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Research article

Transcriptomic response to elevated water temperatures in adult migrating Yukon River Chinook salmon (*Oncorhynchus tshawytscha*)

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Chinook salmon (*Oncorhynchus tshawytscha*) declines are widespread and may be attributed, at least in part, to warming river temperatures. Water temperatures in the Yukon River and tributaries often exceed 18°C, a threshold commonly associated with heat stress and elevated mortality in Pacific salmon. Untangling the complex web of direct and indirect physiological effects of heat stress on salmon is difficult in a natural setting with innumerable system challenges but is necessary to increase our understanding of both lethal and sublethal impacts of heat stress on populations. The goal of this study was to characterize the cellular stress response in multiple Chinook salmon tissues after acute elevated temperature challenges. We conducted a controlled 4-hour temperature exposure (control, 18°C and 21°C) experiment on the bank of the Yukon River followed by gene expression (GE) profiling using a 3′-Tag-RNA-Seq protocol. The full transcriptome was analysed for 22 Chinook salmon in muscle, gill and liver tissue. Both the 21°C and 18°C treatments induced greater activity in genes associated with protein folding (e.g. HSP70, HSP90 mRNA) processes in all tissues. Global GE patterns indicate that transcriptomic responses to heat stress were highly tissue-specific, underscoring the importance of analyzing multiple tissues for determination of physiological effect. Primary superclusters (i.e. groupings of loosely related terms) of altered biological processes were identified in each tissue type, including regulation of DNA damage response (gill), regulation by host of viral transcription (liver) and regulation of the force of heart contraction (muscle) in the 21°C treatment. This study provides insight into mechanisms potentially affecting adult Chinook salmon as they encounter warm water during their spawning migration in the Yukon River and suggests that both basic and more specialized cellular functions may be disrupted.

Key words: Chinook salmon, transcriptome analysis, heat stress, Yukon River

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Introduction

One of the most dramatic changes to freshwater habitats has been increases in water temperatures (Hinzman *et al*., 2005; Crozier *et al*., 2008; Kaushal *et al*., 2010). Pacific salmon (*Oncorhynchus spp*.) are cold-water species with adult life stages that are particularly sensitive to high water temperatures during spawning migrations (McCullough, 1999; Pörtner and Farrell, 2008). Upper thermal limits for induction of negative effects in migrating adult Pacific salmon are in the range of 18°C to 23°C (McCullough, 1999; Strange, 2010). Even sublethal elevated temperatures can influence salmon biology, including effects on metabolism, susceptibility to disease, acceleration of senescence and timing of life history events such as upstream migration and spawning (Jeffries *et al*., 2014a; Groot and Margolis, 1991; Carter, 2005). Temperature impacts are thought to be cumulative and positively correlated to the duration and severity of the exposure, potentially reducing survival to spawning (Elliott, 1981). As salmon do not feed during their spawning migrations, they must enter rivers with all the energy reserves needed to reach their spawning grounds (Brett, 1995). High temperatures and associated increases in metabolic rates put salmon at risk of energy depletion and mortality before reaching spawning grounds as warmer temperatures result in higher energy use (Hasler *et al*., 2012).

Gene transcription is the process by which information from the DNA template of a particular gene is transcribed into messenger RNA (mRNA) and eventually translated into a functional protein. The amount of mRNA of a particular gene is dictated by a number of extrinsic and intrinsic factors, including stimuli such as heat stress, infectious agents, toxin exposure, trauma or malignant transformation (Bowen *et al*., 2012). Untangling the complex web of direct and indirect physiological effects of heat stress on salmon can be assisted by examining transcription of genes associated with multiple biological processes, including immune function, protein folding, protein synthesis, metabolism, oxidative stress and ion transport, all of which are affected by high water temperatures (Akbarzadeh *et al*., 2018; Mesa *et al*., 2002; Tomalty *et al*., 2015; Miller *et al*., 2009; Jeffries *et al*., 2012; Jeffries *et al*., 2014a). Moreover, the alteration of transcription can be predictive of mortality prior to spawning (Miller *et al*., 2011; Jeffries *et al*., 2012).

Heat stress triggers a range of adaptive physiological and cellular mechanisms, including the cellular stress response (CSR), a 'tiered' response, with early and later phase changes in gene expression (GE) that appears to be conserved among all vertebrates (Logan and Buckley, 2015) (Fig. 1). The CSR facilitates the protection of cellular macromolecules through molecular chaperoning, the reallocation of metabolic resources away from homeostatic functions and toward stress responses, the reversible arrest of the cell cycle and, in cases of more extreme stress, programmed cell death through apoptosis (Logan and Buckley, 2015). A key component of the CSR to heat stress is the induction of heat shock proteins (HSPs) which re-fold heat damaged proteins and prevent their cytotoxic aggregation to protect against cellular damage and death (Logan and Buckley, 2015; Bouchama *et al*., 2017; Shi *et al*., 2019). Identification and characterization of temperature-induced CSR will identify genes for monitoring heat stress and aid in understanding the mechanisms linking sublethal temperatures to premature mortality (Jeffries *et al*., 2014a). Premature mortality is the sum of *en route* mortality in migration corridors and prespawn mortality on the spawning grounds.

We conducted manipulative experiments to identify the genes responding to heat stress in Yukon River Chinook salmon (*Oncorhynchus tshawytscha*) and subsequently determine the mechanisms causing the relationships between temperature and CSR. Air and water temperatures have been warming throughout Alaska (Hinzman *et al*., 2005; Kocan *et al*., 2009) with summer water temperatures in the Yukon River and tributaries often exceeding 18°C and occasionally approaching or exceeding 20°C (Zuray *et al*., 2012; Carlson and Edwards, 2017). Chinook salmon declines are widespread in the Arctic–Yukon–Kuskokwim region and prompted the Alaska Board of Fisheries to classify Yukon River Chinook salmon as a 'stock of yield concern' beginning in 2000 (Krueger and Zimmerman, 2009). Cool water refuges are less likely in well-mixed rivers such as the Yukon River (Zuray *et al*., 2012; Jensen *et al*., 2013) and provide less opportunity for behavioural adaptation that occurs in other systems (Mathes *et al*., 2010; Hasler *et al*., 2012). Pre-spawning mortality, *en route* mortality and negative sublethal effects on migrating adult salmon can be very high when conditions approach or are within the upper thermal limits (Keefer *et al*., 2018; Mathes *et al*., 2010; Hinch *et al*., 2012; Jeffries *et al*., 2014a; Teffer *et al*., 2018). Mortality can exceed 90% in some cases (Hinch *et al*., 2012). Premature adult mortality during their spawning migrations can have dramatic population-level effects for Pacific salmon (Hinch *et al*., 2012; Bowerman *et al*., 2016).

Herein, we describe the use of a gene-based diagnostic, RNAseq, to identify the physiological effects of heat stress on migrating, adult, Chinook salmon. Identification of biological processes and genes associated with heat stress will provide insight into the mechanisms and potential manifestations of high temperatures and provide the basis for development of a system for early identification of heat compromised wild Chinook salmon. Pre-spawning mortality appears to be linked to high temperatures through the loss of aerobic scope, energy depletion (Eliason *et al*., 2011; Hasler *et al*., 2012), decreased immune function (i.e. increased susceptibility to disease) and altered reproductive output (Connon *et al*., 2018). Based on temperature stress studies in Pacific salmon,

