, plausibly triggered by a

shift in dissolved oxygen between the ocean and river, was the dominant response to freshwater entry in gill. A suite of common gene ontologies relating to haemoglobin activity were significantly enriched in fish entering freshwater from either migration route in gill, including 'oxygen carrier', 'oxygen transport' and 'gas transport' (Fig. S6A,B). Alpha and beta subunits of the oxygen-carrying protein haemoglobin were uniformly downregulated between both JS and JDFS and the freshwater sampling site at W, indicating that this reaction to river entry was conserved between migration routes (Fig. 4D). Common transcriptional responses to freshwater between migration routes, such as that observed for haemoglobin, were atypical. Only 66 of the 305 genes significantly differentially expressed between the ocean and river (union of JDFS vs. W and JS vs. W) were common to fish taking either marine migration route (Fig. 2A). Five of these genes annotated as different subunits of haemoglobin. Significant differences in the expressions of alpha and beta haemoglobin were absent in liver.

Quantitative real-time polymerase chain reaction-based validation of candidate genes

We performed additional qPCR-based expression analyses on four genes shown to be significantly differentially expressed between marine and freshwater sampling sites in the microarray analysis: Hsp47, CIRP, haemoglobin alpha and haemoglobin beta. These four candidates were selected because of adaptive roles during heat stress, osmotic stress and oxygen fluctuations, respectively, and because they exhibited higher fold-changes relative to other genes involved in responses to the environment, which further implicates their importance. Concordance between qPCR- and microarray-based expressions for these four genes was high (Fig. S8). CIRP was significantly downregulated in freshwater in both tissues between either migration route ($P < 0.05$), as it was in the microarray analysis. The significant upregulation of Hsp47 between JS and W in gill also validated microarray results ($P < 0.05$). Also in agreement with microarray data, qPCR analyses demonstrated that both alpha and beta subunits of haemoglobin were significantly downregulated between JDFS and W in gill ($P < 0.05$). While qPCR demonstrated that both haemoglobin subunits were also downregulated between JS and W, this trend was not significant ($P > 0.05$).

Linking gene expression to freshwater survival

To empirically evaluate the impact of the environment on survival, a combination of transcriptomic profiling and telemetry was used to determine whether differences in gene expression at the time of sampling in Mission influenced survival during subsequent upstream migrations. In a previous study by our group using a similar technique based on sockeye returning to spawn in 2006, an unsupervised principal components analysis was used to identify major trajectories of variation in gene expression and test for their associations with survival (Miller *et al.* 2011). Miller *et al.* (2011) demonstrated that a shared set of genes were significantly correlated with premature mortality in three populations: Lower Adams ('late-run') sockeye sampled in the ocean, Scotch Creek sockeye ('early-summer run') sampled in the lower river and Weaver Creek sockeye ('late-run') sampled at the spawning grounds. However, the migration success of the single summer-run stock investigated, Chilko, was not negatively impacted by this gene expression profile. These data suggest that the summer-run fish sampled here may respond differently to variables encountered along migration routes.

Principal components analysis resolved the same gene expression signature in the 2005 summer-run stocks sampled here that in 2006 was associated with premature mortality in early- summer and late-run stocks. However, as was the case in 2006, no significant relationship with mortality was detected (T. Sierocinski L. Donnison, S. Li, K. Kaukinen, A. Schulze, K. M. Miller, P. Pavlidis, unpublished data). We therefore shifted our focus to isolating other genes that impact performance. A supervised *t*-test revealed that 175 genes were differentially expressed between presumed mortalities and successful migrants at $P < 0.01$ (Fig. 5A). Although a corresponding false-discovery rate (FDR) of 71% indicates that the majority of these 175 genes represent false positives, approximately 51 of these genes (i.e. 29%) represent true positives and may be predictive of salmon survival in freshwater (Fig. S9). A narrow distribution of *P*-values within this gene expression signature made it unfeasible to isolate genes with a higher probability of being false positives by filtering for statistically stronger *P*- or *q*-values (159 of the 175 significant genes have *P*-values between 0.001 and 0.01). Given this underlying uncertainty, we utilize all 175 genes as a means of estimating whether the environmental factors identified as driving physiological change between the two marine sampling sites and between marine and freshwater sampling sites (i.e. temperature, salinity, pathogen exposure and dissolved oxygen) are potentially influencing survival. Additional statistical tools, including principal components and survival analyses, are then used to further interrogate the 175 gene set as a means of isolating genes with a higher probability of influencing survival.

