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Evidence of prevalent heat stress in Yukon River Chinook salmon

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Abstract

Migrating adult Pacific salmon (*Oncorhynchus spp.*) are sensitive to warm water (> 18 °C) with a range of consequences from decreased spawning success to early mortality. We examined the proportion of Yukon River Chinook salmon (*O. tshawytscha*) exhibiting evidence of heat stress to assess the potential that high temperatures contribute to freshwater adult mortality in a northern Pacific salmon population. Water temperatures greater than 18 °C have occurred almost annually in the Yukon River and correspond with low population abundance since the 1990s. Using gene transcription products and heat shock protein 70 biomarkers validated by field experiment we identified heat stress in half of Chinook salmon examined (54%, n = 477) across three main-stem locations and three tributaries in 2016–2017. Biomarkers tracked wide variation in water temperature (14–23 °C) within a tributary. The proportion of salmon with heat stress differed between years at four of the six locations, with more prevalent heat stress in the warmer year. This work demonstrates that warming water temperatures are currently affecting northern populations of Pacific Salmon.

Keywords: Alaska, climate warming, gene transcription, heat shock protein, heat stress, Pacific salmon, thermal stress

Introduction

Air temperatures in the Arctic have warmed by more than 2 °C since the late 19th century, disproportionally higher than the 0.8 °C rise globally during the same time period (Post et al., 2019). Recently, the rate of Arctic warming has accelerated with 0.75 °C of the 2 °C increase occurring in just the last decade (Post et al., 2019). Indeed, the five warmest years on record globally have all occurred since 2015 (NOAA and NASA 2020), and in Alaska four of the five warmest years have occurred since 2014. The negative effects of warming on Arctic specialist species have been widely anticipated and increasingly well-documented (reviewed in Post et al., 2019). A less obvious result of disproportionate and accelerating warmth at high latitudes could be negative effects on more generalist, broadly distributed Northern hemisphere fish and wildlife species encountering unsuitable temperatures at their northern range extent. Pacific salmon (*Oncorhynchus spp.*) are an ideal taxon for examining the possibility that fish and wildlife species are already encountering unsuitable habitats at their northern range extents. There is a long history of Pacific salmon thriving at northern latitudes with a wide range of climatic conditions (Finney et al. 2000), they occur over a broad geographic distribution and the negative effects of heat stress in the southern parts of their range have been well documented (Crozier et al. 2008; Hinch et al. 2012). Heat stress is a cellular and physiological stress response that can cause mortality and results from warming beyond suitable temperatures. High water temperatures may cause mortality through several mechanisms, including increased virulence of pathogens (McCullough 1999; Kocan et al. 2009; Miller et al. 2011), steep increases in metabolic rate that outstrip energy resources (Rand et al. 2006), and an oxygen demand that exceeds the heart's capacity to deliver oxygen (Farrell et al. 2008; Eliason et al. 2013). We

hypothesized that water temperatures are already warm enough to induce heat stress in migrating adults near the northern range extent of Pacific salmon in Alaska's subarctic Yukon River.

The Yukon River is a subarctic, seasonally ice-covered, transboundary river originating in Canada. It flows through the U.S. state of Alaska and terminates in the Bering Sea. The watershed area (approximately 850,000 km^2) is the fifth largest in North America and supports important Pacific salmon fisheries (Revenga et al. 1998; Brabets et al. 2000). Despite the high latitude (~ 62 to 67 °N) and ice-covered winters, summer water temperatures have consistently exceeded typical thresholds associated with stress and elevated mortality in migrating adult Pacific salmon (>18 °C) at lower latitudes (~ 44 to 50 °N) (McCullough 1999; Wagner et al. 2005; Crossin et al. 2008; Keefer et al. 2008; Mathes et al. 2010; Strange 2010; Hinch et al. 2012; Bowerman et al. 2016). Indeed, water temperature in the Yukon River during spawning migration met or exceeded 18 °C in 85% of years, 19 °C in 70% of years, and 20 °C in 40% of years (n = 23 years; 1996–2019, except no data in 2006) at Pilot Station, Alaska (Figure 1). Surprisingly, the peak summer water temperatures for the Yukon River are generally similar to the Fraser River (Hinch et al. 2012), despite the Fraser River being ~ 3,000 km further south. Fraser River sockeye salmon (*O. nerka*) have been used as a model system for assessing heat stress and related mortality that can exceed 90% in the warmest cases (reviewed by Hinch *et al.*, 2012). Even water temperature of 18 °C can double adult sockeye salmon mortality rates compared to cooler temperatures if the exposure is prolonged (i.e., weeks) (Crossin et al. 2008). Mortality of migrating and spawning adults due to heat stress can undermine escapement-based management practices and exacerbate population declines if a large component of the individuals counted do not successfully reproduce.

Chinook salmon (*O. tshawytscha)* have been a management focus in the Yukon River and broader Arctic-Yukon-Kuskokwim (AYK) region of Alaska and Canada since a population decline occurred in the late 1990s and failed to recover (Kruger and Zimmerman 2009; ADF&G 2013; AYK-SSI 2013). The causes of the initial population decline and failure to recover are unclear (Kruger and Zimmerman 2009; ADF&G 2013; AYK-SSI 2013). Low Chinook salmon numbers are a hardship for virtually all residents in the region given their key role in the local economies, food security, and culture (ADF&G 2013). In contrast to other regions with declining salmon populations across North America, freshwater and marine habitats are largely intact in the AYK region with little development and sparse human populations (Brabets et al. 2000). Evidence of heat stress in Yukon River salmon is limited to anecdotal observations of listless behavior during warm water periods (generally > 20 °C) and the Pacific salmon mortality event that occurred across Alaska in 2019, where carcasses of various species were observed along migration corridors with eggs retained (i.e., *en route* mortality) (various observations from LEONetwork.org and media stories)(Westley In Press).

Cellular and physiological biomarkers of heat stress precede obvious behavioral changes and death, which provide an early warning of heat stress that is often predictive of death (Miller et al. 2011; Jeffries et al. 2012). Changes in gene transcription (mRNA) and protein expression of heat shock proteins are natural biomarkers of cellular stress that may provide information for assessing the prevalence of heat stress in wild populations (Iwama et al. 1998; Basu et al. 2002; Deane and Woo 2011; Jeffries et al. 2012). Many genes and proteins are highly conserved among taxa and allow for comparisons across a wide array of species (Welch 1993; Feder and Hofmann 1999; Basu et al. 2002). These techniques require experimental validation studies to demonstrate the response of specific genes or proteins to high temperatures and establish the

specific signatures that reflect heat stress. Validations with model laboratory species and more southern wild populations have supported the use of these biomarkers to identify heat stress in salmonids (Feder and Hofmann 1999; Basu et al. 2002; Iwama 2004; Crossin et al. 2008; Chadwick and McCormick 2017).

Here, we conducted an experimental temperature manipulation with a subset of wild Yukon River Chinook salmon to validate heat stress biomarkers and subsequently estimated the proportion of adult Chinook salmon with heat stress biomarkers across six collection locations in the Yukon River watershed that were sampled in two years (2016 and 2017) with different water temperatures. While differences in water temperatures were small between the two years, water temperatures in July of both years were predominantly > 18 °C and in a range where the Pacific salmon heat stress response is known to be extremely sensitive. We classified each Chinook salmon as heat stressed or not using thresholds of muscle gene transcription levels and heat shock protein 70 (HSP70) abundance developed from the experiment. Gene transcription levels were measured for a panel of 12 genes associated with heat shock (*HSP27, HSP70, HSP90*), oxidative stress and detoxification *(AHR, CYP1A, SOD, MT-A*), immune system function (*AHR, tbx21, Gata3, Mx1, IFNa*), and metabolism (*leptin*) (Table 1). The gene transcription and protein biomarkers were examined jointly because they provide different snapshots of the heat stress response as gene transcription precedes protein translation and the combination of the two biomarkers may also help differentiate between moderate and severe heat stress (Lund et al. 2002; Lewis et al. 2016).

Methods

Temperature Manipulation Experiment

A temperature manipulation experiment was conducted in the lower Yukon River, adjacent to the Alaska Department of Fish and Game's (ADF&G) test fishery site (N 61.94716° W 162.84161°) at the beginning of the spawning migration in June, when ambient water temperatures were cooler in order to ensure that individuals had not already experienced temperatures near or above heated treatment conditions. Pilot Station is a mixed stock and population fishery and genetics (single nucleotide polymorphisms) estimates the contributions of three major groups during the experiment (mid-June 2018) were approximately 10% U.S. Lower Yukon, 40% U.S. middle Yukon, and 50% Canada (West and Prince 2019) such that the experimental results are unlikely to be specific to a particular stock or population. Genetic stock assignments are not available for individual Chinook salmon. Across the Yukon River basin, 183 separate Chinook salmon spawning areas are known with 32 areas being major producers, suggesting the potential for a high number of genetic populations (Brown et al. 2017).

Sample size was determined in consultation with ADF&G managers and limited given the low population size of Yukon River Chinook salmon. Briefly, up to three individual Chinook salmon were acquired daily from the ADF&G test fishery across nine consecutive days (n = 27) and randomly assigned to one of three tanks: control (~15 °C), 18 °C, or 21 °C. The treatment temperatures were selected because they already occur with some frequency in the main-stem Yukon as well as some tributaries and headwater streams (Zuray 2010; Carlson and Edwards 2017; Conitz 2018; Koch et al. 2020) (Figure 1) and spanned the temperature range associated with heat stress, decreased migration performance, and increased mortality based on review of literature (McCullough 1999; Goniea et al. 2006; Crossin et al. 2008; Farrell et al. 2008; Keefer et al. 2008; Jeffries et al. 2012; Martins et al. 2012). Water temperatures of 18 °C and 21 °C are likely near the thresholds for detecting heat stress and the upper temperature limit, respectively,

for migrating Chinook salmon (McCullough 1999). Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (NAS 2011). This work was approved by the USGS Alaska Science Center Animal Care & Use Committee (ACUC 2018-04) and under a permit from the Alaska Department of Fish and Game (SF2018-132).

Treatment tanks were 587 L polyethylene stock tanks with a liquid propane fueled heater to raise temperature, electric aquarium heaters to maintain temperature, circulation pumps to prevent thermal stratification, aerators to supplement dissolved oxygen, and temperature loggers to record water temperature every five minutes. Each experimental run lasted approximately six hours with one or two hours of heating (18 °C and 21 °C, respectively) and a minimum of four hours at the treatment temperature. Prior experiments indicate that four hours is sufficient to elicit gene transcription and protein responses to heat stress (Buckley et al. 2006). The rate of temperature rise to treatment temperatures was 3.71 ± 1.31 °C (mean ± SD) with a mean hold temperature of 18.0 °C in the low heat stress treatment (range = 17.3 – 18.6 °C) and 20.9 °C in the high heat stress treatment (range = 19.8 – 22.0 °C). Individuals in the control group were held at a near consistent ambient water temperature until the completion of the heat treatments each day. Details of the heating and control system used for these experiments can be found in Donnelly et al. (In Press).

All fish were sacrificed immediately following the four-hour period at the target temperature by cranial stunning. All individuals in the control and 18 °C groups survived the trial, but one individual that began the 18 °C trial failed to acclimate to the tank and was released. Fifty-six percent (n = 9) of those in the 21 °C treatment group survived and provided samples for this study. Mortality in the 21 °C treatment is not surprising given that 21 °C has been suggested as the upper thermal limit for migrating Chinook salmon (McCullough 1999).

Experimental mortality is discussed in greater detail in Donnelly et al. (In press). Individuals that survived the 21 °C treatment were not especially lethargic or moribund and similar in appearance and behavior to fish in other treatments. Twenty-two individuals were used in the experimental results with nine in the control group, eight in the 18 °C treatment group, and five in the 21 °C treatment group. Samples consisted of three muscle biopsy plugs taken from the white dorsal muscle above the lateral line and posterior to the operculum. Muscle tissue was chosen because it can be sampled non-lethally in adult salmonids with minimal training by those already staffing monitoring sites. All samples were immediately placed in microcentrifuge tubes and then into a liquid nitrogen dry shipper. All tissue samples were stored at –70 °C or below until laboratory work was conducted to assess the gene transcription and protein abundance.

Experimental Laboratory Analyses

Genes are denoted in italics (e.g., *HSP70*) and proteins are denoted in plain font followed by the word "protein" (e.g., HSP70 protein) for clarity throughout. The particular genes selected for this study are relatively well-studied with heat shock genes selected for their specific response to elevated water temperatures and the remaining genes selected for their broad response to a variety of stressors (references in Table 1). Gene transcription was measured using quantitative real-time polymerase chain reaction (qPCR) assays of mRNA at the U.S. Geological Survey Western Ecological Research Center in Davis, CA, USA. Total RNA was extracted from ground muscle tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen; www.qiagen.com). To remove contaminating genomic (g)DNA, extracted total RNA was treated with 10 U µl−1 of RNase-free DNase I (DNase, Amersham Pharmacia Biotech Inc.; www.apbiotech.com) at room temperature (20-30°C) for 15 min. The extracted RNA was stored in a -80 °C freezer until analyzed.