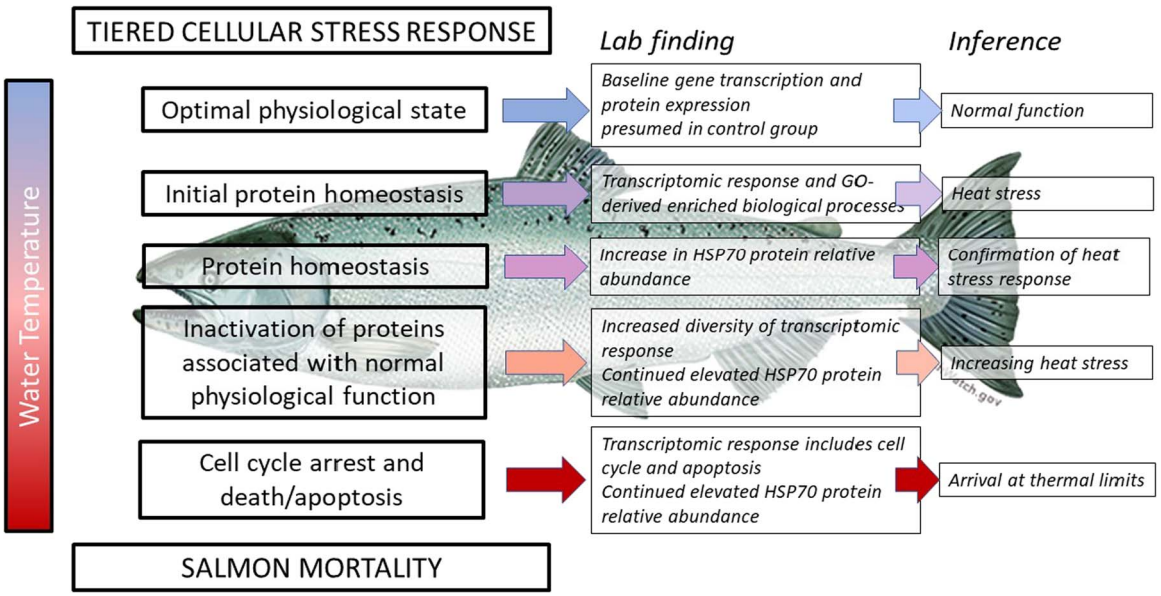

Figure 1: Conceptual model of a heat-induced cellular stress response (CSR) as it applies to our system. The CSR facilitates the protection of cellular macromolecules through molecular chaperoning, the reallocation of metabolic resources away from homeostatic functions and toward stress responses, the reversible arrest of the cell cycle and, in cases of more extreme stress, programmed cell death through apoptosis (Logan and Somero 2011, Jeffries *et al*., 2012, Logan and Buckley, 2015). A key component of the CSR to heat stress is the induction of heat shock proteins (HSPs) which re-fold heat damaged proteins and prevent their cytotoxic aggregation to protect against cellular damage and death (Logan and Buckley, 2015; Bouchama *et al*., 2017; Shi *et al*., 2019).

we anticipated identifying biological processes influenced by heat stress to include HSP, immune function, oxidative stress and metabolism (Akbarzadeh *et al*., 2018; Wurster *et al*., 2005; Mesa *et al*., 2002; Tomalty *et al*., 2015; Miller *et al*., 2009; Jeffries *et al*., 2012; Jeffries *et al*., 2014b). However, this study differs from others in that it was conducted near the northern range extent of the species where the warm water temperatures that exceed important thresholds for stress and mortality (McCullough, 1999) are likely more recent in an evolutionary perspective with less opportunity for genetic adaptation over generations as compared to lower latitude locations. While *in situ* freshwater temperature records across northern latitudes are too sparse and short to confirm the assumptions that warm water temperatures are a recent phenomenon in northern latitudes and the rate of warming in rivers and streams is more rapid than that of lower latitudes, several other observations suggest that this is the case including the faster pace of temperature increase at northern latitudes among air temperatures and lake surface water, earlier ice breakup and recent loss of seasonally persistent ice and snow that would have previously maintained cool summer water temperatures (Reist *et al*., 2006; Brabets and Walvoord, 2009; Lisi *et al*., 2015; O'Reilly *et al*. 2015; Pavelsky and Zarnetske, 2017; Post *et al*., 2019). Additionally, no current studies examining the CSR of heat stress in adult Chinook salmon were found in the literature. Our objectives were to conduct a thermal exposure experiment, its efficacy validated by heat shock protein 70 (HSP70) induction, (Basu *et al*., 2002), and subsequently elucidate potential causes of premature mortality resulting from acute exposure to elevated temperature in migrating adult Yukon River Chinook salmon.

Materials and methods

Yukon River Chinook salmon were collected under the US Geological Survey Alaska Science Center ACUC 2018-04 and the Alaska Department of Fish and Game Fish (ADF&G) Resource Permit for Scientific Collection Purposes SF2018-132.

Controlled temperature experiment

Because protocols for implementing experiments in remote field settings were lacking, we developed an experimental protocol for holding adult Chinook salmon while manipulating water temperature in a remote field setting described in Donnelly *et al*. (In press). Briefly, the experiment was conducted on the bank of the Yukon River at N 61.94716° W 162.84161° adjacent to the ADF&G's test fishery site located ~1 km upstream of the community of Pilot Station, Alaska. This experiment was conducted in mid-June (13–21 June 2018) prior to the seasonal peak of water temperature in the Yukon River to ensure the fish had not previously experienced water temperatures. Fish were captured in gillnets with a mesh

size of 10.2 cm and transferred to polyvinyl chloride holding tubes in a live well aboard a skiff filled with water directly from the river (∼14°C) for transport to the experimental tank. In an effort to minimize stress induced by gillnet capture, we avoided individuals that were 'gilled' with the net wrapped tightly under the operculum in favour of individuals that were minimally tangled in the mesh and quickly removed. The net sets were ∼8 min in duration, and fish were immediately transferred to experimental tanks such that the time from initial capture to experimental tanks was a maximum of 57 min. There were no statistically significant differences among transit times by treatment group as determined by analysis of variance (ANOVA; $F = 0.378$, $P = 0.69$) (NCSS, Statistical and Power Analysis Software, 2020, Kaysville, Utah, USA). Average transit times were 6 minutes for the control fish, 11 minutes for the 18°C fish and 12 minutes for the 21°C fish (Donnelly et al., In press). As a result of these precautions, all individuals included in this experiment appeared vigorous. Individually, fish were placed in one of three 587-L oval polyethylene stock tanks (High Country Plastics, model W-155) filled with river water at ambient temperature (∼14°C) for a short acclimation period of at least 30 min before water was warmed in two of the tanks to either a low-heat (mean of 18°C) or high-heat (mean of 21°C) temperature treatment. In order to minimize potential stress from conditions in the field (i.e. confinement) we used an acclimation time shorter than that normally used in controlled laboratory experiments. Low- and high-heat temperatures were selected for this study based on the available literature for heat stress in salmonids (Miller *et al*., 2009; Strange, 2010; Hasler *et al*., 2012; Hinch *et al*., 2012; Jeffries *et al*., 2014a) which already occur with some frequency in the watershed. The low-heat treatment temperature of 18°C is near the threshold for detecting thermal stress, and the high-heat treatment temperature of 21°C is likely near the upper temperature limit for migrating Chinook salmon (McCullough, 1999). The target rate of heating for the low- and high-heat stress trials was 4°C hr^{-1} to minimize total fish holding time out of concern that prolonged confinement would reduce survival. This rate of temperature increase has been used in other heat stress studies in Chinook Salmon (Clark *et al*., 2008) and is similar to a temperature shift that a salmon would experience when moving between water masses such as the transition from marine waters in the Bering Sea to river waters in western Alaska or a transition from the mainstem Yukon River to a tributary (Martin *et al*., 1986). Indeed, Pacific salmon routinely move across thermally heterogenous habitats at the surface and with depth including watersheds with temperature differences of up to 7°C between rivers (Goniea *et al*., 2006; Keefer *et al*., 2015) and diurnal vertical migrations associated with abrupt water temperature shifts of 8°C (Roscoe *et al*., 2010). Temperatures in control and treatment tanks were maintained within narrow ranges that did not overlap among control (12.9–15.9°C; mean, 14.4°C), low-stress (17.3–18.6°C; mean, 18.0°C) and high-stress treatments (19.8–22.0°C; mean, 20.9°C) following the warming period.

All individuals in the control and low-heat groups survived to the end of the trial, but one individual that began the low-heat trial failed to acclimate to the tank and was released. Only 56% ($n = 9$) of those in the high-heat group survived and provided samples used in this whole transcriptome analysis. Experimental mortality in the high-heat treatment was likely related to water temperatures being near the upper thermal limit for migrating Chinook salmon (McCullough, 1999) and is discussed in more detail in Donnelly *et al*. (In press). The length of the 22 Chinook salmon that survived the experiment ranged from 392 to 879 mm (mid-eye to fork measurement) with a mean length of 733 mm (SD, 110 mm). All fish were upstream migrating adults including a single precocial male.

All fish were sacrificed immediately prior to tissue sampling at the end of the four-hour target-temperature period. Gill, muscle and liver tissues were collected from each fish, placed immediately into a cryovial and stored in a dry shipper supercooled with liquid nitrogen. The samples were kept at −80°C from 25 June 2018 until 10 July 2018 then transported via dry shipper to University of California at Davis where they were stored at −80°C until tissue preparation.