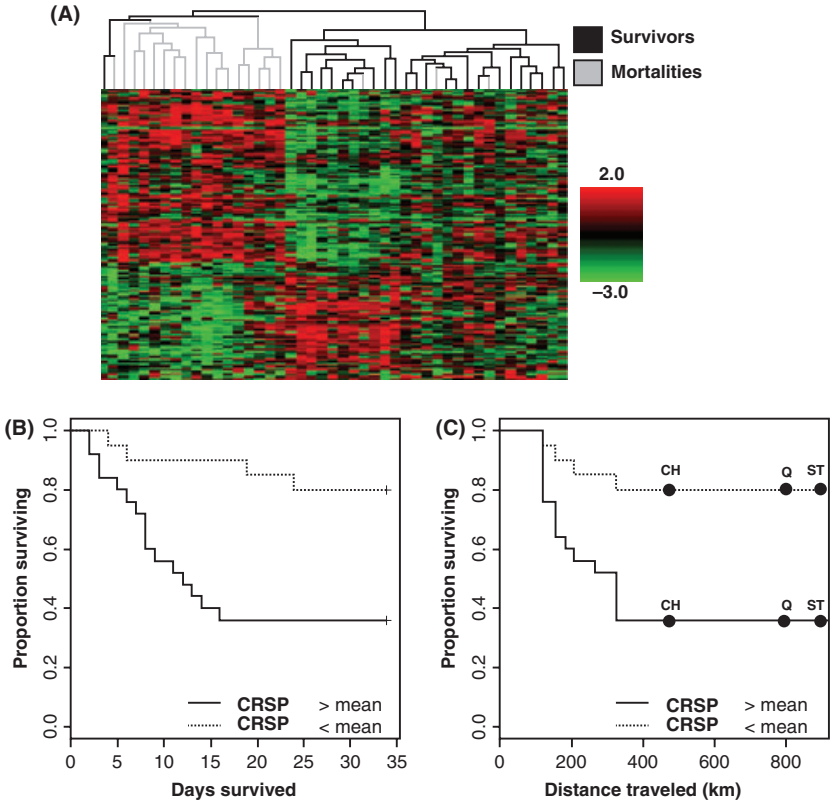


Fig. 5 The relationship between gene expression and survival in migrating sockeye salmon. (A) Pearson correlation hierarchical cluster illustrating expression patterns for each individual fish (columns) across all genes differentially expressed (rows) between survivors (black) and mortalities (grey) created using GeneSight 4.1 software (BioDiscovery Inc.). Parametric survival analyses showing the significant effect of cofactor required for Sp1 transcriptional activation (CRSP) on days survived (B) and distance travelled (C) in the river for radio-tagged sockeye salmon. Fish with expression values lower than the experimental mean for CRSP survived significantly longer and migrated significantly further in the river than fish whose expression values were higher than the mean. Closed circles denote approximate distance travelled to be considered a successful migrant for each of the three stocks investigated (CH = Chilko, Q = Quesnel, ST = Stuart).

Genes associated with shifts in temperature, salinity and pathogen exposure were present within the 175 genes differentially expressed between successful migrants and premature mortalities (Fig. S9). Two genes correlated with osmotic stress were upregulated in survivors relative to premature mortalities, namely the ion transporters cGMP-gated cation channel alpha-1 and $Na^{+}/K^{+}/Ca^{+2}$-exchange protein 6. Nitric oxide synthase, which modifies blood flow in the gill and is regulated by both oxygen levels (Swenson *et al.* 2005) and salinity (Evans 2002), was also differentially expressed. Temperature-sensitive molecular chaperones such as Hsp10 and heat shock cognate 70 protein were also differentially expressed between survivors and mortalities. Salmon hyperosmotic protein 21 (Shop21), part of an ubiquitin–ligase complex induced upon exposure to osmotic or heat stress (Pan *et al.* 2002), was upregulated in survivors. Nine genes involved in pathogen defence were downregulated in surviving fish relative to mortalities.