A standard cDNA synthesis was performed on 2 µg of RNA template from each salmon. Reaction conditions included 4 units reverse transcriptase (Omniscript, Qiagen, Valencia, CA), 1 µM random hexamers, 0.5 mM each dNTP, and 10 units RNase inhibitor, in RT buffer (Qiagen, Valencia, CA). Reactions were incubated for 60 min at 37 °C, followed by an enzyme inactivation step of 5 minutes at 93 °C, and then stored at –20 °C until further analysis.

Briefly, 1 µl of cDNA was added to a mix containing 12.5 µl of QuantiTect Fast SYBR Green® Master Mix [5mM Mg^{2+}] (Qiagen, Valencia, CA), 0.5 µl each of forward and reverse sequence specific primers (Table 2), and 10.5 µl of RNase-free water; total reaction mixture was 25 µl. The primers for *HSP27*, *HSP70*, and *HSP90* are specific to the inducible forms of these genes. The reaction mixture cDNA samples for each gene of interest and reference genes were loaded into MicroAmp Fast Optical® 96 well reaction plates in duplicate and sealed with optical sealing tape (Applied Biosystems, Foster City, CA). Reaction mixtures containing water, but no cDNA, were used as negative controls.

Amplifications were conducted on a QuantStudio 3 Real-time Thermal Cycler (Applied Biosystems, Foster City, CA), using the QuantStudio 3 software. Reaction conditions were as follows: an initial hold stage of 95 °C for 20 s, 40 cycles of 95 °C for 1 s, and 60 °C for 20 s. The melt curve was 95 °C for 1 s, 60 °C for 20 s, and 0.3 °C/s temperature increase, and then 95 °C for 1 s.

We analyzed qPCR data using normalized values calculated as the threshold cycle (C_T) of the reference gene subtracted from the C_T of the gene of interest where C_T is the amplification cycle that allows for detection (Bustin 2002). Because samples with inherently higher numbers of transcripts require fewer amplification cycles for detection, lower normalized values indicate that

more transcripts are present. A change in normalized value of 2 is approximately equivalent to a 4-fold change in the amount of the transcript.

HSP70 protein abundance of the inducible form was analyzed at the U.S. Geological Survey S.O. Conte Anadromous Fish Research Laboratory, Turners Falls, MA, USA. Muscle from the dermal punch was separated from subdermal fat and skin and weighed to the nearest milligram. All tissues were thawed and homogenized with a 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. 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). Dilution titration for tissue homogenates was completed to establish the range of linearity. A total of 10 µg of muscle protein was loaded per sample. Two lanes were reserved on each gel for a standard consistent tissue preparation as reference to control for blot-to-blot variation and to allow for comparison across all treatments and locations. Following electrophoresis, proteins were transferred to Immobilon polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mMTris, 192 mM glycine buffer, pH 8.3. Equal loading was verified by reversible total protein staining with Ponceau S. Samples with unequal loading or alternate banding patterns were removed from analysis. 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 1:25,000 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 1:10 000 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 (ECL) solution A (396 µMcoumaric 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 are represented as a ratio to the mean standard value that we refer to as HSP70 relative abundance.

Statistical Analyses of controlled experiment

Heat stress was inferred through differences between fish held in the control and elevated temperature treatments that allow for high classification accuracy (correct classification > 75%) for both the gene transcript levels and HSP70 protein abundance. Following separate statistical analysis for the two biomarkers, heat stress classifications based on gene transcription and HSP70 protein were considered jointly with indications of heat stress in both biomarkers interpreted as more severe stress (Lund et al. 2002; Lewis et al. 2016).

A linear discriminate analysis (LDA) was used to identify the combination of muscle gene transcript levels that best classified individuals among the control and two elevated temperature treatments. LDA was chosen as the data reduction technique as it explicitly attempts to model the difference among *a priori* groups. Specifically, the 'lda' function in the MASS package of R (Venables and Ripley 2002) was implemented to identify a linear combination of

the mRNA transcript values from genes that results in maximum separation between the centers of the groups while minimizing variation within the groups.

Data for all genes were examined to assess statistical assumptions of normal distribution and multicollinearity. The muscle mRNA transcript data for *leptin* were removed because concurrent investigations of similar data in Yukon River Chinook salmon that were not part of the experiment revealed a violation of normal distribution due to a high frequency of individuals without detectable levels of *leptin* and the need for any model developed from the experimental results to be applicable to the broader population of Chinook salmon. *MT-A* was also removed from the analysis due to strong positive correlations ($r > 0.60$) with both *HSP27* and *IFNa* that were not dependent on a single influential point (i.e., outlier).

Given the small experiment sample size, a sequential reduction in the number of genes in the LDA was preformed to limit the number of genes used in the model and reduce overfitting. The sequential reduction was based upon gene transcript data that was centered and scaled prior to analysis so that coefficients reflected the influence of each gene in the model. The least influential gene with the coefficient closest to zero (mean of LD1 and LD2 coefficients weighted by the variation attributed to each LD) was dropped from the analysis sequentially until the classification rate fell below the desired threshold of 75%. The model that used the fewest number of genes and still maintained a classification rate of >75% was considered the preferred model. HSP70 protein abundance was plotted and visually examined for the location of a threshold that best separated fish among treatments. The mean HSP70 abundance was compared among treatments using a one-way analysis of variance (ANOVA).

Application of experimentally-derived heat stress thresholds

Muscle biopsy samples were collected from 477 live Chinook salmon during their spawning migration in the Yukon River watershed during summer 2016 and 2017 (Table 3). The timing of sample collection varied by sites to generally align with the peak of spawning migration at each location. Collection locations were annual management assessment projects or subsistence fishing efforts that varied in capture methods. Chinook salmon were sampled in Alaska at three locations on the main-stem Yukon River and three tributaries. Main-stem sites sample a mix of populations, while tributary locations presumably sample a single population. Genetic stock identification is not routine at all collection sites, nor are genetic assignments available for individual fish or designed to identify the spawning population (West and Prince 2019). This work was approved by the USGS Alaska Science Center Animal Care & Use Committee (ACUC 2017-08) and under permits from the Alaska Department of Fish and Game (SF2016-186 and SF2017-167).

Main-stem Yukon River locations were near the mouth at Emmonak (ADF&G test fishery gillnets), subsistence fish wheels in the middle portion of the main-stem Yukon River (Rapids Fish Wheel operated by Stan Zuray and others), and just before the Canadian border at Eagle (ADF&G test fishery gillnets). Individuals captured at the Rapids fish wheel are predominantly Canadian stocks, as there are only two major spawning areas in the U.S. beyond there, and all individuals captured at Eagle are Canadian stocks (Eiler et al. 2014; Brown et al. 2017). Upstream migration in the main-stem occurs primarily in June and July. Tributary collections occurred at weirs in the East Fork Andreafsky and Gisasa rivers (U.S. Fish and Wildlife Service) and electrofishing in the Chena and Salcha rivers (ADF&G). Fish collected in the Chena and Salcha rivers were grouped together for analysis because both are components of the Tanana River with similar migration routes and temperature histories. Sampling at all

tributary locations occurred primarily in July. In all cases, muscle biopsies were taken within minutes of capture, including at the Rapids fish wheel where individuals were sampled directly from the capture basket and not the live-well holding box. This approach assumes that capture, short handling time (minutes), and differences in capture methods described here have no effect on the cellular stress biomarkers used because they require hours for response (Lund et al. 2003; Buckley et al. 2006). To date, there is no evidence that physical stressors and confinement can induce heat shock proteins in fish, nor does cortisol (which does respond rapidly to stressors) have direct effects on heat shock proteins (Deane and Woo 2011). Moreover, the experimentally-derived heat stress classifications are developed by contrasting control fish to heated fish. Thus, heat stress classifications are primary related to the water temperature while minimizing the potential effects of capture and confinement that are more likely in tissue samples collected hours after capture and confinement.

All laboratory analyses of gene transcription and HSP70 protein were conducted in an identical fashion as above for the experimental fish. The preferred LDA model for classifying experimental fish to their respective treatments was then applied to gene transcript data from the field sampled fish to classify each individual as grouping with either control fish that were not heat stressed, 18 °C treated fish, or 21 °C treated fish. Only the experiment data were used to estimate the model coefficients. Because the gene transcript C_T values of the field sampled fish likely differed in their gene-specific means and standard deviations from the experiment fish, the classifications of field sampled fish were based on coefficients from a model that was refit with transcript data that was not centered and scaled from experimental fish.

Assessment of experimentally-derived models to identify heat stress in field sampled fish

A subset of field sampled fish (n = 477), just those from the East Fork Andreafsky site collected in 2016 and 2017 (n = 86), were used to assess the experimentally-derived thresholds to identify heat stress. At this location, individuals have spent enough time in freshwater (~200 rkm over several days at a minimum) to encounter warm water but have not traveled so far that a reasonable assessment of their temperature history is difficult. At sites further upstream in the Yukon River watershed, spans stretching hundreds of river kilometers lack water temperature data and preclude a reasonable assessment of temperatures previously encountered. Moreover, the individuals captured at this location experienced a wide range of temperatures, including seasonal increases that rose to > 23°C in 2016. The East Fork Andreafsky is one of only two major spawning populations that are downstream from the experimental location and therefore could not have contributed to the sample used in the experiment.

The relationships between results from each individuals heat stress biomarkers were compared to the three-day maximum water temperature at the capture location (maximum temperature on the day of and two days prior to capture based upon U.S. Fish and Wildlife Service water temperature data collected at East Fork Andreafsky River weir every 15 min). The three-day maximum temperature from the capture location is a reasonable metric for recent temperature history given that the capture location on the East Fork Andreafsky River is just 43 rkm from the main-stem Yukon River. While lower Yukon River Chinook salmon could travel that distance in about 1–1.5 days (Eiler et al. 2015), warm water temperatures are known to decrease migration speeds by ~ 50% in Columbia River Chinook salmon (Goniea et al. 2006) and may result in a travel time closer to three days if speed reductions with warming temperatures are comparable. To assess the strength of gene transcription data to identify heat stress caused by warm temperatures, the three-day maximum temperature was compared to the

LD1 value calculated for each fish captured in the Andreafsky River using linear regression. LD1 values were predicted from each individual's gene transcription data using coefficients estimated during the experiment. LD1 captured the majority (70%) of the variability in the gene transcription data used to assigned individuals among the control and experimental treatments (see results). Similarly, we plotted the HSP70 protein abundance relative to the three-day maximum temperature. Next, we tested whether the presence of elevated HSP70 abundance was related to the three-day maximum temperature using logistic regression (values > threshold set to 1 and values ≤ threshold set to 0).

Heat stress proportions and water temperatures across the Yukon River watershed.

Each field sampled fish was classified as heat stressed or not based on gene transcription levels and HSP70 protein abundance. An individual was classified as stressed if the LDA equation developed from experiment fish classified the individual with 18 or 21 °C treated Chinook salmon or if their HSP70 protein abundance exceeded 0.14, the experimental-derived threshold. The proportion of individuals with evidence of heat stress in either biomarker was calculated for each location and year. The proportion of individuals with evidence of heat stress were compared between years at each site using a z-test for independent proportions.

Water temperature data were available from the main-stem Yukon River and the three tributaries where Chinook salmon were captured in 2016 and 2017. Main-stem Yukon River water temperatures were measured hourly by ADF&G in the lower river near Pilot Station on the left and right sides of the river using HOBO Pro v2 data logger. In the East Fork Andreafsky and Gisasa rivers, water temperatures were measured every 15 and 20 min, respectively, by U.S. Fish and Wildlife Service at the weirs using HOBO Pro v2 data loggers. In the Chena River, water temperatures were measured every 15 min by the U.S. Geological Survey at the gage station near

Two Rivers, AK (station number 15493000). Monthly mean water temperatures were compared in each river between years using a Welch's t-test for unequal variances for qualitative comparison to interannual differences in the heat stress proportions. All data and metadata associated with this study is publicly available in von Biela and Donnelly (2020) and von Biela et al. (2020).