HSP70 protein abundance

HSP70 protein abundance was analysed at the US Geological Survey Silvio O. Conte Anadromous Fish Research Laboratory, Turners Falls, MA, USA. HSP70 protein was quantified by Western immunoblotting as previously outlined by Chadwick *et al*. (2015) with modification as follows. Muscle from the dermal punch was separated from subdermal fat and skin and weighed to the nearest milligram. Liver and gill tissues were also weighed to the nearest milligram. All tissues were thawed and homogenized with Kontes Pestle Pellet handheld homogenizer (Thermo Fisher Scientific, Hampton, NH, USA) in 10 volumes of SEID (150 mM sucrose, 10 mM EDTA and 50 mM imidazole, pH 7.3 plus 0.1% deoxycholic acid). Homogenates were centrifuged at 3000 x g for 7 min at 4°C. A portion of the resulting supernatant was immediately diluted with an equal volume of 2 × Laemmli buffer, heated for 15 min at 65°C and stored at −80°C for later analysis by western blotting. A small volume of supernatant was used to determine total protein concentration in quadruplicate using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Hampton, NH, USA). Thawed samples were run on a 7.5% SDS-PAGE gel along with Precision Plus protein standards at 5 µg in a reference lane (Bio-Rad Laboratories, Hercules, CA, USA). To account for overall difference in HSP protein abundance between tissues, 10 µg of muscle protein was loaded per sample, whereas 5 µg of gill per sample and 4 µg of liver protein per sample were loaded in each lane. Two lanes were reserved on each gel for a standard consistent tissue preparation reference to control for blot-to-blot variation. This standard consistent preparation was used for all three tissue types to allow for comparison across treatments and tissues. Following electrophoresis, proteins were

transferred to Immobilon polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mM Tris, 192 mM glycine buffer, pH 8.3. Equal loading was verified by reversible total protein staining with Ponceau S. PVDF membranes were blocked with 5% non-fat dry milk in PBST (phosphate buffered saline plus 0.1% Triton X-100) for 1 h at room temperature, rinsed in PBST and exposed to primary polyclonal antibody specific for the inducible form of salmonid HSP70 (AS05061; Agrisera, Vannas, Sweden) at 1:25000 dilution in PBST with 5% non-fat dry milk for 1 h at room temperature. After rinsing in PBST, blots were exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:10000 in PBST and 5% non-fat dry milk for 1 h at room temperature. Blots were washed in PBST and incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent solution A (396 µM coumaric acid, 2.5 µM luminol, 100 mM Tris, pH 8.5) and ECL B (0.018% H_2O_2, 100 mM Tris, pH 8.5), and then digitally imaged and quantified (Syngene PXi, GeneTools, Frederick, MD, USA). All blots were normalized to the internal standard consistent tissue preparation and per µg of tissue loaded and are represented as a ratio to the mean standard value that we refer to as HSP70 relative abundance. HSP70 protein abundance was plotted and examined for the location of a threshold that best separated fish between the control and 21°C treatments within each tissue type. The mean HSP70 abundance was compared among treatments using a one-way ANOVA with each tissue type.

Transcriptome analysis

Total RNA was extracted from pulverized tissues using the RNeasy Lipid Tissue Mini Kit (Qiagen; www.qiagen.com). To remove contaminating genomic (g)DNA, the spin columns were treated with 10 U μl^{-1} of RNase-free DNase I (DNase, Amersham Pharmacia Biotech Inc.; www.apbiotech.com) at 20°C for 15 minutes. RNA was then stored at −80°C pending further analyses. After the RNA was extracted, further clean-up was performed on the RNA to remove any residual DNA. For the muscle samples, a range of 3 μl^{-7} µl was aliquoted and mixed with RNase/DNase free water to reach a total volume of 87.5 µl, to reach a final concentration of 100 ng/µl in a volume of 10 µl or more. For both liver and gill, only 1 µl of RNA was aliquoted and mixed with 86.5 µl of RNase/DNase free water. The samples were then treated with the RNase-free DNase I kit. Once this was completed the samples were treated using the Zymo RNA Clean & Concentrator-25 kit. The samples were then sent to the UC Davis Genome Center DNA Technologies Core Facility for further DNase treatment followed by a microbead clean-up to further purify the RNA.

The total RNA samples were DNAse digested in a volume of 50 µl with two units of RNAse-free DNAse I (NEB, Ipswich, MA) in the accompanying DNAse buffer at 37°C for 10 min. The digestion reaction was stopped and cleaned up by the addition of 90 µl RNAClean XP beads (Beckman Coulter, Brea, CA) according to the protocol of the manufacturer. The RNA was eluted from the beads in 12 µl molecular biology grade water. Quality assurance of total RNA showed Bioanalyzer RIN scores ≥7 and enough material for library preparation.

GE profiling was carried out using a 3′-Tag-RNA-Seq protocol. Barcoded sequencing libraries were prepared using the QuantSeq FWD kit (Lexogen, Vienna, Austria) for multiplexed sequencing according to the recommendations of the manufacturer using both the UDI-adapter and UMI Second-Strand Synthesis modules (Lexogen). The fragment size distribution of the libraries was verified via micro-capillary gel electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The libraries were quantified by fluorometry on a Qubit fluorometer (Life Technologies, Carlsbad, CA) and pooled in equimolar ratios. Up to 48 libraries were sequenced per lane on a HiSeq 4000 sequencer (Illumina, San Diego, CA) with single-end 100 bp reads.

Raw reads were processed with HTStream v.1.0 (https://ibest.github.io/HTStream/) to perform raw sequence data QA/QC and remove adapter contamination and low-quality bases/sequences. On average, there were 4.3 M reads per sample, and 4.2 M (98%) remaining after trimming. Of those, on average, 7.3% did not map to the salmon genome. On average, 93% of the trimmed reads aligned to the Chinook salmon genome, and 42% of the trimmed reads uniquely aligned to a Chinook salmon gene. The trimmed reads were aligned to the Chinook salmon genome v.1.0 (NCBI Assembly Accession GCF_002872995.1) with NCBI gene annotation release 100 using the aligner STAR v. 2.7.0e (Dobin *et al.*, 2013) to generate raw counts per gene.

In order to characterize the molecular CSR from acute exposure to elevated temperatures, we conducted differential expression (DE) analyses using limma-voom in R (limma version 3.34.9, edgeR version 3.20.9, R 3.4.4). Prior to analysis, genes with fewer than 3 counts per million reads in all samples were filtered, leaving 16 908 genes. The model fitted in limma included effects for temperature (control, 14°C; low stress, 18°C; high stress, 21°C), tissue, the interaction between temperature and tissue, sex and RNA extraction batch. Fish were treated as a random effect. All fold change comparisons were calculated as contrasts between temperature treatments within this model. Standard errors of log fold changes were adjusted for within-fish correlations.

Differentially expressed genes were annotated based on Gene Ontology (GO) pathway analysis. GO annotations explain the function of a particular gene and are created by associating a gene or gene product with a GO term. Together, these statements comprise a 'snapshot' of current biological knowledge describing gene functions at the molecular level, location in the cell of these functions and what biological processes (pathways, programs) it helps to carry out. Therefore, multiple functions of individual genes can be accommodated by association with these three classes of GO terms: cellular component, molecular function and biological

process. A particular gene can have any number of associated annotations in any of those categories. Additionally, the GO is structured in a hierarchy in which there are general terms (higher levels of hierarchy) and more specific terms (lower levels of hierarchy) for a given gene or its product.

GO enrichment analyses were conducted using Kolmogorov–Smirnov tests to compare DE *P*-values annotated and not annotated with a given GO term, implemented using the Bioconductor package topGO, version 2.30.1. A GO term is a group of genes associated with a cellular biological process that are predefined by the GO bioinformatics initiative using a controlled vocabulary. GO terms describe three main aspects of the biological domain, molecular function, cellular component and biological process. In this paper we focus mainly on biological process. GO terms were analysed using ReVIGO (http://revigo.irb.hr/), a web server that summarizes long lists of GO terms by finding a representative subset of the terms. A simple clustering algorithm that relies on semantic similarity measures was used to summarize GO terms. ReVIGO then visualizes the non-redundant GO term set to assist in interpretation (Supek *et al.*, 2011). This analysis identifies GO terms which are statistically over- or under-represented and describes some important underlying biological process or behaviour and groups them into larger superclusters. Superclusters are plotted using a treemap figure that visualizes each over- or under-represented process as a rectangle with the rectangle size in proportion to the evidence of biological process enrichment. Throughout the results and discussion sections, a statistically over or underrepresented GO term will be referred to as an enriched biological process.