Parametric survival analysis was performed to isolate genes significantly influencing survival in the river. First, principal components analysis was employed to identify a subset of genes responsible for the highest proportion of the variation in the 175 gene expression signature. PC1 accounted for 31.4% of the observed variation and was significantly correlated with both distance travelled in the river and days survived in the river. Rotation values, a measure of how much each gene is affecting each principal component, were then used to identify genes having the greatest effect on PC1. Only the five genes with the highest rotation values were used in the survival analyses in an attempt to isolate genes whose expression both accounted for a large proportion of the total variation and clearly delineated survivors from mortalities. Two of the top five genes with the highest rotation values could not be annotated. The remaining three genes were the global transcriptional regulator cofactor required for Sp1 transcriptional activation (CRSP), the ion channel protein cGMP-gated cation channel alpha-1 and prolipoprotein, whose function could not be accurately determined. Expression values for all five genes were used as explanatory variables in the survival analysis. Parametric survival analyses with stepwise removal of nonsignificant explanatory variables revealed that only CRSP was significantly predictive of survival. Fish with expression values lower than the experimental mean for CRSP survived significantly longer (Fig. 5B) and migrated significantly further (Fig. 5C) in the river than fish whose expression values were higher than the mean [$F_{(1,42)} = 8.00$, $P = 0.005$]. Although fish tagged and sampled at a later date survived better than those tagged earlier [$F_{(1,42)} = 7.68$, $P = 0.006$], the effect of

CRSP was independent of sampling date [$F_{(1,40)} = 0.88$, $P = 0.35$].

Discussion

Transcriptomic profiling of wild sampled Fraser River sockeye salmon was used to evaluate the relationship between the environment and physiology during spawning migration. This is the first demonstration of the potential effects of environmental variables at a genome level in wild salmon. Our results show that the environment is a major driver of physiological change in migrating salmon. Responses to the environment were apparent in both tissues investigated. In gill, transcriptional reactions to the environment were visible between all three sampling sites, while in liver, responses to the environment were prominent between JS and W. Livers sampled in JDFS exhibited levels of interindividual variability in gene expression that exceeded the variability occurring between sample sites. Although fish sampled in JDFS were from two genetically distinct stocks, the numbers of each stock sampled at JDFS did not differ greatly from other sampling sites in liver, suggesting that the variability in gene expression was not linked to increased genetic diversity or population-specific physiological adaptations. Alternatively, JDFS may represent an area of widespread physiological change in liver and fish happened to be sampled across the spectrum of this change. Considering the liver plays a primary role in lipid metabolism and salmon rely upon stored fat reserves to fuel migration, some fish sampled within JDFS may have already initiated a metabolic shift related to energy utilization, thus leading to heterogeneity in overall sample gene expression.

Salmon experience shifts in a suite of abiotic and biotic variables during migration, including salinity, pH, water chemistry, toxicants, pathogens, temperature, dissolved oxygen, microbial and algal communities. However, significant differential expression of key biomarkers and enrichment of ontologies relating to temperature, salinity and pathogen exposure within marine and freshwater environments suggest that these three environmental variables most strongly influence salmon physiology. Furthermore, differential expression of transcripts associated with temperature, salinity and pathogen exposure between survivors and premature mortalities hints that these environmental factors also influence survival in the river. Changes in dissolved oxygen between the ocean and river habitats also emerged as a potentially important environmental factor. While salmon utilizing different migration routes varied considerably in their transcriptional reactions to freshwater, altering the capacity for oxygen transport was a highly conserved response suggesting an important physiological function.