Results

Temperature Manipulation Experiment

Heat stress was inferred through differences in gene transcription of individuals in both the 18 and 21 °C treatment groups compared to control group. The mean and standard error for all normalized qPCR data that reflected gene transcript levels from each experimental treatment were calculated to visualize input data used in the LDA (Figure 2). The LDA produced two linear combinations of the continuous predictor variables, LD1 and LD2, and established a threshold that classified individuals based on their location in the ordination of LD1 and LD2. Data from the ten genes that met statistical assumptions (*HSP27*, *HSP70, HSP90, Gata3, tbx21, MX1, IFNa, AHR, SOD, and CYP1A)* classified individuals by treatment with 82% accuracy with LD1 accounting for 61% and LD2 account for 39% of the variability. Sequential model reduction to include fewer genes resulted in a preferred model with six genes (*HSP70, HSP90, Gata3, IFNa, AHR, and SOD)* with a classification rate among the three treatments of 77% (Figure 3A). Misclassification of individuals by the preferred model always involved the intermediate 18 °C treatment. No individuals from the control were classified with the 21 °C fish or *vice versa*. Among individuals in the control group, classification was accurate in 88% of individuals with one misclassification to the 18 °C treatment. Similarly, 80% of individuals from the 21 °C treatment were correctly classified and only a single fish was misclassified to the 18 °C

409 treatment. Classification rate was lowest among fish from the 18 °C treatment at 63% with one
410 individual grouped with the control and two individuals grouped with the 21 °C treatment. If the
411 classifications are summarized as more simplistic control or heat treated (18 and 21 °C
412 combined), 91% of individuals are correctly classified by the LDA.

413 The LDA based upon centered and scaled gene transcript data resulted in the following
414 coefficients with LD1 accounting for 70% of the variability across the six genes and LD2
415 account for the remaining 30%:

416 $$LD1 = 0.83HSP70 + 1.97HSP90 + 0.88Gata3 - 2.06IFNa - 0.04AHR - 1.96SOD \quad (1)$$

417 $$LD2 = -1.06HSP70 - 0.07HSP90 - 0.07Gata3 - 7.04IFNa + 3.08AHR - 1.54SOD \quad (2)$$

418 Higher normalized qPCR values used in the analysis indicate less mRNA transcript. For
419 example, positive coefficients of LD1 indicate a higher normalized value from qPCR moving
420 from left to right along the LD1 axis, but less mRNA transcript. The first linear discriminate
421 axis, LD1, of the six gene model accounted for 70% of the variation in the genes examined and
422 primarily separated individuals from the 21 °C treatment group from both the 18 °C treatment
423 and control groups (Figure 3). The positive coefficients and relative position of fish from the
424 treatments in Figure 3, indicated that 21 °C treatment group had more transcript for the genes
425 *HSP70, HSP90*, and *Gata3*, and less transcript for *IFNa* and *SOD* compared to control and 18 °C
426 groups. The second linear discriminate axis, LD2, accounted for the remaining 30% of the
427 variation in the gene transcript data and primarily separated fish between the 18 °C treatment and
428 control groups (Figure 3). Coefficients indicated that fish from the 18 °C treatment group had
429 more transcript from *HSP70, IFNa,* and *SOD* (i.e., negative coefficients indicate a lower
430 normalized qPCR value and more gene transcript, moving upward along the LD2 axis in Figure

3), but less *AHR* compared to the control group. Coefficients for the genes *HSP90* and *Gata3* were near zero for LD2 and indicated little influence in distinguishing between the 18 °C treatment and control groups.

The reduced six gene model was refit with data that was not centered and scaled for application to Chinook salmon collected across the Yukon River watershed that were not part of the experiment. The formula for LD1 and LD2 fitted by the analysis was:

$$LD1 = 0.67HSP70 + 1.08HSP90 + 0.13Gata3 - 0.36IFNa - 0.02AHR - 1.27SOD \quad (3)$$

$$LD2 = -0.87HSP70 - 0.04HSP90 - 0.01Gata3 - 1.23IFNa + 1.09AHR - 1.00SOD \quad (4)$$

HSP70 protein abundance distinguished fish in the 21° C treatment group and was higher compared to both the 18 °C treatment and control groups in muscle (ANOVA; F = 4.95, P = 0.02) (Figure 3B). The threshold that separated HSP70 protein values between control and 21 °C fish with the fewest misclassifications was 0.14 (Figure 3B). The threshold for HSP70 protein from muscle tissue resulted in correct classifications for 93% of individuals overall, with 100% correct classifications of fish in the control group and 80% correct classification of fish in the 21 °C fish treatment group. Only the 21 °C treatment group elicited a detectable response in both gene transcription and the HSP70 protein.

Assessment of experimentally-derived model to identify heat stress

Among Chinook salmon captured in the East Fork Andreafsky River, the three-day maximum water temperature was related to the LD1 value of gene transcript data calculated based on coefficients that were fit using only fish from the experiment (equation 3) (linear regression, y = -0.92x + 17.6, t = -9.46, df = 84, r^2 = 0.510, $P < 0.001$) (Figure 4A). When the continuous LD1 variable is used to classify the heat stress status of each Andreafsky River fish,

453 an abrupt shift is observed between 20 and 22 °C where individuals switch from being primarily
454 categorized as unstressed (e.g., similar to experimental control group) to all individuals
455 categorized as high heat stress fish (e.g., similar to the 21 °C treatment group) (Figure 4A).
456 Water temperature was also related to the presence of elevated HSP70 protein abundance
457 (logistic regression, deviance = -55.7, df = 1, $P < 0.001$) (Figure 4B). Each degree of increase in
458 temperature resulted in a 4.3-fold increase in the odds of elevated HSP70 (log-odds ratio = 1.46).
459 The three-day maximum water temperature associated with a 50% probability of elevated HSP70
460 protein was 17.8 °C.

461 *Heat stress proportions and water temperatures across the Yukon River watershed*

462 Gene transcription and HSP70 protein abundance results (Figure 5) were available for
463 477 Chinook salmon captured in the Yukon River and three spawning tributaries (Table 3).
464 Across all capture locations and both study years, heat stress was identified using gene
465 transcription and protein biomarkers in more than half of Chinook salmon examined (54%, n =
466 477). The overall evidence of heat stress was summed across individuals for which heat stress
467 was only indicated by gene transcription (15%), only indicated by HSP70 protein (20%), or
468 indicated by both biomarkers (19%).

469 The proportion of Chinook salmon with evidence of heat stress was higher in 2017 than
470 2016 at the three locations further upstream: Rapids Fish Wheel (18% in 2016 vs 97% in 2017, z
471 = 4.45, $P < 0.001$), the Chena River (45% in 2016 vs 64% in 2017, z = 3.12, $P = 0.002$), and
472 Eagle (38% in 2016 vs 64% in 2017, z=2.12, $P = 0.028$) (Figure 6). Among individuals captured
473 at the East Fork Andreafsky River, heat stress was more common in 2016 (98% in 2016 vs 18%
474 in 2017, z = -4.90, $P < 0.001$) (Figure 6). The proportion of fish with heat stress was similar

between years for fish captured at Emmonak (31% in 2016 vs 34% in 2017, z = 0.30, P = 0.764) and in the Gisasa River (66% in 2016 vs 77% in 2017, z = 1.08, P = 0.282).

Water temperatures in the main-stem Yukon River were warm during both years, although not extremely so in the context of the last decade (Figure 1). Main-stem mean water temperatures at Pilot Station were similar between the two years in June (Table 4,t-test, t = 0.65, df = 2701, P = 0.517) and August (t-test, t = 0.40, df = 1612, P = 0.693), but differed in July (t-test, t = -13.6, df = 2964, P < 0.001) with July 2017 being warmer than July 2016 (Table 4). Water temperatures were also warmer in 2017 than 2016 in the Gisasa River during July (t-test, t = -32.8, df = 2213, P < 0.001) and Chena River during June (t-test, t = -12.4, df = 4952, P < 0.001), July (t-test, t = -53.6, df = 4553, P < 0.001), and August (t-test, t = -38.9, df = 3749, P < 0.001). In contrast, water temperatures at the East Fork Andreafsky River weir were warmer in 2016 during the second half of June (data only available for June 15 to 30; t-test, t = 32.4, df = 2553, P < 0.001) and July (t-test, t = 21.5, df =4334, P < 0.001).

Discussion

This study provides evidence that heat stress is prevalent in migrating adult Chinook salmon near their northern range extent in the Yukon River. Given the established links between heat stress and increased mortality, warm water temperatures may already contribute to population-level consequences for Yukon River Chinook salmon and the failure to recover from declines that began more than two decades ago. Because water temperature data are limited prior to population declines in the late 1990s, the possibility that warming temperatures contributed to the initial decline is difficult to assess. Our experiment identified gene transcription and HSP70 protein response consistent with heat stress at two water temperatures (18 and 21 °C) that regularly occur during the spawning migration. The experimental fish response was used to train

biomarker classification models that subsequently identified field-captured individuals as heat stressed or not and allowed heat stress to be summarized as a proportion. More than half of the field-captured Chinook salmon had evidence of heat stress in at least one biomarker. The proportion of heat stressed fish varied between years at most locations with higher heat stress proportions estimated in the warmer year. Between the two biomarkers, the HSP70 protein is a more straight-forward, cost-effective tool for identifying and monitoring heat stress in future studies, and gene transcription results provided a deeper understanding of how warm temperatures disrupt processes in the body and helped identify potential mechanisms linking heat stress to mortality (see Bowen et al. (In Press) as well).

Temperature Manipulation Experiment

Gene transcript biomarkers successfully identified a cellular response to 18 and 21 °C, while the HSP70 protein response only distinguished fish in the 21 °C treatment group. The differences in mRNA and protein response identified here largely agree with previous studies of Pacific salmon in southern regions of their range, namely the upregulation of heat shock genes and proteins and differential immune system response (Evans et al. 2011; Miller et al. 2011, 2014; Jeffries et al. 2014). This study is the first to identify a cellular stress response to elevated temperature from a high latitude Pacific salmon population.

An increase in the transcription and protein abundance of HSPs is the hallmark of the heat shock response (Lindquist 1986). Thus, it was no surprise that *HSP70* and *HSP90* were influential in distinguishing heat stress in muscle tissue or that the HSP70 protein alone was able to correctly classify individuals to the 21 °C treatment in all cases. The transcription and protein abundance of HSPs exhibit a ubiquitous increase in response to elevated water temperature in past studies regardless of tissue type (Palmisano et al. 2000; Basu et al. 2002; Iwama 2004;

Buckley et al. 2006; Evans et al. 2011; Lapointe et al. 2011; Nakano et al. 2014). A differential response between the HSP genes was evident between the 18 and 21 °C treatment groups with both *HSP70* and *HSP90* contributing to classification of the 21 °C group, *HSP90* responded more strongly based upon the magnitude of the coefficients in the model. In contrast, the 18 °C treatment group was distinguished by elevated *HSP70* in the LDA model with little influence of *HSP90*.

Immune system genes were differentially expressed in the muscle tissue of 18 and 21 °C treatment groups compared to control fish. Immune system responses to heat stress have often been documented in Pacific salmon and include examples from gill and cardiac tissue (McCullough et al. 2009; Evans et al. 2011; Miller et al. 2011; Jeffries et al. 2012; Anttila et al. 2014). The immune response in muscle tissue has been less frequently studied in fish, but muscle is known to be immunologically active (Valenzuela et al. 2017) and is a common site of disease and infection in Pacific salmon (Meyers et al. 2019). Immune system genes retained in the final model were related to two pathways of T helper (Th) cells, Th1 immune response (*IFNa*) and Th2 immune response (*Gata3*) (Citations in Table 1). Within our results, an influence of the Th1 immune gene *IFNa* was consistent with upregulation at 18 °C, but downregulation at 21 °C. The Th2 immune gene *Gata3* was upregulated at 21 °C, but no clear influence was present at 18 °C. The upregulation of Th1 immune response seen in the 18 °C treatment is typical of acute stressors and reflects an appropriate immune defense, while the Th2 response seen in the 21 °C treatment is typical of a chronic response and immune suppression that can be detrimental (Tort 2011). Still, data reflected the changes in just two of many genes that are part of the immune system and did not provide a full picture of the immune system response. Concurrent results from an analysis of the entire transcriptome for individuals included in this experiment offers

more insight to the complexity of the immune system response to warming temperatures (Bowen et al. Accepted).

Immune system genes may respond to temperature if warming increases activity of latent pathogens in the body of the fish (Crossin et al. 2008; Miller et al. 2011, 2014). Many pathogens occur at chronic low levels in fish under normal circumstances and are poised to respond when conditions are more suitable (Arkoosh et al. 2004; Miller et al. 2014). Although there are likely many pathogens present, the most studied pathogen in Yukon River Chinook salmon is the parasite *Ichthyophonus hoferi* that causes inflammation in several tissues including muscle (Kocan et al. 2004; AYK-SSI 2013). Disease progression for fish infected with *I. hoferi* is known to be faster in warmer water (Kocan et al. 2004), but it is not clear if the short (~ 6 h) duration of this experiment would allow enough time for the response of a latent pathogen. Without an independent assessment of pathogen load or even a synoptic examination of pathogens in Yukon River Chinook salmon, interpreting the cause of differential responses among immune system genes following exposure to heat is speculative. Still, the response of the immune system and immune genes is a consistent feature in heat stressed salmon (McCullough et al. 2009; Evans et al. 2011; Miller et al. 2011; Jeffries et al. 2012; Anttila et al. 2014) and warrants further study about the potential for pathogens to exacerbate stress and mortality with warming in Alaska Pacific salmon.