Results

The HSP70 protein abundance and transcriptomes for muscle, gill and liver tissue were analysed for 22 Chinook salmon (9 held at control temperatures, 8 held at 18°C, 5 held at 21°C). HSP70 protein abundance for fish in the 21°C treatment was higher compared to both control and 18°C treatment in muscle (ANOVA; $F = 4.95$, $P = 0.02$), liver (ANOVA; $F = 49.7$; $P < 0.001$) and gill (ANOVA; $F = 5.94$, $P = 0.01$) tissue (Fig. 2). When minimizing misclassifications, HSP70 protein relative abundance of 0.014 in muscle tissue and 0.100 in liver tissue separated the control and 21°C treatment response (Fig. 2). Mean HSP70 levels in the 21°C treatment were ∼17-fold higher in liver (0.046 in control vs 0.78 in 21°C), ∼7.8-fold higher in muscle (0.0084 in control vs 0.066 in 21°C) and ∼3.5-fold higher in gill (0.077 in control vs 0.27 in 21°C) compared to controls, with minimal change for the 18°C treatment; the magnitude of increase in HSP70 corresponds roughly to the number of genes that are increased in each tissue.

Transcription was influenced by temperature treatment and tissue. The number of significantly DE genes differed among tissues and temperature treatments (Table 1). Numbers of significantly DE genes increased within tissue groups

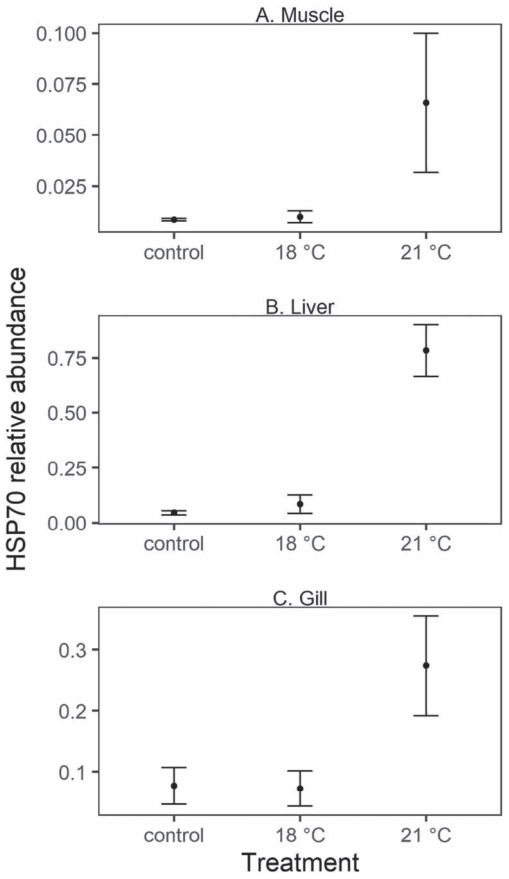

Figure 2: The mean (± SE error bars) abundance of HSP70 protein in muscle (A), liver (B) and gill (C) tissue from fish held in three different experimental temperatures: control (river ambient), 18°C or 21°C.

for comparisons of 18°C vs control to 18°C vs 21°C, and finally, 21°C vs control. The liver was the most temperature-responsive tissue based on the number of DE genes, followed by the gill and then muscle (Table 1).

Significantly enriched biological processes were distributed uniquely according to temperature treatment and tissue type (Table 2, Appendices 1 and 2). Enriched biological processes were identified in gill, liver and muscle in the low-heat treatment salmon (18°C) in comparison with control salmon (Fig. 3, Appendix 1). For 18°C salmon, enriched biological processes were broadly associated with protein processing in all tissues (Table 2). DE genes related to protein processing in 18°C salmon included HSP90, but not HSP70. Enriched biological processes in gill included SMAD protein signal transduction, protein localization to pro-autophagosomal structure and protein folding. In liver, enriched biological processes included endoplasmic reticulum (ER) to Golgi vesicle mediated transport, and protein K11-linked ubiquitination. SMADS comprise a family of proteins that are linked with cells critically important for regulating cell development and growth.

Figure 3: REVIGO treemaps of enriched biological processes due to heat stress in migrating adult Chinook salmon in gill (left), liver (center) and muscle (right) tissue. Each rectangle is a single supercluster representative of biological processes that were enriched between control (river ambient) and 18°C treated (top) or control and 21°C treated (bottom) Chinook salmon. Superclusters of loosely related biological processes are visualized with different colours. Across all tissue types, 21 unique superclusters were enriched with heat stress and nearly all superclusters appear in only a single tissue type. Only the protein folding supercluster (A, white) is shared across tissue types. Larger rectangles indicate superclusters with more evidence of biological process enrichment due to heat stress. Size of the rectangles reflects the *P*-value of the GO term. Letters refer to supercluster names that would not be legible on the figure: A, protein folding; B, circadian rhythm; C, positive regulation of mRNA binding; D, regulation of developmental pigmentation; E, pigmentation; F = response to UV-C; G, rhythmic process; H, chaperone mediated protein folding requiring cofactor; I, regulation of circadian rhythm; and J, reproductive process.

Enriched biological processes in muscle tissue included SRP-dependent co-translational protein targeting to membrane, and cytoplasmic translation. SRP is a cytosolic particle that transiently binds to the (ER) signal sequence in a nascent protein, to the large ribosomal unit and to the SRP receptor in the ER membrane.

Enriched biological processes were identified in gill, liver and muscle in salmon exposed to high heat treatment (21°C) in comparison with control salmon (Table 1, Fig. 3, Appendix 2). Once again, protein processing was identified as an enriched biological process including HSP90 as a DE gene across all three tissue types and HSP70 as a DE gene in gill and

Table 1: The number of significantly DE genes by comparison (i.e. 18°C vs control, 21°C vs 18°C, 21°C vs control) and by tissue type (i.e. Gill, Liver, Muscle) with adjusted $P < 0.05$

Comparison	Tissue	Number of DE genes
18°C vs control	Gill	1
21°C vs 18°C	Gill	28
21°C vs control	Gill	340
18°C vs control	Liver	100
21°C vs 18°C	Liver	667
21°C vs control	Liver	1822
18°C vs control	Muscle	45
21°C vs 18°C	Muscle	75
21°C vs control	Muscle	93

muscle. In gill tissue we identified an enrichment of processes associated broadly with positive regulation of DNA damage response signal transduction by p53 class mediator, telomere maintenance by telomerase, parathyroid gland development, secretory granule localization and protein folding (Fig. 3). To a lesser degree, enriched processes in gill tissue included processes associated with inflammation and immune system activation, T lymphocyte activation functions, inflammatory response to antigenic stimulus, regulation of cytokine production involved in inflammatory response, regulation of natural killer cell mediated cytotoxicity, as well as activation of innate immune response. We identified enriched biological processes in liver associated with positive regulation by the host of viral transcription, muscle organ development, negative regulation of DNA binding, and protein folding. Of note, in liver tissue we also identified enriched biological processes associated with response to organic cyclic compound. In muscle tissue we identified enriched biological processes associated broadly with phosphatidylinositol metabolism, regulation of the force of heart contraction (including cardiac muscle hypertrophy in response to stress), positive regulation of reactive oxygen species biosynthesis, and protein folding.

Discussion

We characterized differences in transcriptome expression with heat stress across tissues (gill, liver, muscle) and elevated temperatures (18°C, 21°C) in migrating adult Yukon River Chinook salmon to elucidate potential causes of premature mortality. The 18°C and 21°C treated Chinook salmon reflect two distinct phases in the tiered CSR. First, the comparison between control and 18°C treated Chinook salmon describes the transcriptome response that precedes the HSP70 protein response (from optimal physiological state to protein homeostasis; Fig. 1). Second, a comparison between control and 21°C treated Chinook salmon elucidates the heat stress response that occurs following the elevation of the HSP70 protein response (inactivation of proteins associated with normal physiological function; Fig. 1). The transcriptome response within each of these phases was distinct with a more narrowly defined 18°C response focused on altered protein processing (similar among tissues) in contrast to the 21°C response that was characterized by alterations to a wide diversity of biological processes within and specific to each tissue type examined. It should be noted that fish experienced gill netting and handling that may have resulted in alterations in GE. Since all of the fish, including controls, experienced the same level of disturbance, this should not have been an important factor in discovering temperature related differences in GE. We cannot rule out the possibility, however, that some of the differences in GE that we observed is an interaction with temperature and prior handling stress.