Divergent responses to the marine environment between migration routes

Migration route is not considered in salmon management models, and JS and JDFS have been assumed to represent similar habitats. Contrary to this perception, the largest transcriptional divergence occurred between fish sampled in each marine environment. An underlying genetic cause for this major divergence in gene expression is unlikely, as fish from three different salmon stocks were sampled at each marine site, indicating that members of the same population are utilizing different routes. Alternatively, our data indicate that JS and JDFS are distinct habitats that differentially affect the physiology of migrating salmon. Key transcriptional differences between fish sampled in JS and JDFS suggest that salinity, temperature and pathogen exposure represent environmental variables that differ between migration routes. Forty-seven genes associated with these variables were significantly differentially expressed exclusively between marine sampling sites in gills, and ontologies relating to each were also significantly enriched.

Given that migration times from sampling sites within JS or JDFS to W are similar, the differential expression of genes correlated with shifts in temperature, salinity or pathogen exposure is either indicative of transcriptional responses to differing ocean conditions between migration routes or a reflection of how route-specific environmental variables temporally alter preparatory changes in gene expression that increase fitness upon river entry. Most plausibly, the expression of genes related to temperature or pathogen exposure is a response to differing ocean conditions between migration routes. JDFS appears to experience larger magnitude shifts in temperature than does JS (R. Thomson, personal communication, Institute of Ocean Sciences, Sidney, BC, Canada), while salmon migrating through JDFS avoid possible exposure to pathogens stemming from fish aquaculture operations in JS (Krkošek *et al.* 2007). However, divergent osmoregulatory responses between migration routes are more likely a function of route-specific environmental conditions temporally influencing preparations for freshwater. Prolactin, a renowned freshwater-adapting hormone known to be expressed in fish gills (Imaoka *et al.* 2000; Sakamoto & McCormick 2006; Boutet *et al.* 2007), was significantly elevated in JDFS-sampled fish relative to JS (Fig. 3B), but not significantly differentially expressed between either ocean sampling site and freshwater. Increased expression of a freshwater-

adapting hormone exclusively in the marine environment provides compelling evidence that fish are preparing for river entry well in advance of encountering freshwater. In support of this conclusion, in 2006, 40% of migrating adult sockeye collected in the marine waters off the mouth of the Fraser River and subsequently held in sea water perished within 1-week, while direct transfer to freshwater resulted in only a 15% mortality rate (Cooperman *et al.* 2010). Migrating adult sockeye have also been shown to decrease AT-Pase activity, plasma osmolality and chloride ion levels while still in the marine environment (Shrimpton *et al.* 2005). Collectively, these data indicate that salmon extensively prepare for freshwater entry hundreds of kilometres from the river mouth, but fish utilizing distinct migration routes vary temporally in these preparations. Differences in the extent of the Fraser River plume (river water driven into the marine environment by wind) or freshwater inputs from adjacent rivers may drive asynchronous osmoregulatory preparations in fish migrating through JS or JDFS.

Responses to the freshwater environment

Temperature. Migrating adult salmon are exposed to the warmest and most variable temperatures encountered during their life history (Hague *et al.* 2010). Exposure to high river water temperatures can have profound negative effects on survival and have frequently been attributed to increased mortality during migration (Macdonald 2000; Crossin *et al.* 2008). Heat stress is especially pertinent for the salmon sampled in this study that live near the southern boundary of the geographic distribution of sockeye and initiate upstream migration when river temperature is at its maximum (i.e. summer-run stocks). A 2 °C elevation in average peak summer temperatures over the past 40 years has exacerbated heat stress within the Fraser River, with waters projected to warm an additional 2 °C over the next few decades (Morrison *et al.* 2002; Morrison & Foreman 2005). Such dramatic shifts in river temperature exemplify the need to assess how current thermal regimes are affecting salmon physiology and whether salmon possess the physiological plasticity to respond to additional warming (Martins *et al.* 2010).