Warming was associated with differential expression of a gene closely linked with contaminants (*AHR* and *SOD*) (Wheelock et al. 2005; Arellano-Aguilar et al. 2009; Erdoğan et al. 2011; Uno et al. 2012). *AHR* transcripts were lower in the 18 °C treatment, while *SOD* transcripts declined with warming and contributed to distinguishing fish from both the 18 and 21 °C treatments. Decreased expression of genes related to detoxification of xenobiotics has been

associated with heat stress (Veldhoen et al. 2010; Lapointe et al. 2011; Tomalty et al. 2015), as well as hypoxia (Rahman and Thomas 2012), and pathogens (Reynaud et al 2008). Although the mechanisms involved are unclear, decreased expression of genes involved in detoxification may simply indicate a reallocation of resources to the more substantial stressor.

Assessment of experimentally-derived models to identify heat stress

The experimentally-derived models for gene transcription and the HSP70 protein used to identify heat stress performed well in an independent assessment using Chinook salmon captured in a lower Yukon River tributary, the East Fork Andreafsky River. Salmon captured when water temperatures had recently exceeded 21 °C were all identified by the model as being similar to 21 °C treated fish based on gene transcription response (represented by LD1, Figure 4A). Moreover, differences in gene transcription were related to recent maximum water temperature across the whole range of observed temperature values. Elevated HSP70 protein was also strongly related to water temperature (Figure 4B) with an inflection point separating unstressed and heat stress individuals near 18 °C, the presumed heat stress threshold for migrating adult Pacific salmon based on our literature review (see introduction).

Very few individuals in the East Fork Andreafsky had gene transcription results similar to 18 °C treated fish despite water temperature data suggesting that several individuals recently experienced water temperatures between 18 and 21 °C (Figure 4A). In the experiment, a lower level of heat stress could be detected in the gene transcript response but not the HSP70 protein response, the opposite appeared to be true in the assessment of Andreafsky fish. A HSP70 protein response identified heat stress for nearly all individuals captured when water temperatures had recently exceeded 18 °C (98%, n = 40) in the tributary. It appears that prolonged exposure to temperatures near 18 °C (or increased time since exposure) may have

resulted in an HSP70 protein response in field sampled Chinook salmon, which was not apparent in the acute experiment. This hypothesis is supported by laboratory studies that indicate that HSP70 protein abundance first appears after 2 hours of exposure to elevated temperature and peaks and stays elevated for 24–72 hours (Lund et al. 2003). This possibility is also supported by evidence of increased *HSP70* mRNA levels in 18 °C treated individuals that would presumably have resulted in more HSP70 protein in time. The gene transcription signature that defined our 18 °C treated individuals, captured by LD2, may be relatively fleeting. Indeed, a gene transcription response similar to that of 18 °C treated individuals was rarely (5%, n = 477) observed in field sampled Chinook salmon across all locations in the Yukon River watershed.

Heat stress prevalence

Heat stress was prevalent (>50% of individuals) at all locations in at least one of the two study years, except near the river mouth at Emmonak (Figures 5 & 6). Lower and consistent rates of heat stress for individuals passing through Emmonak agree with the cooler water temperatures that occur early in the migration during June. Heat stress was prevalent in 2017 at all three upper watershed locations (Rapids, Chena River, and Eagle), but not 2016, in agreement with warmer temperatures in the main-stem Yukon River (Figure 6). Within the East Fork Andreafsky River, a lower Yukon River tributary, an opposite inter-annual difference was observed consistent with the local water temperatures in the tributary. The lack of coherence in water temperature interannual variation among rivers is likely related to differences in the hydrology and atmospheric conditions across the distinct regions of the watershed (Brabets and Walvoord 2009). The East Fork Andreafsky River differs from the rest of the watershed in terms of its lower elevation and gradient (Olsen et al. 2010) and closer proximity to the Bering Sea that

612 results in more of a maritime climate as compared to middle and upper river tributaries (Brabets
613 et al. 2000).

614 Results for Chinook salmon captured in the Chena River may be among the most
615 surprising and informative given that water temperatures are often cool in the Chena, Salcha, and
616 greater Tanana River watershed owing to a higher influence of cold groundwater and glacier
617 melt (Brabets et al. 2000; Walvoord and Striegl 2007). It appears that prolonged (weeks)
618 exposure to warmer water temperatures in the main-stem Yukon River during the spawning
619 migration was still detectable when individuals were captured in the Chena River's cooler water
620 further upstream. Chinook salmon captured in the Chena River have spent ~24 days in the main-
621 stem Yukon River (~1,100 km) and ~11 days in the cooler Tanana and Chena rivers (~500 km)
622 assuming a migration speed of 45.8 km d^{-1} for Chena River Chinook salmon (Eiler et al. 2015).
623 Note that individuals may spend more or less time in each of these rivers given that migration
624 speeds are known to decline with warm temperature (Goniea et al. 2006) and as migration
625 progresses (Eiler et al. 2015).

626 Similarity in results among fish captured from the three sites (Rapids, Chena River, and
627 Eagle) further upstream in the Yukon River watershed suggests that long stretches of shared
628 migration corridors may synchronize the influence of heat stress among populations. Synchrony
629 in any attribute of Pacific salmon across populations has usually been interpreted as a reflection
630 of their shared conditions during ocean residency (e.g., ocean temperature, feeding conditions),
631 as the natal freshwater habitats of each population can have dramatic differences in
632 environmental conditions (Hare et al. 1999; Mueter et al. 2002). Synchrony as a result of shared
633 migration corridors is rarely considered because this phase is short relative to the lifespan of the

fish, many attributes are set prior to river entry (e.g., body length), and not all watersheds have a long, shared migration route like the Yukon River.

In addition to thermal stress, heat stress biomarkers can be induced by a variety of environmental factors (Deane and Woo 2011), including social stressors and lack of food (Currie et al. 2010). In our studies the wide variation in heat stress rates between study years within locations provided additional confidence that heat stress classifications were robust to changes in gene transcription and HSP70 during the spawning migration that are associated freshwater entry, sexual maturation, and senescence (Evans et al., 2011; Miller et al., 2011; Carey et al., 2019). Our sampling focused on collections that occurred in migration corridors and used only live fish sampled within minutes of capture. The thresholds developed here for identifying heat stress are likely inappropriate for Chinook salmon captured on spawning grounds or collected as carcasses. Among the locations included in this study, the applicability of these thresholds is most uncertain for individuals collected near Emmonak. This location is the closest to the river mouth where fish undergo a major physiological shift from saltwater to freshwater that may result in shifts to gene transcription and HSP70 protein abundance (Evans et al. 2011). Indeed, any detection of heat stress at this location is suspect because water temperatures were cool (generally < 15 °C) at the time of collection and there was no reason to suspect previous exposure to warm water temperatures at this time of year. Moreover, gene transcription and HSP70 protein values are just above the threshold for heat stress detection (Figure 5).

The Gisasa River was the only location where heat stress was prevalent in both study years and no inter-annual difference in heat stress proportion was detected despite warmer temperatures in 2017. The high heat stress proportions in both years may simply reflect that Chinook salmon reach the Gisasa River weir at the seasonal peak of water temperatures in mid-

July. The largest observed Pacific salmon mortality event within the Yukon River watershed during the 2019 heatwave was also located within the same portion of the Yukon watershed (Koyukuk River) in mid-July with chum salmon that have similar migration timing (Westley In Press. The only other site with a strong overlap in sample collections and peak water temperatures was in the much cooler Chena River, where individuals likely move into cooler tributary waters prior to the seasonal maximum temperature in the main-stem Yukon River in mid-July.

Here, we did not have the data necessary to consider the effects of population on the response of heat stress biomarkers. Previous studies have indicated that populations with a long-term history of warmer water temperatures have physiological adaptations that moderately increase thermal tolerance and performance (Eliason et al. 2011). The potential for similar population-specific adaptations to spawning migration temperatures appears unlikely within the Yukon River Chinook salmon because nearly all Yukon River Chinook salmon populations encounter warm water during their upstream migrations. Thus, the variability in water temperature experience among populations might be too weak to elicit or detect variability in thermal tolerance in Yukon River Chinook salmon. Still, we cannot rule out the effects of variability in water temperatures of spawning and rearing habitats on thermal tolerances among populations.

Conclusions

The evidence presented here was consistent with routine, prevalent, heat stress for Chinook salmon near their northern range extent in the Yukon River. Three major findings support this conclusion. First, the experiment and field results from the East Fork Andreafsky River agreed with the general presumption that water temperatures near 18 °C approximate the

threshold of heat stress. Second, water temperatures routinely exceeded this threshold during the spawning migration each July over the last two decades. Third, gene transcription and HSP70 protein levels identified prevalent heat stress during field sampling in 2016 and 2017, years when outward signs of heat stress were nearly nonexistent. The cellular stress response used to identify heat stress in this study is an adaptive response that can protect cellular function for just a limited amount of time in a relatively narrow temperature range. To this point, heat stress often predicts *en route* and prespawn mortality, or reduced reproductive success because warm temperatures often exceed the protection afforded by the cellular stress response (McCullough 1999; Quinn et al. 2007; Keefer et al. 2008; Mathes et al. 2010; Strange 2010; Hinch and Martins 2011; Hinch et al. 2012; Bowerman et al. 2016). In light of the findings presented here, the unusual mortality of Pacific salmon species along migration routes during the record-breaking warmth and low water level of 2019 (e.g., Westley In Press) are not surprising. The mortality observations provide additional evidence that water temperatures are already high enough to cause mortality among Pacific salmon populations near their northern range extent. The apparent absence of carcasses in previous years does not infer a lack of mortality because carcasses initially sink at death and are rarely observable in large turbid rivers (Farrell et al. 2008) like the Yukon River. Indeed, the low water levels may be a key reason that carcasses were observed in 2019 and not previous years with warm water temperatures.

This work has immediate implications for management considerations and research priorities, particularly in the context of projections that universally forecast continued warming (Post et al. 2019). This study is the first to confirm that freshwater temperatures are stressful to Pacific salmon migrating in a northern river. Results here raise concerns that unaccounted mortality may result in an overestimation of Pacific salmon spawning success in warm water

703 years. Spawning abundances are the primary data used to assess management success in
704 escapement-based management. In fact, the productivity (log recruits per spawner) of Chinook
705 salmon populations across several southcentral Alaska watersheds, was recently shown to
706 decline steeply when spawning water temperatures exceed 18 °C, the same water temperature
707 threshold identified for heat stress in this study (Jones et al. 2020). More research is needed that
708 links water temperature, heat stress, and mortality in northern rivers to assess the sensitivity of
709 population dynamics to environmental conditions during the spawning migration. Useful studies
710 for assessing the severity and scale of this threat include expanding water temperature and heat
711 stress monitoring to additional northern watersheds, simple surveys of egg retention on spawning
712 grounds (e.g., prespawn mortality), large-scale tagging studies to estimate *en route* mortality
713 rates, physiological experiments to identify lethal temperature and dissolved oxygen thresholds
714 (e.g., aerobic scope).

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Literature Cited

ADF&G. 2013. Chinook salmon stock assessment and research plan, 2013. *In* Alaska Department of Fish and Game, Special Publication No. 13-01, Anchorage, AK.

Anttila, K., Eliason, E.J., Kaukinen, K.H., Miller, K.M., and Farrell, A.P. 2014. Facing warm temperatures during migration: Cardiac mRNA responses of two adult *Oncorhynchus nerka* populations to warming and swimming challenges. J. Fish Biol. **84**(5): 1439–1456. doi:10.1111/jfb.12367.

Arellano-Aguilar, O., Montoya, R.M., and Garcia, C.M. 2009. Endogenous Functions and Expression of Cytochrome P450 Enzymes in Teleost Fish: A Review. Rev. Fish. Sci. **17**(4): 541–556. doi:10.1080/10641260903243487.

Arkoosh, M.R., Clemons, E., Kagley, A.N., Stafford, C., Glass, A.C., Jacobson, K., Reno, P., Myers, M.S., Casillas, E., Loge, F., Johnson, L.L., and Collier, T.K. 2004. Survey of pathogens in juvenile salmon *Oncorhynchus spp.* migrating through Pacific Northwest estuaries. J. Aquat. Anim. Health **16**(4): 186–196. doi:10.1577/H03-071.1.

AYK-SSI. 2013. Arctic-Yukon-Kuskokwim Chinook Salmon research action plan: Evidence of decline of Chinook Salmon populations and recommendations for future research. *In* Arctic-Yukon-Kuskokwim Sustainable Salmon Initiative, Anchorage, AK.

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., and Iwama, G.K. 2002. Heat shock protein genes and their functional significance in fish. Gene

295: 173–183. doi:10.1016/S0378-1119(02)00687-X.

von Biela, V.R., and Donnelly, D.S. 2020. Water temperature and dissolved oxygen measured during a manipulative thermal challenge experiment for adult salmonids, Yukon River, Alaska, 2018. U.S. Geological Survey data release, https://doi.org/10.5066/P9ECW77M.

von Biela, V.R., McCormick, S.D., Bowen, L., Regish, A., and Waters, S. 2020. Gene transcription and heat shock protein 70 abundance results from migrating adult Chinook salmon, Yukon watershed, 2016-2017. U.S. Geol. Surv. data release, https//doi.org/10.5066/P9Y0IZH2.