We identified a lack of broad-scale diversity of enriched biological processes in the transcriptome responses of fish held at 18°C across all three tissues (Fig. 3); many processes were associated with cellular processing (Quinn *et al.*, 2011). However, transcriptome results from the three tissues of fish held in 21°C water identified alterations in diverse cellular processes (DNA damage and telomere maintenance in the gill; protein folding in all tissues), immune challenges (liver tissue), and more specific changes in energy sources to muscle tissue and heart function (muscle tissue). Our results align with findings in studies of other Pacific salmon species and ectotherms (Akbarzadeh *et al.*, 2018; Mesa *et al.*, 2002; Tomalty *et al.*, 2015; Miller *et al.*, 2009; Jeffries *et al.*, 2012; Jeffries *et al.*, 2014a). The only biological process influenced by heat stress across all sampled tissue types was protein folding. Alterations in protein folding are a hallmark heat stress response that includes the HSP chaperones (Wali and Balkhi, 2016).

HSP70 protein validation of experiment

HSP70 induction confirmed a heat stress response in the 21°C treatment. The magnitude of response was greatest in the liver, followed by the muscle and then gill. There was also substantially more variation in the gill. Tissue-dependent responses are common in biomarkers of cellular stress as each tissue has specialized functions. The reduced response in gill tissue HSP70 is at odds with a number of other studies that have used heat stress biomarkers in gill (Iwama, 1999; Jeffries *et al.*, 2013; Tomalty *et al.*, 2015; Akbarzadeh *et al.*, 2018). It is unclear if a longer heat stress exposure or longer time course prior to tissue sampling may have resulted in a more distinct heat stress response in gill tissue. Response timing of HSP70 protein is known to be tissue specific with longer delays in gill tissue (Lewis *et al.*, 2016). Chinook salmon held in the 18°C treatment did not demonstrate a significant elevation in HSP70 protein in any tissue type after a 4 h treatment. Still, HSP70 proteins were elevated in migrating adult Chinook salmon that had recently experienced maximum water temperatures ≥18°C,

Table 2: The top three significant enriched biological processes (i.e. GO terms) associated with DE genes relative to warm water temperature exposures (18°C, 21°C) in comparison with the experimental control group (river ambient)

Treatment	Tissue	Biological processes	*P*-value
18°C			
	Gill	Cell aging	0.00035
		SMAD protein signal transduction	0.00056
		Definitive hemopoiesis	0.00074
	Liver	ER to Golgi vesicle-mediated transport	1.4e-05
		Cell cycle	8.2e-05
		Negative regulation of transcription, DNA-templated	0.00029
	Muscle	Translation	1.8e-19
		Cytoplasmic translation	1.9e-07
		Proteasomal ubiquitin-independent protein catabolic process	3.4e-05
21°C			
	Gill	Protein folding	4.2e-07
		Positive regulation of mRNA binding	0.00013
		Telomere maintenance via telomerase	0.00045
	Liver	Positive regulation by host of viral transcription	0.00033
		Positive regulation of translation	0.00049
		Negative regulation of transcription from RNA polymerase II promoter	0.00051
	Muscle	Ventricular cardiac muscle tissue morphogenesis	1.1e-05
		Regulation of the force of heart contraction	1.1e-05
		Phosphatidylinositol metabolic process	4.7e-05

likely for periods longer than 4 h or more than 4 h prior. It appears likely that the experimental time course (4 h) was not long enough for the HSP70 protein response to be apparent in our 18°C treatment, whereas mRNA levels were more rapidly upregulated as demonstrated by the substantial number of protein folding genes upregulated within 4 h at 18°C.

Transcriptome response preceding HSP70 protein increase in 18°C Chinook salmon

The transcriptome of 18°C treated Chinook salmon revealed a distinct, but relatively narrow set of enriched biological processes broadly associated with protein processing and cell/tissue homeostasis (Fig. 3, Appendix 1). Often changes in the enrichment of biological processes involved in the CSR can begin to be detected at temperatures below where whole organism effects can be observed (Jeffries *et al*., 2012). The major transcriptional overlap in functional response to 18°C exposures across tissues was protein folding, consistent with other studies (Iwama *et al*., 1999; Basu *et al*., 2002; Huang *et al*., 2018). Although HSP90 gene transcripts were identified as differentially transcribed between 18°C and control fish, HSP70 gene transcripts were not.

Enriched biological processes specific to gill tissue in 18°C treated salmon were the SMAD protein signal transduction and cell aging superclusters. SMAD signal transduction is involved with cell proliferation, differentiation and death (Moustakas *et al*., 2001) and is associated with production of HSP90 (Yan *et al*., 2018). Cell aging and oxidative stress are well-known effects of thermal stress in salmon (Nakano *et al*., 2014).

The primary supercluster associated with liver in salmon exposed to 18°C was negative regulation of transcription—DNA templated. This, in effect, describes transcriptomic plasticity, which has been associated with survival outcomes in fish subjected to chronic and acute stress (Logan and Buckley, 2015; Wellband and Heath, 2017).

The primary superclusters associated with muscle tissue in salmon exposed to 18°C were SRP-dependent co-translational protein targeting to membrane and translation, both associated with protein processing. Co-translational protein targeting is an essential and evolutionarily conserved pathway for delivering proteins to the proper cellular membrane (Zhang *et al*., 2010). The translation supercluster contains those biological processes associated with translating mRNA into proteins.

Similarities in heat stress response across tissues in 21°C Chinook salmon

The major transcriptional overlap in functional response to temperature across tissues were several biological processes associated with protein processing in the cell's ER consistent with other studies (Iwama *et al*., 1999; Basu *et al*., 2002; Huang *et al*., 2018). The HSP70 and HSP90 genes were responsive across all tissue types examined as expected given that the resulting chaperone proteins bind to misfolded proteins and are the most well-known component of the cell's protein processing response to heat stress (Iwama *et al*., 1999; Basu *et al*., 2002). Indeed, this study relied on the elevation of the HSP70 protein to confirm that heat stress had occurred. Our whole transcriptome approach provided a more complete picture of the heat stress response in the ER and revealed that most key genes relating to function of the ER were upregulated in the 21°C fish. These included lumenal chaperones to promote correct protein folding such as protein disulphide isomerase and dnaJ and BiP (Huang *et al*., 2018; Rebl *et al*., 2018), which deliver misfolded proteins for ubiquitin-dependent degradation (Plemper *et al*., 1997). Ubiquitin is key to degradation of misfolded proteins, which, when accumulated, can impair ER function and lead to cell death (Goldberg, 2003). Altered expression of genes (ubiquitin, nuclear factor erythroid 2-related factor 2, ERO-1) playing key roles in maintaining the redox state within cells were also identified (Ramsden and Gallagher, 2016). The down-regulation of key genes in the mitigation of heat stress in our study (ATF-6 alpha, ubiquitin, nuclear factor erythroid 2-related factor 2) may suggest that these salmon were transitioning from adaptive regulation to injury in response to heat stress (Xia *et al*., 2017).

Tissue-specific responses to heat stress in 21°C Chinook salmon

Global GE patterns indicate that transcriptomic responses to heat stress were highly tissue specific (Fig. 3). Tissue-specific responses likely relate to the specific physiological role of each tissue in the fish, a phenomenon that has been found in other studies of heat stress in ectotherms (Dietz and Somero, 1993; Buckley and Somero, 2008; Nuez-Ortín *et al*., 2018). Additionally, it has been hypothesized that the specialized functions of different tissues could make some tissues more or less susceptible to disruptions from heat stress (Guisbert *et al*., 2013). The most significant superclusters of biological processes associated with each tissue type are discussed below.

Gill response to heat stress in 21°C Chinook salmon

Gill tissue supports spawning by moving oxygen from the water to the blood stream for delivery throughout the body. The gill is also involved in ion and water regulation, acid-base balance and nitrogenous waste (ammonia) excretion. In gill tissue we identified an enrichment of processes associated broadly with positive regulation of DNA damage response and signal transduction by p53 class mediator and telomere maintenance by telomerase (Fig. 3). Positive regulation of DNA damage response and signal transduction by p53 class mediator is a supercluster of biological processes associated with mitigation of DNA damage [DNA damage responses (DDR)]. In studies on juvenile Chinook salmon, Tomalty *et al*. (2015) found similar enrichment of DDR processes in fish exposed to increased temperatures. Studies on heat stress in pufferfish (*Takifugu obscurus*) and cold stress in tilapia (*Oreochromis niloticus*) and zebrafish (*Danio rerio*) have also identified enrichment of DDR processes (Cheng *et al*., 2018; Hu *et al*., 2016) suggesting that DDR response are common when fish encounter unsuitable water temperatures.