The induction of Hsps and other molecular chaperones is considered a hallmark of the highly conserved heat shock response (HSR) (Lindquist 1986; Feder & Hofmann 1999). The trajectory of change among molecular chaperones in gill and liver tissue (where the majority of transcripts were upregulated in river-sampled fish), combined with the relatively large number of molecular chaperones differentially expressed and functionally enriched in freshwater, suggests that temperatures frequently encountered within the Fraser River may exceed evolved thresholds for triggering a HSR in adult sockeye salmon. Temperature measurements at the time of sampling support this hypothesis and indicate an increase of 7–8 °C between ocean and river sampling sites. The 17 °C water temperature at the time of sampling in W exceeds thermal optima for oxygen consumption during exercise in sockeye salmon, affirming that heat stress is likely to occur at this temperature (Lee *et al.* 2003). Furthermore, molecular chaperones whose expressions were elevated in the marine environment and remained elevated upon river entry would not be detected as significantly differentially expressed but could, nonetheless, contribute to adaptive responses to temperature in the river. Hsp60, Hsp70 and Hsp70a were all significantly differentially expressed between ocean sampling sites in gill tissue. While not all molecular chaperones were induced in freshwater, even in cases of acute thermal stress in laboratory-controlled experiments, a proportion of chaperone genes are significantly downregulated (Buckley *et al.* 2006).

While the induction of molecular chaperones indicates that salmon possess adaptive mechanisms to cope with heat stress, once this class of proteins has been elevated, organisms typically possess a limited ability to respond to further increases in temperature (Tomanek 2010; Logan & Somero 2011). These data indicate that migrating sockeye possess a limited capacity to respond to further warming (Farrell *et al.* 2008; Pörnter & Farrell 2008). While the ultimate outcome of river and ocean warming on Fraser River sockeye remains unclear, if warming continues, there is little doubt that the survival of some sockeye populations will be in jeopardy (Eliason *et al.* 2011). Thus, the current challenge is to determine whether the rates and extents of adaptation across populations will allow Fraser River sockeye to endure the current warming trend.

Salinity. Unlike very recent shifts in thermal regimes that may deviate from evolved norms, the saltwater to freshwater transition required for successful migration may represent an environmental variable to which salmon have evolved strong adaptive mechanisms. Given a complete reversal of osmotic gradients, we hypothesized that changes in osmoregulatory gene expression would be prevalent in gill tissue sampled between saltwater and freshwater. Contrary to this hypothesis, minimal transcriptional change with regard to osmoregulation occurred between marine and freshwater sampling sites. For example, osmotic acclimatization typically culminates through the modification of the abundance or activity of ion-transporting proteins (Tang & Lee 2007); however, only a single ion transporter was

differentially expressed between marine and freshwater habitats in gill. Unfortunately, the Na^+, K^+ transporting-ATPase α1a isoform thought to be fundamental to freshwater acclimatization in salmonids was not spotted on this version of the array (Richards *et al.* 2003; Shrimpton *et al.* 2005). However, even if the differential expression of this gene is assumed, limited change in gene expression relating to ion transport is occurring in freshwater. In comparison, seven genes involved in ion transport were differentially expressed between ocean sampling sites in gill. Despite the paucity of transcriptional change related to osmoregulation in freshwater, CIRP emerged as potentially important. CIRP increases in juvenile salmon exposed to hyper-osmotic stress and is thought to facilitate the translation of osmotically regulated genes during the transition from the river to the ocean (Pan *et al.* 2004). The reduced expression of CIRP in freshwater here is consistent with this function and suggests that limiting the production of certain osmoregulatory proteins is important to freshwater acclimatization in migrating adult salmon. Nonetheless, taken in the context of strong evidence for preparatory adjustments made in the marine environment, the preponderance of evidence indicates that migrating salmon undergo limited osmoregulatory change at the transcriptional level in the final 300 km or so prior to river entry or in the first few days of freshwater occupancy. Major osmoregulatory shifts in gene expression required to increase fitness in freshwater may then occur primarily in the marine environment. An important caveat is that minimal osmoregulatory change at the transcriptional level does not preclude the absence of adaptive osmoregulatory processes. Post-translational shifts in the activity of ion-transporting proteins play key roles in freshwater acclimatization in salmonids (Shrimpton *et al.* 2005).