Bowen, L., Biela, V.R. von, McCormick, S.D., Waters, S., Durbin-Johnson, B., Britton, M., Settles, M., Donnelly, D.S., Laske, S., Carey, M.P., Brown, R.J., Larson, S., and Zimmerman, C.E. (In Press). Transcriptomic response to elevated water temperatures in adult migrating Yukon River Chinook salmon (*Oncorhynchus tshawytscha*). Conserv. Physiol.

Bowerman, T., Keefer, M.L., and Caudill, C.C. 2016. Pacific salmon prespawn mortality: patterns, methods, and study design considerations. Fisheries **41**(12): 738–749. doi:10.1080/03632415.2016.1245993.

Brabets, T.P., and Walvoord, M. a. 2009. Trends in streamflow in the Yukon River Basin from 1944 to 2005 and the influence of the Pacific Decadal Oscillation. J. Hydrol. **371**(1–4): 108–119. Elsevier B.V. doi:10.1016/j.jhydrol.2009.03.018.

Brabets, T.P., Wang, B., and Meade, R.H. 2000. Environmental and hydrologic overview of the Yukon River basin, Alaska and Canada. *In* U.S. Geological Survey, Water Resources Investigations Report 99-4204.

768 Brown, R.J., von Finster, A., Henszey, R.J., and Eiler, J.H. 2017. Catalog of Chinook Salmon
769 spawning areas in Yukon River basin in Canada and United States. J. Fish Wildl. Manag.
770 **8**(2): 558–587. doi:10.3996/052017-JFWM-045.

771 Buckley, B.A., Gracey, A.Y., and Somero, G.N. 2006. The cellular response to heat stress in the
772 goby Gillichthys mirabilis: a cDNA microarray and protein-level analysis. J. Exp. Biol. **209**:
773 2660–2677. doi:10.1242/jeb.02292.

774 Bustin, S.A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-
775 PCR): Trends and problems. J. Mol. Endocrinol. **29**(1): 23–39. doi:10.1677/jme.0.0290023.

776 Carlson, J.G., and Edwards, O.N. 2017. Abundance and run timing of adult Pacific salmon in the
777 East Fork Andresfsky River, Yukon Delta National Wildlife Refuge, Alaska, 2016. *In* U.S.
778 Fish and Wildlife Service, Alaska Fisheries Data Series 2017-5.

779 Chadwick, J.G., and McCormick, S.D. 2017. Upper thermal limits of growth in brook trout and
780 their relationship to stress physiology. J. Exp. Biol. **220**(21): 3976–3987.
781 doi:10.1242/jeb.161224.

782 Conitz, J.M. 2018. Abundance and run timing of adult Pacific slamon in the East Fork
783 Andreafsky River, Yukon Delta National Wildlife Refuge, Alaska, 2017. Alaska Fish. Data
784 Ser. **2018–8**: 22.

785 Copeland, D.L., Duff, R.J., Liu, Q., Prokop, J., and Londraville, R.L. 2011. Leptin in teleost
786 fishes: an argument for comparative study. Front. Physiol. **2**(June): Article 26.
787 doi:10.3389/fphys.2011.00026.

788 Crossin, G.T., Hinch, S.G., Cooke, S.J., Welch, D.W., Patterson, D.A., Jones, S.R.M., Lotto,

A.G., Leggatt, R.A., Mathes, M.T., Shrimpton, J.M., Van Der Kraak, G., and Farrell, A.P. 2008. Exposure to high temperature influences the behaviour, physiology, and survival of sockeye salmon during spawning migration. Can. J. Zool. **86**: 127–140. doi:10.1139/Z07-122.

Crozier, L.G., Hendry, A.P., Lawson, P.W., Quinn, T.P., Mantua, N.J., Battin, J., Shaw, R.G., and Huey, R.B. 2008. Potential responses to climate change in organisms with complex life histories: evolution and plasticity in Pacific salmon. Evol. Appl. **1**: 252–270. doi:10.1111/j.1752-4571.2008.00033.x.

Currie, S., LeBlanc, S., Watters, M.A., and Gilmour, K.M. 2010. Agonistic encounters and cellular angst: Social interactions induce heat shock proteins in juvenile salmonid fish. Proc. R. Soc. B Biol. Sci. **277**(1683): 905–913. doi:10.1098/rspb.2009.1562.

Deane, E.E., and Woo, N.Y.S. 2011. Advances and perspectives on the regulation and expression of piscine heat shock proteins. Rev. Fish Biol. Fish. **21**(2): 153–185. doi:10.1007/s11160-010-9164-8.

Donnelly, D.S., Von Biela, V.R., McCormick, S.D., Laske, S.M., Carey, M.P., Waters, S., Bowen, L., Brown, R.J., Larson, S., and Zimmerman, C.E. (In Press). A manipulative thermal challenge protocol for adult salmonids in remote field settings. Conserv. Physiol.

Eiler, J.H., Evans, A.N., and Schreck, C.B. 2015. Migratory patterns of wild Chinook salmon *Oncorhynchus tshawytscha* returning to a large, free-flowing river basin. PLoS One **10**(4): 1–33. doi:10.1371/journal.pone.0123127.

Eiler, J.H., Masuda, M.M., Spencer, T.R., Driscoll, R.J., and Schreck, C.B. 2014. Distribution, Stock Composition and Timing, and Tagging Response of Wild Chinook Salmon Returning

to a Large, Free-Flowing River Basin. Trans. Am. Fish. Soc. **143**(6): 1476–1507. doi:10.1080/00028487.2014.959997.

Eliason, E.J., Clark, T.D., Hague, M.J., Hanson, L.M., Gallagher, Z.S., Jeffries, K.M., Gale, M.K., Patterson, D.A., Hinch, S.G., and Farrell, A.P. 2011. Differences in thermal tolerance among sockeye salmon populations. Science (80-.). **332**(6025): 109–112. doi:10.1126/science.1199158.

Eliason, E.J., Clark, T.D., Hinch, S.G., and Farrell, A.P. 2013. Cardiorespiratory collapse at high temperature in swimming adult sockeye salmon. Conserv. Physiol. **1**(1): 1–19. doi:10.1093/conphys/cot008.

Erdoğan, O., Ceyhun, S.B., Ekinci, D., and Aksakal, E. 2011. Impact of deltamethrin exposure on mRNA expression levels of metallothionein A, B and cytochrome P450 1A in rainbow trout muscles. Gene **484**(1–2): 13–17. doi:10.1016/j.gene.2011.05.026.

Evans, T.G., Hammill, E., Kaukinen, K., Schulze, A.D., Patterson, D.A., English, K.K., Curtis, J.M.R., and Miller, K.M. 2011. Transcriptomics of environmental acclimatization and survival in wild adult Pacific sockeye salmon (*Oncorhynchus nerka*) during spawning migration. Mol. Ecol. **20**: 4472–4489. doi:10.1111/j.1365-294X.2011.05276.x.

Farrell, A., Hinch, S.G., Cooke, S.J., Patterson, D.A., Crossin, G.T., Lapointe, M., and Mathes, M.T. 2008. Pacific salmon in hot water: applying aerobic scope models and biotelemetry to predict the success of spawning migrations. Physiol. Biochem. Zool. **81**(6): 697–709. doi:10.1086/592057.

Feder, M.E., and Hofmann, G.E. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. **61**: 243–282.

833 Finney, B.P., Gregory-Eaves, I., Sweetman, J., Douglas, M.S.V., and Smol, J.P. 2000. Impacts of
834 climatic change and fishing on Pacific salmon abundance over the past 300 years. Science
835 (80-.). **290**(5492): 795–799. doi:10.1126/science.290.5492.795.

836 Goniea, T.M., Keefer, M.L., Bjornn, T.C., Peery, C.A., Bennett, D.H., and Stuehrenberg, L.C.
837 2006. Behavioral thermoregulation and slowed migration by adult fall Chinook salmon in
838 response to high Columbia river water temperatures. Trans. Am. Fish. Soc. **135**(November
839 2015): 408–419. doi:10.1577/T04-113.1.

840 Hare, B.S.R., Mantua, N.J., and Francis, R.C. 1999. Inverse production regimes : Alaska and
841 west coast Pacific salmon. Fisheries **24**(1): 6–14.

842 Hinch, S.G., Cooke, S.J., Farrell, A.P., Miller, K.M., Lapointe, M., and Patterson, D.A. 2012.
843 Dead fish swimming: A review of research on the early migration and high premature
844 mortality in adult Fraser River sockeye salmon *Oncorhynchus nerka*. J. Fish Biol. **81**: 576–
845 599. doi:10.1111/j.1095-8649.2012.03360.x.

846 Hinch, S.G., and Martins, E. 2011. A review of potential climate change effects on survival of
847 Fraser River sockeye salmon and an analysis of interannual trends in en route loss and pre-
848 spawn mortality. *In* Cohen Commission Technical Report 9. Vancouver, British Columbia,
849 Canada.

850 Iwama, G.K. 2004. Are hsps suitable for indicating stressed states in fish? J. Exp. Biol. **207**(1):
851 15–19. doi:10.1242/jeb.00707.

852 Iwama, G.K., Thomas, P.T., Forsyth, R.B., and Vijayan, M.M. 1998. Heat shock protein
853 expression in fish. Rev. Fish Biol. Fish. **8**: 35–56.

Iwama, G.K., Vijayan, M.M., Forsyth, R.B., and Ackerman, P.A. 1999. Heat shock proteins and physiological stress in fish. Am. Zool. **39**(6): 901–909. doi:10.1093/icb/39.6.901.

Jeffries, K.M., Hinch, S.G., Sierocinski, T., Clark, T.D., Eliason, E.J., Donaldson, M.R., Li, S., Pavlidis, P., and Miller, K.M. 2012. Consequences of high temperatures and premature mortality on the transcriptome and blood physiology of wild adult sockeye salmon (*Oncorhynchus nerka*). Ecol. Evol. **2**(7): 1747–1764. doi:10.1002/ece3.274.

Jeffries, K.M., Hinch, S.G., Sierocinski, T., Pavlidis, P., and Miller, K.M. 2014. Transcriptomic responses to high water temperature in two species of Pacific salmon. Evol. Appl. **7**: 286–300. doi:10.1111/eva.12119.

Jones, L.A., Schoen, E.R., Shaftel, R., Cunningham, C.J., Mauger, S., Rinella, D.J., and St. Saviour, A. 2020. Watershed-scale climate influences productivity of Chinook salmon populations across southcentral Alaska. Glob. Chang. Biol. (August 2019): 1–18. doi:10.1111/gcb.15155.

Keefer, M.L., Peery, C.A., and Heinrich, M.J. 2008. Temperature-mediated en route migration mortality and travel rates of endangered Snake River sockeye salmon. Ecol. Freshw. Fish **17**: 136–145. doi:10.1111/j.1600-0633.2007.00267.x.

Kocan, R., Hershberger, P., Sanders, G., and Winton, J. 2009. Effects of temperature on disease progression and swimming stamina in Ichthyophonus-infected rainbow trout, *Oncorhynchus mykiss* Walbaum. J. Fish Dis. **32**: 835–843. doi:10.1111/j.1365-2761.2009.01059.x.

Kocan, R.M., Hershberger, P.K., and Winton, J. 2004. Ichthyophoniasis: an emerging disease of Chinook salmon (*Oncorhynchus tshawytscha*) in the Yukon River. J. Aquat. Anim. Health **16**: 58–72. doi:10.1577/H03-068.1.

876 Koch, J.C., Records, M.K., Striegl, R., and Walvoord, M.A. 2020. Water Level, temperature, and
877 discharge of headwater streams in the Yukon River basin, Alaska, 2016 and 2017.

878 Kruger, C., and Zimmerman, C.E. 2009. Pacific salmon: ecology and management of Western
879 Alaska's populations. American Fisheries Society, Bethesda, MD.

880 Lapointe, D., Pierron, F., and Couture, P. 2011. Individual and combined effects of heat stress
881 and aqueous or dietary copper exposure in fathead minnows (*Pimephales promelas*). Aquat.
882 Toxicol. **104**(1–2): 80–85. doi:10.1016/j.aquatox.2011.02.022.

883 Lewis, M., Götting, M., Anttila, K., Kanerva, M., Prokkola, J.M., Seppänen, E., Kolari, I., and
884 Nikinmaa, M. 2016. Different relationship between hsp70 mRNA and hsp70 levels in the
885 heat shock response of two salmonids with dissimilar temperature preference. Front.
886 Physiol. **7**(NOV): 1–12. doi:10.3389/fphys.2016.00511.