Enriched biological processes included telomere maintenance by telomerase. Telomeres protect chromosome ends from degradation; variation in telomere length in fish has been linked to extreme environmental temperatures, which can result in oxidative stress that accelerates telomere attrition, cellular ageing and increases disease risk (Debes *et al*., 2016). In a study of free-ranging young-of-the-year brown trout (*Salmo trutta*), Debes *et al*. (2016) found telomere-length variation is associated with both past temperature and growth, although causal relationships among temperature, growth, oxidative stress and cross-sectional telomere length still remain largely unknown. Simide *et al*. (2016) did find that Siberian sturgeon (*Acipenser baerii*) exposed to increased temperatures had 15% shorter telomeres. However, few studies have explored the causal relationship between stress and telomere length, or the molecular mechanisms underlying that relationship (Romano *et al*., 2013).

Liver response to heat stress in 21°C Chinook salmon

The primary role of the liver in adult migrating salmon is likely mobilizing and metabolizing stored lipid resources as fuel (Johnson *et al*., 2013). Several enriched biological processes in the liver tissue of 21°C fish were related to responses to virus, potentially indicative of latent viral infection at the population level. These biological processes included activation of interferon gamma (IFNγ) production as well as involvement of major histocompatibility complex class I, both considered hallmarks of defence against viruses (Goldsby, 2003). Miller *et al*. (2011) found an intensification of inflammatory, apoptotic and Th1 immune stimulation (i.e. genes with known linkages to anti-viral activity) in Fraser River sockeye salmon gill tissue (*O. nerka*). It is well-known that stress plays a role in fish disease outbreaks and that many infectious agents are opportunistic and do not impact survival unless fish are also challenged by other stressors (Wedemeyer *et al*., 1970; Barton *et al*., 1985; Miller *et al*., 2014). In fish, most viruses are unapparent or cause mild disease under normal circumstances, congruent with long-term latency (Miller *et al*., 2017).

Muscle response to heat stress in 21°C Chinook salmon

Skeletal muscle is the major locomotor tissue that propels salmon upstream to their spawning grounds. In muscle tissue we identified enriched biological processes associated broadly with phosphatidylinositol metabolism, regulation of the force of heart contraction (including cardiac muscle hypertrophy in response to stress), positive regulation of reactive oxygen species biosynthesis and protein folding. Phosphatidylinositol metabolism reflects energy use from amino acids rather than glucose, the preferred energy source (Nuez-Ortín *et al*., 2018). A shift toward amino acid use during upstream migration of adults has previously been indicated in the gill tissue of juvenile Chinook salmon (Tomalty *et al*., 2015). Increasing water temperatures cause an increase in metabolic rate, leading to a state of metabolic remodelling to compensate for increased energy demand and an increased energy deficit induced under elevated temperatures, potentially shifting to an increased dependence on amino acids rather than glucose (e.g. reliance on metabolism of muscles rather than fat) (Kullgren *et al*., 2013; Nuez-Ortín *et al*., 2018).

Surprisingly, many of the enriched biological processes in the white skeletal muscle of heat stressed fish were related to regulation of the force of heart contraction (cardiac muscle contraction pathway and adrenergic signalling in cardiomyocytes pathway). It is not clear if this response reflects a process that is occurring locally in the skeletal muscle or if transcriptomic signatures from cardiac tissue can be reflected by the muscle tissue as is known to be the case with cardiac tissue and blood (Chaussabel, 2015). Still, this result is notable because a fishes' ability to cope with warm temperatures can be limited by cardiorespiratory performance and the delivery of oxygen to locomotor tissues by the frequency of heart contractions (Eliason *et al*., 2013). Supra-optimal temperatures result in an unsustainable increase in oxygen demand by tissues and decrease on oxygen supply as with reduced cardiac output and subsequent arterial oxygen supply (Anttila *et al*., 2014). Other studies have shown that exposure to high temperatures in fish can cause alterations in cardiorespiratory performance, myocardial morphology and expression, and phosphorylation of structural genes and proteins (Jørgensen *et al*., 2014). Cardiac collapse and death have been shown to start at 21–23°C in Atlantic salmon (*Salmo salar*) and 25°C in Chinook salmon from more southern populations (Clark *et al*., 2008; Anttila *et al*., 2014). Cardiac acclimation to increased temperatures has also been shown to occur, which is preceded by cardiac remodelling of tissue composition and morphology, assumed to at least partially compensate for the decreased power-generating ability at elevated temperature (Jørgensen *et al*., 2014; Clark *et al*., 2008).

Synthesis

This study described two distinct steps (protein homeostasis and inactivation of proteins associated with normal physiological function) in the CSR response to heat stress across three distinct tissue types, identifying evidence of disruption to both basic and more specialized functions. Nearly all the enriched biological processes are consistent with cumulative mortality processes that occur over days and weeks and are related to the increased costs of migrating in warmer temperatures. Among the increased costs are continued transcription of genes responsible for mitigating the effects of heat stress (Graham *et al*., 2010) and the reallocation of nutrients and energy from one portion of an individual's resource budget to other metabolic functions, which can be significant (Romero *et al*., 2009). The indirect involvement of heart contraction processes also suggests the possibility of a more sudden cause of mortality, the collapse of aerobic scope and failure of the cardio-respiratory system to deliver adequate oxygen to the brain (Farrell *et al*., 2008; Eliason *et al*., 2011). Mitigation of stressors imposes demands on animals above those normally required to sustain life during a life history period already characterized by the extreme energy demands of upriver migration and reproduction. In this context, it should not be surprising that warm water temperatures result in reduction of fitness via decreased reproductive capability and premature mortality that can contribute to population declines over generations (Graham *et al*., 2010; Martin *et al*., 2010; Connon *et al*., 2018).

Subsequent to our thermal exposure experiment, Alaska experienced a state-wide pattern of record-breaking heat (summer of 2019) that was associated with geographically widespread *en route* mortality of migrating adult Pacific salmon across all species (various reports available at leonetwork.org and media reports) during a prolonged period of warm water temperatures akin to our 21°C treatment for several days. Results from our study suggest that unusual mortality in 2019 could have been the result of several proximate causes of mortality given the wide range of genes and pathways involved. Given the projections of continued warming across the northern range extent of Pacific salmon (Post *et al*., 2019), the cellular heat stress response described here will likely become more common in the wild.

This research demonstrates the broad effects of temperature stress on Pacific salmon physiology and underscores the importance of analyzing multiple tissues for determination of physiological effect. These results provide valuable information to help inform scientists and managers regarding how warm temperatures can affect the physiology of migrating adult Pacific salmon and help identify new avenues of study. Identifying the potential for heat stress to contribute to mortalities can enhance effective management of this escapement-based fishery that relies upon counts of migrating adults and assumes that nearly all migrating adults successfully spawn.

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Appendix 1: Top 40 significant ($P \leq 0.05$) enriched biological processes (Gene Ontology (GO) convention) in Chinook salmon exposed to 18°C compared with controls in gill, liver, and muscle tissue. Columns include a numeric GO ID, annotated GO term to describe the cellular process, the number of annotated genes associated the GO term (N), and raw *P*-value.