Pathogen exposure. Salmon entering freshwater encounter a set of pathogens not present in the marine environment, and transcriptomic profiles of river-sampled sockeye present substantial evidence that freshwater pathogens are affecting salmon physiology. Salmon are likely responding to multiple pathogens in freshwater because genes representing both cellular and humoral immune responses were significantly differentially expressed (Iwama & Nakanishi 1996). The Fraser River harbours several disease-causing agents that could elicit such responses and have been previously correlated with premature mortality in sockeye salmon. The myxosporean parasite *Parvicapsula minibicornis* is acquired by salmon in the Fraser River estuary and hypothesized to influence the migratory behaviour and survival of Fraser River sockeye salmon (Bradford *et al.* 2010). Bacterial infections such as *Renibacterium salmoninarum* (Bell *et al.* 1984) and *Flavobacterium psychrophilum* are also known to cause disease in salmon in freshwater (Nematollahi et al. 2003).

Tissue-specific expression patterns for genes involved in immune responses indicate that gills and liver may be differentially affected by or differ in their total exposure time to pathogens. In gills, immune-related genes significantly differentially expressed between the ocean and river were almost uniformly upregulated, while liver contained a mixture of up- and downregulated transcripts. As gill tissue is a primary route of entry for many fish pathogens (Becker & Speare 2007) and interfaces directly with the external environment, it is likely that a more rapid response to pathogen exposure would occur in this tissue relative to internal organs like the liver. Telemetry suggests that the summer-run fish sampled here do not spend appreciable time in the Fraser River estuary (Crossin *et al.* 2009); hence, freshwater or estuarine pathogen exposure would have been recent and may have not yet spread to other tissues at the time of sampling. Given such clear indication of pathogen exposure, a logical speculation is that pathogen infection is contributing to recent rises in premature mortality (Miller *et al.* 2011). Recent shifts in abiotic variables such as temperature may have altered the host–pathogen balance and triggered increased infection rates (Kocan *et al.* 2009).

Dissolved oxygen. Haemoglobin expression has not previously been shown to change during salmon migrations. However, being that haemoglobin expression decreased in freshwater in fish utilizing either migration route, modulating the capacity for oxygen transport may be critical for optimized physiological performance during freshwater phases of migration. Reduced expression of haemoglobin is likely occurring in the gill blood supply and could therefore reflect diminished blood perfusion of gills and/or decreased haemoglobin gene expression. Given that indices of extensive osmoregulatory change were minimal in freshwater, these haemoglobin changes probably represent a response to increased oxygen concentration in the river (http://www.waterquality.ec.gc.ca). Fish modify gill blood flow in response to changes in ambient oxygen (Nilsson 2007) and haemoglobin levels decrease in the sockeye congener *Oncorhynchus mykiss* when exposed to elevated oxygen concentrations (Jewett *et al.* 1991).

Divergent gene expression between successful migrants and premature mortalities

The differential expression of genes tightly correlated with changes in the environment suggests that migrating

sockeye salmon extensively modulate their transcriptome to increase fitness in freshwater. However, the significant differential expression of transcripts indicative of cellular damage also implies that fish sampled in the lower river exhibit compromises in cellular function that impairs performance early in migration. For example, genes significantly differentially expressed between JS and W in liver were enriched for ontologies related to cell death, which can result when environmental stress levels exceed adaptable limits (Krumschnabel & Podrabsky 2009). Changes in the expressions of apoptotic genes were not observed in response to starvation in the sockeye congener *O. mykiss* using the same microarray platform (Salem *et al.* 2007), suggesting that cell death in the liver is not a consequence of starvation. In addition, genes involved in DNA repair (e.g. growth arrest and DNA damage–inducible protein 45) were significantly differentially expressed between ocean- and river-sampled fish in both tissues (Figs S1–S7). These data suggest that salmon accumulate cellular damage prior to river entry, which may in turn impair their ability to complete up-river migrations.