887 Lindquist, S. 1986. The heat shock response. Annu. Rev. Biochem. **55**: 1151–91. Available from
888 papers2://publication/uuid/F71F0922-523E-40E0-A1A9-2316431D1B66.

889 Lund, S., Lund, M., and Tufts, B. 2003. Red blood cell Hsp 70 mRNA and protein as bio-
890 indicators of temperature stress in the brook trout (Salvelinus fontinalis). Can. J. Fish.
891 Aquat. Sci. **60**(2002): 460–470. doi:10.1139/F03-039.

892 Lund, S.G., Caissie, D., Cunjak, R.A., Vijayan, M.M., and Tufts, B.L. 2002. The effects of
893 environmental heat stress on heat-shock mRNA and protein expression in Miramichi
894 Atlantic salmon (*Salmo salar*) parr. Can. J. Fish. Aquat. Sci. **59**: 1553–1562.
895 doi:10.1139/F02-117.

896 Martins, E.G., Hinch, S.G., Cooke, S.J., and Patterson, D.A. 2012. Climate effects on growth,

phenology, and survival of sockeye salmon (*Oncorhynchus nerka*): A synthesis of the current state of knowledge and future research directions. Rev. Fish Biol. Fish. **22**(4): 887–914. doi:10.1007/s11160-012-9271-9.

Mathes, M.T., Hinch, S.G., Cooke, S.J., Crossin, G.T., Patterson, D.A., Lotto, A.G., and Farrell, A.P. 2010. Effect of water temperature, timing, physiological condition, and lake thermal refugia on migrating adult Weaver Creek sockeye salmon (*Oncorhynchus nerka*). Can. J. Fish. Aquat. Sci. **67**: 70–84. doi:10.1139/F09-158.

McCullough, D.A. 1999. A review and synthesis of effects of alterations to the water temperature regime on freshwater life stages of Salmonids, with special reference to Chinook Salmon. Reg. 10 Water Resour. Assess. Rep. No. 910-R-99-010.

McCullough, D.A., Bartholow, J.M., Jager, H.I., Beschta, R.L., Cheslak, E.F., Deas, M.L., Ebersole, J.L., Foott, J.S., Johnson, S.L., Marine, K.R., Mesa, M.G., Petersen, J.H., Souchon, Y., Tiffan, K.F., and Wurtsbaugh, W.A. 2009. Research in thermal biology: burning questions for coldwater stream fishes. Rev. Fish. Sci. **17**(755239602): 90–115. doi:10.1080/10641260802590152.

Meyers, T., Burton, T., Bentz, C., Ferguson, J., Stewart, D., and Starkey, N. 2019. Diseases of wild and cultured fishes in Alaska. Alaska Department of Fish and Game, Fish Pathology Laboratories.

Miller, K.M., Li, S., Kaukinen, K.H., Ginther, N., Hammill, E., Curtis, J.M.R., Patterson, D.A., Sierocinski, T., Donnison, L., Pavlidis, P., Hinch, S.G., Hruska, K.A., Cooke, S.J., English, K.K., and Farrell, A.P. 2011. Genomic signatures predict migration and spawning failure in wild Canadian salmon. Science (80-.). **331**: 214–217. doi:10.1126/science.1196901.

Miller, K.M., Teffer, A., Tucker, S., Li, S., Schulze, A.D., Trudel, M., Juanes, F., Tabata, A., Kaukinen, K.H., Ginther, N.G., Ming, T.J., Cooke, S.J., Hipfner, J.M., Patterson, D.A., and Hinch, S.G. 2014. Infectious disease, shifting climates, and opportunistic predators: cumulative factors potentially impacting wild salmon declines. Evol. Appl. **7**(7): 812–855. doi:10.1111/eva.12164.

Mueter, F.J., Peterman, R.M., and Pyper, B.J. 2002. Opposite effects of ocean temperature on survival rates of 120 stocks of Pacific salmon (*Oncorhynchus* spp.) in northern and southern areas. **463**: 456–463. doi:10.1139/F02-020.

Nakano, T., Kameda, M., Shoji, Y., Hayashi, S., Yamaguchi, T., and Sato, M. 2014. Effect of severe environmental thermal stress on redox state in salmon. Redox Biol. **2**: 772–776. Elsevier. doi:10.1016/j.redox.2014.05.007.

NAS. 2011. Guide for the care and use of laboratory animals. *In* 8th edition. National Academy of Sciences, Washington, D.C.

NOAA, and NASA. 2020. 2019 was 2nd hottest year on record for Earth say NOAA, NASA. Available from https://www.noaa.gov/news/2019-was-2nd-hottest-year-on-record-for-earth-say-noaa-nasa.

Olsen, J.B., Beacham, T.D., Wetklo, M., Seeb, L.W., Smith, C.T., Flannery, B.G., and Wenburg, J.K. 2010. The influence of hydrology and waterway distance on population structure of Chinook salmon *Oncorhynchus tshawytscha* in a large river. J. Fish Biol. **76**(5): 1128–1148. doi:10.1111/j.1095-8649.2010.02554.x.

Palmisano, A., Winton, J., and Dickhoff, W. 2000. Tissue-Specific Induction of Hsp90 mRNA and Plasma Cortisol Response in Chinook Salmon following Heat Shock, Seawater

941 Challenge, and Handling Challenge. Mar. Biotechnol. **2**(4): 329–338.
942 doi:10.1007/s101260000005.

943 Post, E., Alley, R.B., Christensen, T.R., Macias-fauria, M., Forbes, B.C., Gooseff, M.N., Iler, A.,
944 Kerby, J.T., Laidre, K.L., Mann, M.E., Olofsson, J., Stroeve, J.C., Ulmer, F., Virginia, R.A.,
945 and Wang, M. 2019. The polar regions in a 2°C warmer world. Sci. Adv. **5**: eaaw9883.

946 Quinn, T.P., Eggers, D.M., Clark, J.H., and Rich, H.B. 2007. Density, climate, and the processes
947 of prespawning mortality and egg retention in Pacific salmon (*Oncorhynchus spp.*). Can. J.
948 Fish. Aquat. Sci. **64**: 574–582. doi:10.1139/F07-035.

949 Quintana, F.J., Basso, A.S., Iglesias, A.H., Korn, T., Farez, M.F., Bettelli, E., Caccamo, M.,
950 Oukka, M., and Weiner, H.L. 2008. Control of Treg and TH17 cell differentiation by the
951 aryl hydrocarbon receptor. Nature **453**(7191): 65–71. doi:10.1038/nature06880.

952 Rahman, M.S., and Thomas, P. 2012. Effects of hypoxia exposure on hepatic cytochrome P450
953 1A (CYP1A) expression in Atlantic croaker: Molecular mechanisms of CYP1A down-
954 regulation. PLoS One **7**(7): 1–15. doi:10.1371/journal.pone.0040825.

955 Rand, P.S., Hinch, S.G., Morrison, J., Foreman, M.G.G., MacNutt, M.J., Macdonald, J.S.,
956 Healey, M.C., Farrell, A.P., and Higgs, D.A. 2006. Effects of river discharge, temperature,
957 and future climates on energetics and mortality of adult migrating Fraser River Sockeye
958 Salmon. Trans. Am. Fish. Soc. **135**(3): 655–667. doi:10.1577/t05-023.1.

959 Revenga, C., Murray, S., Abramovitz, J., and A, H. 1998. Watersheds of the world: ecological
960 value and vulnerability. World Resources Institute, Washington, DC.

961 Roberts, A.P., Oris, J.T., and Stubblefield, W.A. 2006. Gene expression in caged juvenile coho

962 salmon (*Oncorhynchys kisutch*) exposed to the waters of Prince William Sound, Alaska.
963 Mar. Pollut. Bull. **52**(11): 1527–1532. doi:10.1016/j.marpolbul.2006.05.016.

964 Robertsen, B. 2018. The role of type I interferons in innate and adaptive immunity against
965 viruses in Atlantic salmon. Dev. Comp. Immunol. **80**: 41–52. Elsevier Ltd.
966 doi:10.1016/j.dci.2017.02.005.

967 Strange, J.S. 2010. Upper thermal limits to migration in adult Chinook Salmon: evidence from
968 the Klamath River Basin. Trans. Am. Fish. Soc. **139**(4): 1091–1108. doi:10.1577/T09-
969 171.1.

970 Tomalty, K.M.H., Meek, M.H., Stephens, M.R., Rincón, G., Fangue, N.A., May, B.P., and
971 Baerwald, M.R. 2015. Transcriptional Response to Acute Thermal Exposure in Juvenile
972 Chinook Salmon Determined by RNAseq. G3 Genes, Genomes, Genet. **5**(7): 1335–1349.
973 doi:10.1534/g3.115.017699.

974 Tort, L. 2011. Stress and immune modulation in fish. Dev. Comp. Immunol.
975 doi:10.1016/j.dci.2011.07.002.

976 Tsan, M.F., and Gao, B. 2004. Heat shock protein and innate immunity. Cell. Mol. Immunol.
977 **1**(4): 274–279.

978 U.S. Geological Survey. 2015. National Hydrography Dataset (ver. USGS National Hydrography
979 Dataset (NHD) Best Resolution for HU4-1909 20151218 HU-4 Subregion FileGDB 10.1
980 (published 20151218)). Available from https://www.usgs.gov/core-science-
981 systems/ngp/national-hydrography/access-national-hydrography-products.

982 Uno, T., Ishizuka, M., and Itakura, T. 2012. Cytochrome P450 (CYP) in fish. Environ. Toxicol.

983 Pharmacol. **34**(1): 1–13. Elsevier B.V. doi:10.1016/j.etap.2012.02.004.

984 Valenzuela, C.A., Zuloaga, R., Poblete-Morales, M., Vera-Tobar, T., Mercado, L., Avendaño-
985 Herrera, R., Valdés, J.A., and Molina, A. 2017. Fish skeletal muscle tissue is an important
986 focus of immune reactions during pathogen infection. Dev. Comp. Immunol. **73**: 1–9.
987 doi:10.1016/j.dci.2017.03.004.

988 Veldhoen, N., Ikonomou, M.G., Dubetz, C., MacPherson, N., Sampson, T., Kelly, B.C., and
989 Helbing, C.C. 2010. Gene expression profiling and environmental contaminant assessment
990 of migrating Pacific salmon in the Fraser River watershed of British Columbia. Aquat.
991 Toxicol. **97**(3): 212–225. Elsevier B.V. doi:10.1016/j.aquatox.2009.09.009.

992 Venables, W., and Ripley, B. 2002. Modern Applied Statistics with S. *In* fourth edi. Springer,
993 New York.

994 Verrier, E.R., Langevin, C., Benmansour, A., and Boudinot, P. 2011. Early antiviral response
995 and virus-induced genes in fish. Dev. Comp. Immunol. **35**(12): 1204–1214. Elsevier Ltd.
996 doi:10.1016/j.dci.2011.03.012.

997 Vignesh, K.S., and Deepe, G.S. 2017. Metallothioneins: Emerging modulators in immunity and
998 infection. Int. J. Mol. Sci. **18**(10). doi:10.3390/ijms18102197.

999 Wagner, G.N., Hinch, S.G., Kuchel, L.J., Lotto, A., Jones, S.R.M., Patterson, D.A., Macdonald,
1000 J.S., Van der Kraak, G., Shrimpton, M., English, K.K., Larsson, S., Cooke, S.J., Healey,
1001 M.C., and Farrell, A.P. 2005. Metabolic rates and swimming performance of adult Fraser
1002 River sockeye salmon (*Oncorhynchus nerka*) after a controlled infection with *Parvicapsula*
1003 *minibicornis*. Can. J. Fish. Aquat. Sci. **62**: 2124–2133. doi:10.1139/F05-126.

1004 Walvoord, M.A., and Striegl, R.G. 2007. Increased groundwater to stream discharge from
1005 permafrost thawing in the Yukon River basin : Potential impacts on lateral export of carbon
1006 and nitrogen. **34**. doi:10.1029/2007GL030216.

1007 Wang, T., Holland, J.W., Martin, S.A.M., and Secombes, C.J. 2010. Sequence and expression
1008 analysis of two T helper master transcription factors, T-bet and GATA3, in rainbow trout
1009 Oncorhynchus mykiss and analysis of their expression during bacterial and parasitic
1010 infection. Fish Shellfish Immunol. **29**(5): 705–715. Elsevier Ltd.
1011 doi:10.1016/j.fsi.2010.06.016.

1012 Welch, W.J. 1993. How cells respond to stress. Sci. Am. **268**(5): 56–64.
1013 doi:10.1038/scientificamerican0593-56.

1014 Wessel, P., and Smith, W.H.F. 1996. A global, self-consistent, hierarchical, high-resolution
1015 shoreline database. J. Geophys. Res. **101**(B4): 8741–8743. doi:10.1029/96JB00104.

1016 West, F., and Prince, D. 2019. Genetic stock identification of Pilot Station Chinook salmon,
1017 2018. Alaska Department of Fish and Game, Division of Commerical Fisheries, Regional
1018 Information Report 3A19-09, Anchorage, AK.

