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 (Onchorhynchus 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 appear 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 tschawytscha) 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 heterogeneous 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 x 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% H2O2, 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⁻¹ 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 RNA Clean 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; high stress, 21°C; and warm stress, 28°C), sex and RNA extraction batch. Fish treatments within each tissue type. The mean HSP70 relative abundance was compared among treatments using a one-way ANOVA with each tissue type.

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 F-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 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.
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 muscle.
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.

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**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 $P < 0.05$.
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                      | 0.0013     |
|           |        | 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 (Plumper 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) 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-Ortin 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 a 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 the epidural 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 lonetwork.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 warm temperatures 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:009612  | 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:0003381 | 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:0070049 | 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:1900440 | 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 | cytosplasmic 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:00333598 | 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 transcripton preinitiation complex assembly | 14   | 0.00157  |
| GO:0031120 | snRNA pseudouridine synthesis                   | 6    | 0.00169  |
| GO:0006260 | DNA replication                                  | 198  | 0.00188  |
| GO:0016310 | phosphorylation                                  | 1669 | 0.002    |
| GO:002252  | 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  |

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      | protein folding                                   | 177  | 4.20E-07 |
| GO:0006457 | positive regulation of mRNA binding              | 10   | 0.00013  |
| GO:1902416 | 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:002252  | 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:00006984 | 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:0070940 | dephosphorylation of RNA polymerase II C-terminal domain              | 8  | 0.00551  |
| GO:0033407 | 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:000738 | 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  |

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| GO ID   | GO Term                                      | N  | P-value  |
|---------|----------------------------------------------|----|----------|
| GO:0055010 | ventricular cardiac muscle tissue morphogenesis | 38 | 0.00000  |
| GO:0002026 | regulation of the force of heart contraction  | 20 | 0.00000  |
| GO:0046488 | phosphatidylinositol metabolic process       | 136| 0.00000  |

(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:0005472 | regulation for 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    |