To determine how the conditional state of fish upon river entry relates to migratory success, we combined transcriptomic profiling and telemetry. We resolved a gene expression signature comprising at least 51 genes that delineated successful migrants from fish dying on route to the spawning grounds. These data support the hypothesis that the conditional state of fish upon river entry has an influence on subsequent survival. While a high corresponding FDR placed constraints on the interpretation of this data, the identity of these genes provides some insight into the link between the environment and survival. For example, the presence of multiple genes associated with temperature, salinity and pathogen exposure suggests that these three environmental variables impact survival during river migration. These data corroborate transcriptomic changes that occurred between lethal sampling sites and reinforce that these variables most strongly influence physiology during spawning migration.

Survival analysis identified CRSP as having a significant effect on survival during river migration in summer-run sockeye salmon. As a very broad-scale regulator of transcription involved in a large number of cellular processes (Ryu *et al.* 1999), the key to CRSP's influence on in-river survival may lie in its ability to modulate the expression of a large number of downstream genes. Identifying CRSP as a potential biomarker of salmon fate represents an important step in developing a biomarker-based conservation strategy. Despite investing approximately $40 million (Canadian dollars) annually in salmon conservation, there is still considerable uncertainty in predicting population dynamics on a yearly basis (Cooke *et al.* 2004; Haeseker *et al.* 2008). The isolation of biomarkers predictive of survival could provide a more accurate means of inferring population trends. Preliminary tests on potential biomarkers indicate that differences in the expression of only 5–10 genes are sufficient to predict survival at a high rate, which will allow managers to meet the speed and cost requirements to implement such a conservation strategy. While to date physiological data has never been incorporated into conservation decision making, given the high current levels of en-route mortality, the recent acknowledgement that Fraser River sockeye salmon populations are in decline and the announcement by the Prime Minister of Canada of a judicial inquiry into these declines, there may be more support now than ever for the implementation of novel tools to aid in conservation efforts.

Summary

The current study presents evidence that the transcriptome of wild migrating adult sockeye salmon is adaptively modulated in response to changing environmental conditions associated with movement from an ocean to a river habitat and that shifts in gene expression relating to the environment probably influence survival. Conspicuous shifts in gene expression associated with temperature, salinity, dissolved oxygen and pathogen exposure imply that these environmental variables most extensively influence salmon performance. The induction of molecular chaperones within the river suggests that freshwater temperature regimes are encroaching on tolerance limits and that salmon possess little surplus physiological plasticity to cope with additional heat stress. Osmoregulatory changes necessary for survival in the river appear largely preparatory at the transcriptional level and occur a considerable distance from freshwater. Increases in dissolved oxygen within the river environment plausibly reduced the expression of multiple isoforms of the oxygen-carrying protein haemoglobin. Finally, dealing with newly acquired pathogens in the freshwater environment may hinder up-river migrations. Combining transcriptomics and telemetry provided a novel means of identifying differences in gene expression between premature mortalities and successful migrants. These data hint that differential responses to the environment affected survival and isolated a potential biomarker predictive of salmon fate in the river. From a broader perspective, this study showcases the utility of high-throughput gene expression to extract insightful information on the conditional state of wild organisms in their natural habitats.

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T.G.E is a physiologist interested in applying genomic tools to questions surrounding climate change. E.H. is an ecologist interested in how differences at the individual level affect the behaviour of populations and communities. K.K. tracks gene expression patterns in the brain and gill of migrating adult and juvenile salmon. A.D.S. uses genetics and genomics to investigate population structure, diet, fitness and responses to the environment in salmon. D.A.P. evaluates the impact of different environmental factors on the migration success of Pacific salmon in fresh water. K.K.E. designs and implements studies to improve the quality and quantity of information available for the management and assessment of Pacific salmon and steelhead stocks. J.M.R.C. uses ecological models to inform decisions on species assessment and management. K.M.M. is interested in the development and application of molecular genetic tools that can be applied towards sustainable fisheries management and aquaculture.

Data accessibility

Microarray expression data: Microarray expression data were deposited in the NCBI Gene Expression Omnibus under series accession GSE 22346.

Supporting information

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

Table S1 Signifcantly differentially expressed features—Gill Johnstone Strait vs. Juan De Fuca Strait (*t*-test $P < 0.01$).

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