1019 Westley, P.A.H. (In Press). Documentation of en route mortality of summer chum salmon in the
1020 Koyukuk River, Alaska and its potential linkage to the heatwave of 2019. Ecol. Evol.

1021 Wheelock, C.E., Eder, K.J., Werner, I., Huang, H., Jones, P.D., Brammell, B.F., Elskus, A.A.,
1022 and Hammock, B.D. 2005. Individual variability in esterase activity and CYP1A levels in
1023 Chinook salmon (*Oncorhynchus tshawytscha*) exposed to esfenvalerate and chlorpyrifos.
1024 Aquat. Toxicol. **74**(2): 172–192. doi:10.1016/j.aquatox.2005.05.009.

1025 Zuray, S. 2010. Rampart rapids full season video monitoring 2010. *In* Annual Report to the
1026 Yukon River Panel, Anchorage, Alaska.

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1029 Table 1. Genes examined with their description, associated pathway, and references. Associated
1030 pathways indicate the function each gene is most associated with and is not comprehensive of all
1031 known roles.

Full name	Short Name	Function	Reference
Heat shock protein 27	*HSP27*	Heat stress chaperone	Basu et al. 2002
Heat shock protein 70	*HSP70*	Heat stress chaperone	Iwama et al. 1999, Tsan and Gao 2004
Heat shock protein 90	*HSP90*	Heat stress chaperone	Iwama et al. 1999, Tsan and Gao 2004
Leptin	*Leptin*	Metabolism: Lipid liberation and storage	Copeland et al. 2011
Th2-specific transcription factor	*Gata3*	Immune system: Initiates Th2 response in the presence of parasites	Wang et al. 2010
Th1-specific interferon alpha, type I	*IFNa*	Immune: Cytokines for viruses; Th1	Robertsen 2018)
Orthomyxovirus resistance gene 1	*Mx1*	Immune: Antiviral activity	Verrier et al. 2011
Th1-specific Tbox transcription factor	*tbx21*	Immune system: Initiates Th1 response in the presence of intracellular pathogens	Wang et al. 2010
Aryl hydrocarbon receptor	*AHR*	Immune: Inflammation and Th differentiation Detox: Oxidative metabolism regulator	Quintana et al. 2008, Veldhoen et al. 2010
Hydrocarbon-inducible cytochrome P4501A	*CYP1A*	Detox: Oxidative metabolism enzyme	Erdoğan et al. 2011
Cyp1aSuperoxide dismutase	*SOD*	Detox: Anti-oxidant enzymes	Roberts et al. 2006
Metallothionein A	*MT-A*	Detox: Oxidative metabolism enzyme	Erdoğan et al. 2011, Vignesh and Deepe 2017

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1035 Table 2. Chinook salmon quantitative real time polymerase chain reaction primers.

Gene	Primer Name	FP1	Primer Name	RP1 rc	Expected Amplicon (bp)
HSP90	Onts HSP90 F1	atgatcgggcagttcggtg	Onts HSP90 R1rc	agtgtcaactttgacagtgaa	140
HSP70	Onts HSP70 F1	gcaccctctcctccagca	Onts HSP70 R1rc	ggtaccgcggaacaggtca	124
CYP1A	Onts CYP1A F1	agacagtccgccaggctc	Onts CYP1A R1rc	agccttgtcggtgctgaag	115
AHR	Onts AHR F	gctccagatgtggtcaagt	Onts AHR R	gagtttgtccaggcgaga	122
HSP27	Onts HSP27 F	ctgacgctgagaaggtga	Onts HSP27 R	tagggcttggtcttgctg	135
MT-A	Onts MT-A F	atcttgcaactgcggtgg	Onts MT-A R	gacagcagtcgcagcaac	253
SOD	Onts SOD F3	gagacaacaccaacggctgt	Onts SOD R3rc	gctcctgcagtcacgttgc	120
IFNg2	Onts IFNg2 F3	tataagatctccaaggaccag	Onts IFNg2 R2rc	ccagaaccacactcatcaac	100
MX1	Onts MX1 F1	ctgatgtggagaagaaaattcg	Onts MX1 R1rc	gcaggtcgatgagtgtgag	128
GATA3	Onts GATA F2	caagcgacgactgtctgca	Onts GATA R3rc	gaccgcaagcgttacacac	118
TBX21	Onts TBX21 F	agtgaaggaggatggttctgag	Onts TBX21 R	ggtgatgtctgcgttctgatag	111
IFNa	Onts IFNa F	cctgccatgaaacctgagaaga	Onts IFNa R	tttcctgatgagctcccatgc	107
Leptin	Onts Lep F1	cttccatagtggagaccatg	Onts Lep R1rc	ggcagcgtgatatcatccag	131
rpL8	Onts rpL8 F	ttggtaatgttctgcctgtg	Onts rpL8 R	gggttgtgggagatgactg	129
EF1a	Onts EF1a F1	gcgtggtatcaccattgaca	Onts EF1a R2rc	ctgagaggtaccagtgatca	120

Table 3. Sample size (n) and length (mean mideye to fork length ± standard deviation in mm) for field sampled Chinook salmon at sites in the Yukon River and tributaries in 2016 and 2017 along with the dates when sample collections occurred. Sample size reflects the number of individuals for which data were obtained for gene transcription and HSP70 protein analyses.

Location	Year	Sampling Dates	n	Length
Main-stem Yukon River				
Emmonak	2016	June 10–18	45	801 ± 113
	2017	June 8 –21	38	773 ± 80.5
Rapids	2016	July 6–7	39	622 ± 111
	2017	July 5–7	30	681 ± 111
Eagle	2016	July 11– August 2	40	790 ± 79.1
	2017	July 16– August 9	33	789 ± 71.5
Tributaries				
East Fork Andreafsky River	2016	June 30– July 14	46	708 ± 89.0
	2017	June 23– July 5	40	648 ± 126
Gisasa River	2016	July 4–18	38	665 ± 97.6
	2017	July 3–20	39	668 ± 105
Chena River	2016	July 13–18	51	676 ± 81.1
	2017	July 18	38	775 ± 66

Table 4. Mean (± standard deviation) and maximum monthly water temperatures (° C) for the main-stem Yukon River and sampled tributaries in June, July and August of 2016 and 2017. Water temperature data for the East Fork Andreafsky River in June are incomplete and begin on June 15. No data are available in June for the Gisasa River or August for the East Fork Andreafsky or Gisasa Rivers. Water temperature records are only collected at the East Fork Andreafsky and Gisasa River when staff are present at weirs to monitor Pacific salmon escapement. * denotes the warmer water temperature in interannual monthly comparisons at each river ($P < 0.05$).

River	Year	June		July		August	
		Mean	Max	Mean	Max	Mean	Max
Yukon River	2016	15.3±1.4	19.6	18.3±0.9	20.1	16.1±0.4	17.6
	2017	15.3±1.8	18.2	18.7±1.0*	20.3	16.1±1.8	18.7
East Fork Andreafsky River	2016	16.2±2.3*	22.2	17.2±2.3*	23.2		
	2017	14.0±1.5	21.7	15.9±1.8	20.5		
Gisasa River	2016			15.2±2.0	20.5		
	2017			17.1±1.5*	21.2		
Chena River	2016	8.67±1.5	12.2	8.28±1.6	13.1	7.66±0.7	9.3
	2017	9.22±1.7*	13.1	10.5±1.3*	13.6	9.05±1.8*	13.7

Figure captions

Figure 1. Water temperatures recorded by the Alaska Department of Fish and Game during the seasonal peak of temperature in July near Pilot Station, Alaska, 1996 – 2019. Data include all measurements made from both river banks. Averages were not calculated as measurement frequency varies across years from one or no measurements made each day by hand (2005 and prior) to consistent and frequent measurements at four-hour (2007 –2011) or hourly (2012–2019) intervals daily. Dashed lines indicate the two experimental temperatures, 18 and 21 °C, shown in this experiment to induce a low and high level of heat stress, respectively. Data available at https://www.adfg.alaska.gov/CF_R3/external/sites/aykdbms_website/Default.aspx

Figure 2. The mean and standard error of normalized qPCR values for twelve genes in muscle tissue of Chinook salmon from individuals held in three different temperatures: control (blue circle, river ambient, ~ 15 °C), 18 °C (orange square), or 21 °C (red triangle). Because lower normalized gene transcript values represent more transcripts present, the y-axis is displayed in reverse order. A change in normalized value of two is approximately equivalent to a four-fold change in the amount of the transcript.

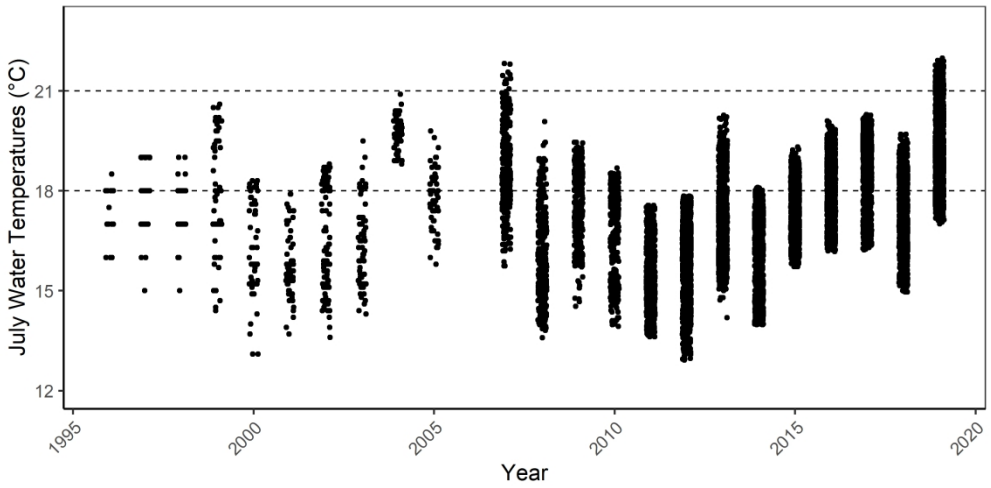
Figure 3. Results from a linear discriminate analysis (A) used to reduce mRNA abundance of six genes (*HSP70, HSP90, Gata3, IFNa, AHR,* and *SOD*) to two linear discriminate axes (LD1 and LD2) and HSP70 protein abundance (B) in muscle tissue of Chinook salmon from individuals held in three different experimental temperatures: control (blue, river ambient, ~ 15 °C), 18 °C (orange), or 21 °C (red). Shapes indicate the model prediction for each individual with either the control (open circles), 18 °C (filled squares), or 21 °C (filled triangles) treatment group. The dashed line in B is the proposed threshold for elevated HSP70 protein that distinguishes heat stress and points in this panel are spread out (i.e., jittered) so that data points are more visible.

Figure 4. The LD1 gene (A) and HSP70 protein abundance (B) plotted against the maximum three day water temperature for Chinook salmon captured at the East Fork Andreafsky River weir during spawning migrations in 2016 and 2017 (n = 86). LD1 gene is calculated based on gene transcript data for each individual fish and gene-specific model coefficients fit based on experiment results ('preferred model'). Symbol color and shape reflects the heat stress classifications based on predictions fit with experiment data. Field-sampled individuals categorized as unstressed were similar to experimental control fish (blue circles) and individuals classified with heat stress were distinguished between heat stress similar to 18 °C experiment group (orange squares, LD1 genes only) or the 21 °C experiment group (red triangles).

Figure 5. Boxplots of LD1 genes (A) and HSP70 protein abundance (B) for spawning Chinook salmon captures across the Yukon River watershed in either 2016 (navy) or 2017 (purple) in relation to results from an experimental temperature manipulation (green). Dashed lines reflect the threshold for identifying heat stress. Heat stress is indicated by values lower than the LD1 threshold and higher than the HSP70 protein threshold. In each boxplot, the horizontal line is the median, the upper and lower ends of the box are the first and third quartiles, and the whiskers extend to the highest and lowest values that are within the 1.5x inter-quartile range. Outliers are not shown.

Figure 6. A map depicting the percent of Chinook salmon in each capture location (black circle) and year with evidence of heat stress. Paired stacked bar charts reflect the heat stress classifications from gene transcript and HSP70 protein for fish captured in summer 2016 (left) and 2017 (right). Fill color reflects the proportion of individuals in each of the four heat stress categories: no evidence of heat stress in either gene transcription or HSP70 protein, green; heat stress identified only in gene transcription, gold; heat stress identified in gene transcription and

elevated HSP70 protein, red; and heat stress identified only by elevated HSP70 protein, purple). Numbers near each bar are the sum across the three categories that identified heat stress presence. The white star is the location of the temperature experiment near Pilot Station, AK, USA. An asterisk (*) prior to the capture location name denotes a significant difference in the heat stress proportion between capture years. Map created in ArcMap 10.7 (ESRI, Redlands, CA, USA) with selected rivers from the National Hydrography Dataset (U.S. Geological Survey 2015) and shorelines from Wessel and Smith (1996).