GO ID	GO Term	N	*P*-value
GILL			
GO:0007569	cell aging	89	0.00035
GO:0060395	SMAD protein signal transduction	25	0.00056
GO:0060216	definitive hemopoiesis	41	0.00074
GO:1904385	cellular response to angiotensin	15	0.00096
GO:0035019	somatic stem cell population maintenance	33	0.00129
GO:0071850	mitotic cell cycle arrest	17	0.00131
GO:0019827	stem cell population maintenance	102	0.00174
GO:0009612	response to mechanical stimulus	167	0.00246
GO:0006457	protein folding	177	0.00264
GO:0031952	regulation of protein autophosphorylation	41	0.00302
GO:0061614	pri-miRNA transcription from RNA polymerase II promoter	48	0.00307
GO:0048793	pronephros development	41	0.00316
GO:0016266	O-glycan processing	24	0.00319
GO:0021983	pituitary gland development	26	0.0035
GO:0034497	protein localization to pre-autophagosomal structure	11	0.00351
GO:0050769	positive regulation of neurogenesis	328	0.00379
GO:0043620	regulation of DNA-templated transcription in response to stress	64	0.0039
GO:1900063	regulation of peroxisome organization	4	0.00428
GO:0014732	skeletal muscle atrophy	5	0.00466
GO:2001014	regulation of skeletal muscle cell differentiation	23	0.00466
GO:0042127	regulation of cell proliferation	1195	0.00475
GO:0003433	chondrocyte development involved in endochondral bone morphogenesis	28	0.00491
GO:0016126	sterol biosynthetic process	59	0.00505
GO:0051222	positive regulation of protein transport	330	0.00512
GO:1900102	negative regulation of endoplasmic reticulum unfolded protein response	13	0.00518
GO:1902213	positive regulation of prolactin signaling pathway	4	0.00519
GO:1900170	negative regulation of glucocorticoid mediated signaling pathway	4	0.00519
GO:0000381	regulation of alternative mRNA splicing, via spliceosome	100	0.00549
GO:0045665	negative regulation of neuron differentiation	184	0.00572
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	926	0.00579
GO:0006071	glycerol metabolic process	23	0.0067
GO:0003148	outflow tract septum morphogenesis	22	0.00673
GO:1900181	negative regulation of protein localization to nucleus	39	0.00716
GO:2000811	negative regulation of anoikis	10	0.00718
GO:0002467	germinal center formation	12	0.00743

(Continued)

Appendix 1: Continued.

GO ID	GO Term	N	*P*-value
GO:0045787	positive regulation of cell cycle	290	0.00789
GO:0035924	cellular response to vascular endothelial growth factor stimulus	47	0.008
GO:0003183	mitral valve morphogenesis	5	0.00863
GO:0042760	very long-chain fatty acid catabolic process	6	0.00864
GO:0002021	response to dietary excess	11	0.00901
LIVER			
GO ID	**GO Term**	**N**	***P* Value**
GO:0006888	ER to Golgi vesicle-mediated transport	148	1.40E-05
GO:0007049	cell cycle	1403	8.20E-05
GO:0045892	negative regulation of transcription, DNA-templated	989	0.00029
GO:0070979	protein K11-linked ubiquitination	33	0.00032
GO:0051301	cell division	539	0.00108
GO:0010498	proteasomal protein catabolic process	460	0.0013
GO:0070427	nucleotide-binding oligomerization domain containing 1 signaling pathway	7	0.00133
GO:0000278	mitotic cell cycle	716	0.00238
GO:0090630	activation of GTPase activity	78	0.00243
GO:1900740	positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway	11	0.00254
GO:0034142	toll-like receptor 4 signaling pathway	27	0.00254
GO:0060212	negative regulation of nuclear-transcribed mRNA poly(A) tail shortening	8	0.003
GO:0035278	miRNA mediated inhibition of translation	23	0.00307
GO:0031663	lipopolysaccharide-mediated signaling pathway	40	0.00315
GO:0032922	circadian regulation of gene expression	66	0.00371
GO:0042742	defense response to bacterium	97	0.00396
GO:0000045	autophagosome assembly	79	0.00416
GO:0070895	negative regulation of transposon integration	3	0.00443
GO:0010875	positive regulation of cholesterol efflux	17	0.0046
GO:0045292	mRNA cis splicing, via spliceosome	50	0.00475
GO:0048715	negative regulation of oligodendrocyte differentiation	7	0.00478
GO:2000480	negative regulation of cAMP-dependent protein kinase activity	9	0.00487
GO:0006457	protein folding	177	0.00496
GO:1990440	positive regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress	15	0.00532
GO:0032053	ciliary basal body organization	3	0.00556
GO:0001568	blood vessel development	628	0.00565
GO:0006621	protein retention in ER lumen	7	0.00568
GO:0021571	rhombomere 5 development	4	0.00603
GO:0021572	rhombomere 6 development	4	0.00603

(Continued)

Appendix 1: Continued.

GO ID	GO Term	N	*P*-value
GO:0022400	regulation of rhodopsin mediated signaling pathway	5	0.00642
GO:0042102	positive regulation of T cell proliferation	51	0.00701
GO:0033500	carbohydrate homeostasis	221	0.00739
GO:0043630	ncRNA polyadenylation involved in polyadenylation-dependent ncRNA catabolic process	4	0.00741
GO:0071050	snoRNA polyadenylation	4	0.00741
GO:1901888	regulation of cell junction assembly	81	0.00764
GO:0019346	transsulfuration	3	0.00765
GO:0009440	cyanate catabolic process	3	0.00765
GO:0000098	sulfur amino acid catabolic process	16	0.00765
GO:0006402	mRNA catabolic process	245	0.0085
GO:0043652	engulfment of apoptotic cell	4	0.0088
MUSCLE			
GO ID	**GO Term**	**N**	***P* Value**
GO:0006412	translation	734	1.80E-19
GO:0002181	cytoplasmic translation	91	1.90E-07
GO:0010499	proteasomal ubiquitin-independent protein catabolic process	28	3.40E-05
GO:0006511	ubiquitin-dependent protein catabolic process	627	0.00018
GO:0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	399	0.00019
GO:0006614	SRP-dependent cotranslational protein targeting to membrane	29	0.00076
GO:0000028	ribosomal small subunit assembly	19	0.00099
GO:0071499	cellular response to laminar fluid shear stress	9	0.00112
GO:0071409	cellular response to cycloheximide	9	0.00112
GO:0060761	negative regulation of response to cytokine stimulus	55	0.00113
GO:0007253	cytoplasmic sequestering of NF-kappaB	8	0.00177
GO:0048713	regulation of oligodendrocyte differentiation	17	0.00185
GO:0060216	definitive hemopoiesis	41	0.00218
GO:1905719	protein localization to perinuclear region of cytoplasm	4	0.00219
GO:0019371	cyclooxygenase pathway	5	0.00259
GO:0000387	spliceosomal snRNP assembly	54	0.00379
GO:0070498	interleukin-1-mediated signaling pathway	25	0.00381
GO:0032418	lysosome localization	59	0.00384
GO:0070427	nucleotide-binding oligomerization domain containing 1 signaling pathway	7	0.00432
GO:0034101	erythrocyte homeostasis	147	0.00481
GO:0070423	nucleotide-binding oligomerization domain containing signaling pathway	24	0.00485
GO:0051302	regulation of cell division	132	0.00493
GO:0090303	positive regulation of wound healing	46	0.00521
GO:0006995	cellular response to nitrogen starvation	10	0.00535

(Continued)

Appendix 1: Continued.

GO ID	GO Term	N	*P*-value
GO:0045475	locomotor rhythm	16	0.00541
GO:0033598	mammary gland epithelial cell proliferation	20	0.00602
GO:0042254	ribosome biogenesis	257	0.0061
GO:0051205	protein insertion into membrane	34	0.00638
GO:0051247	positive regulation of protein metabolic process	1267	0.00642
GO:0090160	Golgi to lysosome transport	8	0.00681
GO:1903671	negative regulation of sprouting angiogenesis	33	0.00698
GO:0000715	nucleotide-excision repair, DNA damage recognition	7	0.007
GO:0035518	histone H2A monoubiquitination	16	0.007
GO:0017156	calcium ion regulated exocytosis	83	0.00738
GO:0071407	cellular response to organic cyclic compound	402	0.00772
GO:0039020	pronephric nephron tubule development	12	0.00775
GO:0035994	response to muscle stretch	27	0.0079
GO:0045899	positive regulation of RNA polymerase II transcriptional preinitiation complex assembly	6	0.00812
GO:0070125	mitochondrial translational elongation	16	0.0083
GO:0010591	regulation of lamellipodium assembly	27	0.0085

Appendix 2: Top 40 significant ($P \leq 0.05$) enriched biological processes (Gene Ontology (GO) convention) in Chinook salmon exposed to 21°C compared with controls in gill, liver, and muscle tissue. Columns include a numeric GO ID, annotated GO term to describe the cellular process, the number of annotated genes associated the GO term (N), and raw *P*-value.

GO ID	GO Term	N	*P*-value
GILL			
GO:0006457	protein folding	177	4.20E-07
GO:1902416	positive regulation of mRNA binding	10	0.00013
GO:0007004	telomere maintenance via telomerase	57	0.00045
GO:1905224	clathrin-coated pit assembly	6	0.00111
GO:0043517	positive regulation of DNA damage response, signal transduction by p53 class mediator	14	0.00157
GO:0031120	snRNA pseudouridine synthesis	6	0.00169
GO:0035459	cargo loading into vesicle	21	0.00182
GO:0006260	DNA replication	198	0.00188
GO:0016310	phosphorylation	1669	0.002
GO:0032252	secretory granule localization	6	0.00221
GO:0045944	positive regulation of transcription from RNA polymerase II promoter	926	0.00246
GO:0045892	negative regulation of transcription, DNA-templated	989	0.00265
GO:0033189	response to vitamin A	7	0.00281

(Continued)

Appendix 2: Continued.