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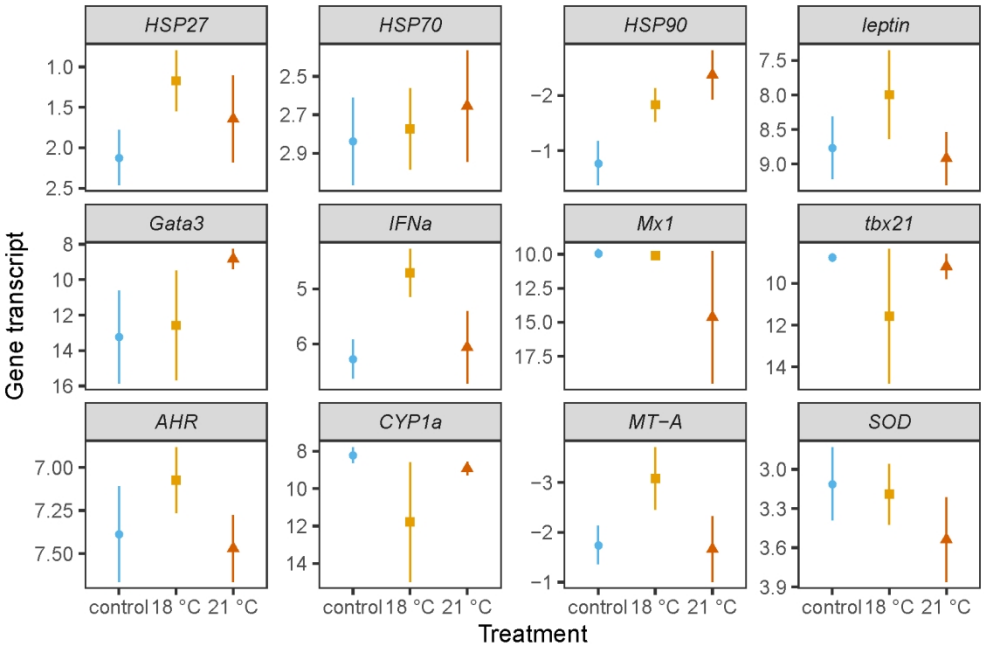

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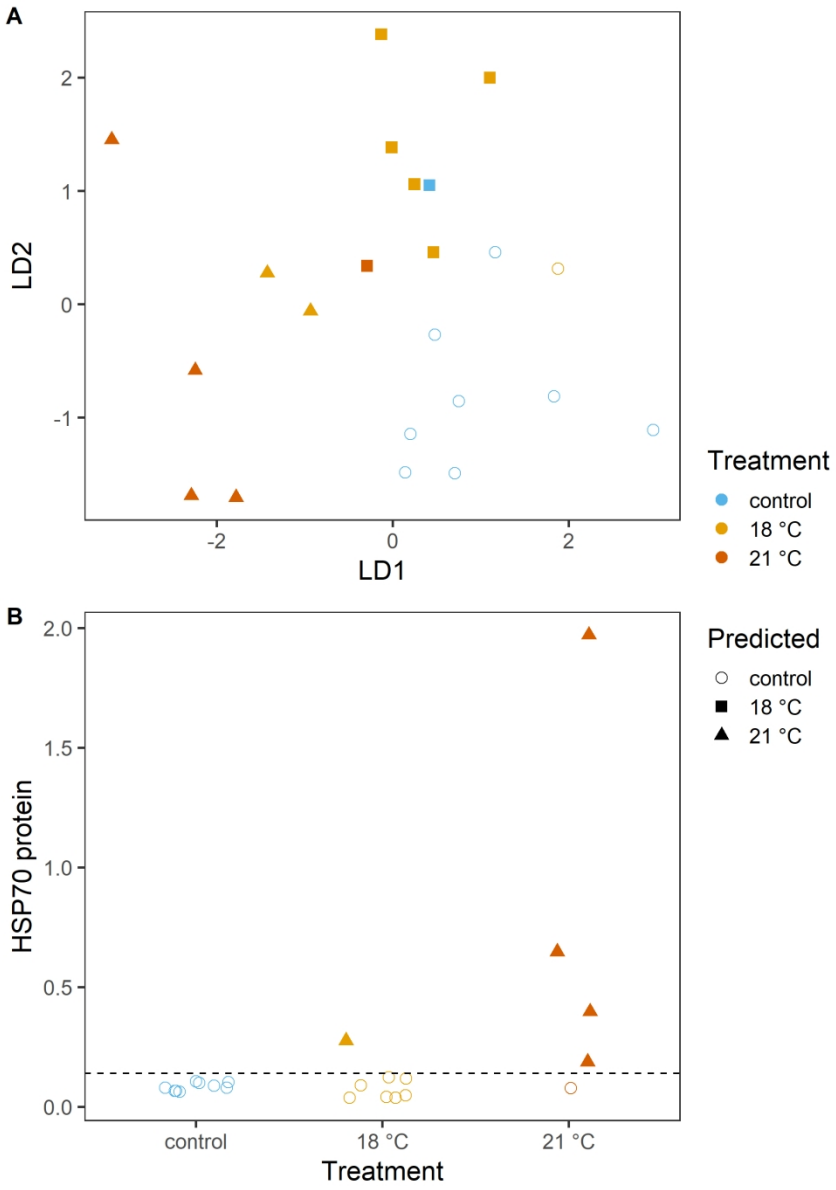

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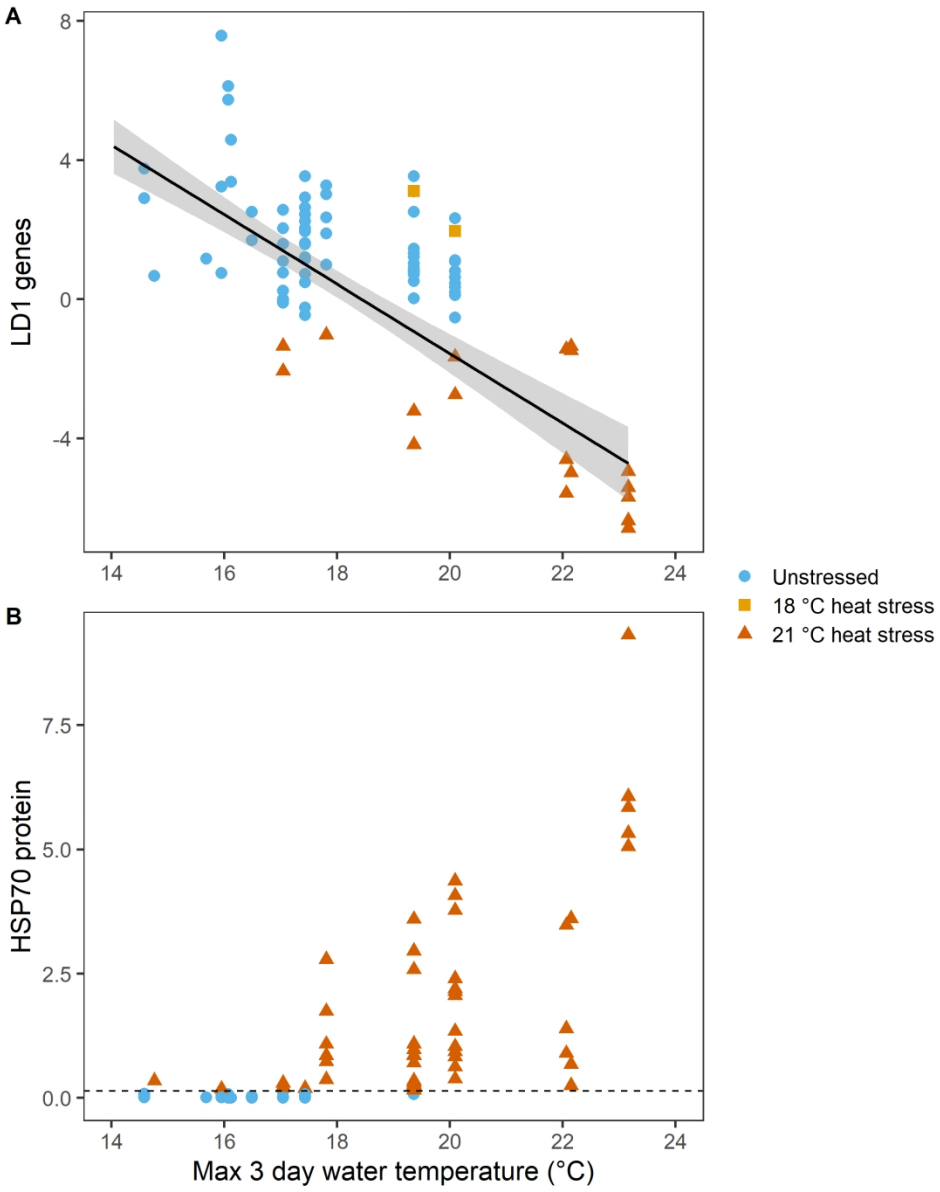

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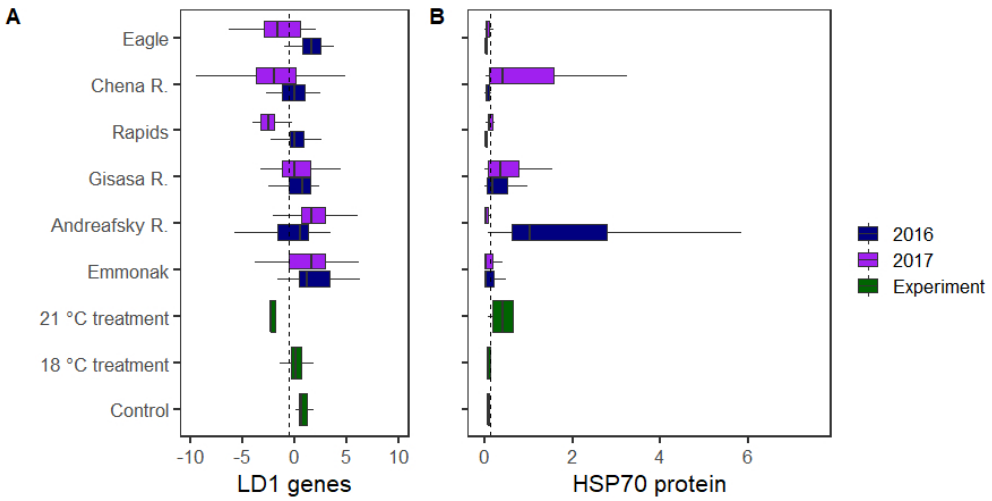

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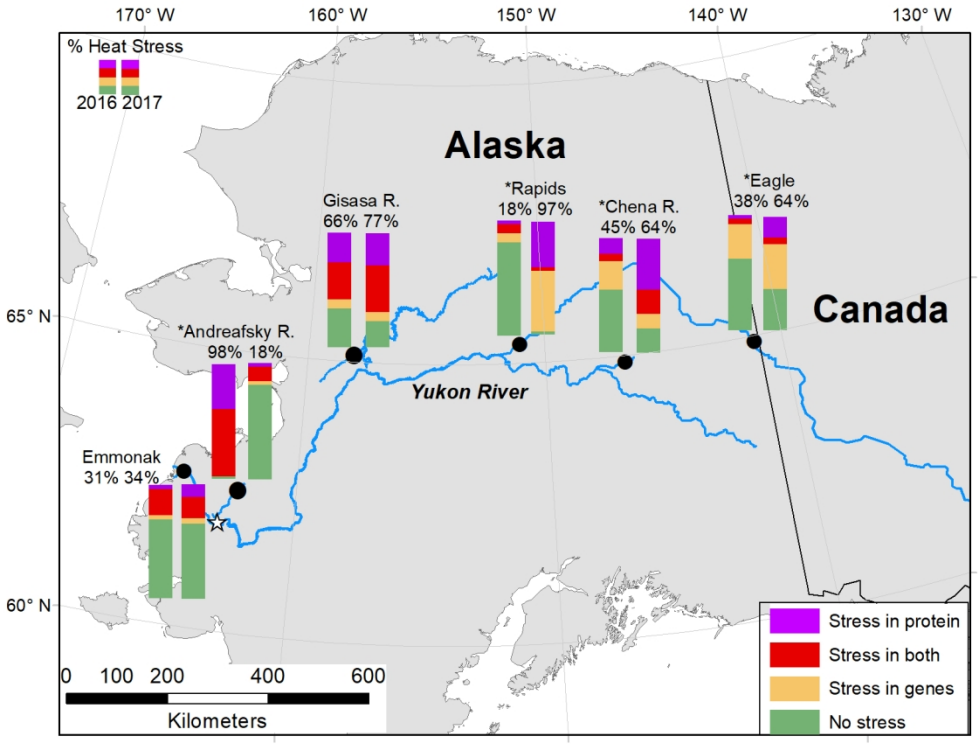

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279x215mm (150 x 150 DPI)