GO ID	GO Term	N	*P*-value
GO:0050829	defense response to Gram-negative bacterium	19	0.00292
GO:0002437	inflammatory response to antigenic stimulus	26	0.00337
GO:0097753	membrane bending	5	0.00346
GO:0032733	positive regulation of interleukin-10 production	19	0.00366
GO:0030521	androgen receptor signaling pathway	47	0.00373
GO:0070828	heterochromatin organization	14	0.0045
GO:0006268	DNA unwinding involved in DNA replication	10	0.00528
GO:1903800	positive regulation of production of miRNAs involved in gene silencing by miRNA	8	0.00546
GO:0002218	activation of innate immune response	140	0.00588
GO:0035774	positive regulation of insulin secretion involved in cellular response to glucose stimulus	19	0.00611
GO:0070973	protein localization to endoplasmic reticulum exit site	8	0.00639
GO:0043484	regulation of RNA splicing	185	0.00676
GO:0050832	defense response to fungus	11	0.00679
GO:0050807	regulation of synapse organization	117	0.00702
GO:0097494	regulation of vesicle size	6	0.00721
GO:0060017	parathyroid gland development	10	0.00727
GO:1901223	negative regulation of NIK/NF-kappaB signaling	59	0.0074
GO:2000045	regulation of G1/S transition of mitotic cell cycle	127	0.00745
GO:0045668	negative regulation of osteoblast differentiation	42	0.00756
GO:0032729	positive regulation of interferon-gamma production	29	0.00762
GO:0010165	response to X-ray	26	0.00791
GO:1902963	negative regulation of metalloendopeptidase activity involved in amyloid precursor protein catabolic process	8	0.00795
GO:0010332	response to gamma radiation	33	0.00802
GO:0007565	female pregnancy	113	0.00808
GO:0009306	protein secretion	381	0.00809
GO:0050873	brown fat cell differentiation	30	0.0084
GO:1900016	negative regulation of cytokine production involved in inflammatory response	41	0.00891
LIVER			
GO ID	**GO Term**	**N**	***P*-value**
GO:0043923	positive regulation by host of viral transcription	8	0.00033
GO:0045727	positive regulation of translation	124	0.00049
GO:0000122	negative regulation of transcription from RNA polymerase II promoter	654	0.00051
GO:0042981	regulation of apoptotic process	1115	0.00081
GO:0043032	positive regulation of macrophage activation	8	0.0009
GO:0045039	protein import into mitochondrial inner membrane	11	0.00126
GO:0016579	protein deubiquitination	170	0.00172
GO:0006457	protein folding	177	0.00182

(Continued)

Appendix 2: Continued.

GO ID	GO Term	N	*P*-value
GO:1904851	positive regulation of establishment of protein localization to telomere	5	0.00226
GO:0032436	positive regulation of proteasomal ubiquitin-dependent protein catabolic process	69	0.00232
GO:0000278	mitotic cell cycle	716	0.0024
GO:0030307	positive regulation of cell growth	109	0.00256
GO:0031115	negative regulation of microtubule polymerization	11	0.00266
GO:0043392	negative regulation of DNA binding	39	0.00323
GO:1903895	negative regulation of IRE1-mediated unfolded protein response	4	0.00372
GO:1904668	positive regulation of ubiquitin protein ligase activity	11	0.00372
GO:0007517	muscle organ development	334	0.00378
GO:0016584	nucleosome positioning	5	0.00385
GO:0010882	regulation of cardiac muscle contraction by calcium ion signaling	17	0.00399
GO:0090312	positive regulation of protein deacetylation	27	0.004
GO:2000234	positive regulation of rRNA processing	5	0.00407
GO:0006984	ER-nucleus signaling pathway	36	0.0048
GO:0032470	positive regulation of endoplasmic reticulum calcium ion concentration	3	0.00481
GO:1903233	regulation of calcium ion-dependent exocytosis of neurotransmitter	3	0.00481
GO:1903515	calcium ion transport from cytosol to endoplasmic reticulum	3	0.00481
GO:0006390	transcription from mitochondrial promoter	13	0.00485
GO:0010225	response to UV-C	12	0.00493
GO:0045736	negative regulation of cyclin-dependent protein serine/threonine kinase activity	23	0.00504
GO:0007049	cell cycle	1403	0.00548
GO:0070940	dephosphorylation of RNA polymerase II C-terminal domain	8	0.00551
GO:0003407	neural retina development	59	0.00554
GO:0032792	negative regulation of CREB transcription factor activity	8	0.00572
GO:0061512	protein localization to cilium	33	0.00573
GO:0000738	DNA catabolic process, exonucleolytic	4	0.00596
GO:0036302	atrioventricular canal development	11	0.00643
GO:0002244	haematopoietic progenitor cell differentiation	112	0.00658
GO:0097192	extrinsic apoptotic signaling pathway in absence of ligand	46	0.00659
GO:1901799	negative regulation of proteasomal protein catabolic process	50	0.00666
GO:0031167	rRNA methylation	11	0.00744
GO:0007221	positive regulation of transcription of Notch receptor target	7	0.00766
MUSCLE			
GO ID	**GO Term**	**N**	***P*-value**
GO:0055010	ventricular cardiac muscle tissue morphogenesis	38	0.0000
GO:0002026	regulation of the force of heart contraction	20	0.0000
GO:0046488	phosphatidylinositol metabolic process	136	0.0000

(Continued)

Appendix 2: Continued.

GO ID	GO Term	N	*P*-value
GO:0014898	cardiac muscle hypertrophy in response to stress	26	0.0003
GO:0014883	transition between fast and slow fiber	15	0.0005
GO:0031449	regulation of slow-twitch skeletal muscle fiber contraction	4	0.0005
GO:0060048	cardiac muscle contraction	99	0.0005
GO:0060326	cell chemotaxis	185	0.0008
GO:1903428	positive regulation of reactive oxygen species biosynthetic process	41	0.0013
GO:0000422	autophagy of mitochondrion	58	0.0013
GO:0051131	chaperone-mediated protein complex assembly	6	0.0014
GO:0060452	positive regulation of cardiac muscle contraction	10	0.0019
GO:0002437	inflammatory response to antigenic stimulus	26	0.0020
GO:0060213	positive regulation of nuclear-transcribed mRNA poly(A) tail shortening	17	0.0021
GO:1902188	positive regulation of viral release from host cell	15	0.0021
GO:0006885	regulation of pH	61	0.0024
GO:0006596	polyamine biosynthetic process	13	0.0024
GO:0014728	regulation of the force of skeletal muscle contraction	5	0.0025
GO:0070814	hydrogen sulfide biosynthetic process	8	0.0026
GO:0006281	DNA repair	387	0.0026
GO:1901389	negative regulation of transforming growth factor beta activation	4	0.0030
GO:0061737	leukotriene signaling pathway	4	0.0031
GO:0003334	keratinocyte development	14	0.0032
GO:0045454	cell redox homeostasis	71	0.0035
GO:0043162	ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway	25	0.0037
GO:0001711	endodermal cell fate commitment	16	0.0038
GO:0010941	regulation of cell death	1229	0.0042
GO:0006310	DNA recombination	194	0.0043
GO:0022414	reproductive process	789	0.0043
GO:0097750	endosome membrane tubulation	8	0.0047
GO:0010510	regulation of acetyl-CoA biosynthetic process from pyruvate	9	0.0047
GO:0032435	negative regulation of proteasomal ubiquitin-dependent protein catabolic process	30	0.0047
GO:0045040	protein import into mitochondrial outer membrane	5	0.0050
GO:0032802	low-density lipoprotein particle receptor catabolic process	9	0.0051
GO:0010501	RNA secondary structure unwinding	8	0.0056
GO:0008277	regulation of G-protein coupled receptor protein signaling pathway	68	0.0065
GO:0060444	branching involved in mammary gland duct morphogenesis	14	0.0065
GO:0051444	negative regulation of ubiquitin-protein transferase activity	17	0.0067
GO:0016197	endosomal transport	336	0.0068
GO:1903774	positive regulation of viral budding via host ESCRT complex	4	0.0072