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Facing warm temperatures during migration: cardiac mRNA responses of two adult *Oncorhynchus nerka* populations to warming and swimming challenges

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The main findings of the current study were that exposing adult sockeye salmon *Onchorhynchus nerka* to a warm temperature that they regularly encounter during their river migration induced a heat shock response at an mRNA level, and this response was exacerbated with forced swimming. Similar to the heat shock response, increased immune defence-related responses were also observed after warm temperature treatment and with a swimming challenge in two different populations (Chilko and Nechako), but with some important differences. Microarray analyses revealed that 347 genes were differentially expressed between the cold (12–13° C) and warm (18–19° C) treated fish, with stress response (GO:0006950) and response to fungus (GO:0009620) elevated with warm treatment, while expression for genes involved in oxidative phosphorylation (GO:0006119) and electron transport chain (GO:0022900) elevated for cold-treated fish. Analysis of single genes with real-time quantitative PCR revealed that temperature had the most significant effect on mRNA expression levels, with swimming and population having secondary influences. Warm temperature treatment for the Chilko population induced expression of heat shock protein (*hsp*) *90α*, *hsp90β* and *hsp30* as well as *interferon-inducible protein*. The Nechako population, which is known to have a narrower thermal tolerance window than the Chilko population, showed even more pronounced stress responses to the warm treatment and there was significant interaction between population and temperature treatment for *hsp90β* expression. Moreover, significant interactions were noted between temperature treatment and swimming challenge for *hsp90α* and *hsp30*, and while swimming challenge alone increased expression of these *hsp*s, the expression levels were significantly elevated in warm-treated fish swum to exhaustion. In conclusion, it seems that adult *O. nerka* currently encounter conditions that induce several cellular defence mechanisms during their once-in-the-lifetime migration. As river temperatures continue to increase, it remains to be seen whether or not these cellular defences provide sufficient protection for all *O. nerka* populations.

Key words: climate change; heat shock protein; *hsp*; immune defence; microarray; temperature tolerance.

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INTRODUCTION

More than 100 genetically different sockeye salmon *Oncorhynchus nerka* (Walbaum 1792) populations in the Fraser River catchment, British Columbia, Canada (Beacham *et al.*, 2005), encounter relatively warm but highly variable river environments during their once-in-a-lifetime upstream migration to spawn. Among these populations, river migration distance varies by >10-fold (from <100 to >1000 km), while the elevation gained during the river migration varies by >100-fold (from 10 to 1200 m; Burgner, 1991; Crossin *et al.*, 2004). Over the course of migration, water temperature can vary considerably and abruptly. It sometimes reaches near critical temperatures of >21° C in the mainstream river, while water temperature could be 10° C or lower in certain spawning areas (Donaldson *et al.*, 2009; Hinch & Martins, 2011). With limited opportunity to behaviourally regulate their body temperature during the migration, due to the limited number of temperature refuges in the mainstream Fraser River (Donaldson *et al.*, 2009), such temperature changes are probably very challenging. Indeed, *en route* and pre-spawn mortality of *O. nerka* have been repeatedly associated with high river temperatures (Hinch & Martins, 2011), raising conservation concerns. Thus, because *O. nerka* are a key species that has great economic and cultural importance to British Columbia (Hinch & Martins, 2011), it is crucial to understand the range of temperature tolerances and the capacity of future populations to meet the challenges of climate change.

A current and unifying hypothesis to mechanistically explain temperature tolerance in fishes is the oxygen and capacity-limited thermal tolerance hypothesis (Pörtner & Farrell, 2008; Pörtner, 2010), which builds from and adds to the well-established fact that the maximal available energy for aerobic activity in fishes is defined by the optimum temperature (T_{opt}) for aerobic scope, which is the maximal difference between routine and maximum oxygen consumption rate (Fry, 1947). While routine metabolic rate typically increases exponentially with temperature in fishes, maximum metabolic rate reaches a plateau near the T_{opt} (Fry, 1947). Thus, aerobic scope decreases at supra-optimal temperatures due to a purported mismatch between an increasing tissue demand for oxygen and the capacity to supply oxygen to tissues (Pörtner & Knust, 2007). The heart plays a significant role in this mismatch because cardiac output and hence arterial oxygen supply decreases at supra-optimal temperatures for aerobic scope in association with the maximum heart rate being reached (Steinhausen *et al.*, 2008; Sandblom *et al.*, 2009; Clark *et al.*, 2011; Eliason *et al.*, 2011, 2013*a*; Gamperl *et al.*, 2011). Understandably, recent studies on salmonids, including *O. nerka*, have set maximum heart rate and other aspects of cardiac design as key distinguishing features in temperature tolerance (Steinhausen *et al.*, 2008; Clark *et al.*, 2011; Eliason *et al.*, 2011; Casselman *et al.*, 2012; Anttila *et al.*, 2013; Eliason *et al.*, 2013*a*). Thus, given the emerging role of the heart in determining temperature tolerance of athletic fishes, this study examined the cardiac transcriptome to elucidate cardiac mRNA responses to temperature and swimming challenges.

The expression of mRNA represents a primary response to environmental change that often, but not always (Korajoki & Vornanen, 2012, 2013), leads to changes in protein expression and cell function. Here, a microarray was used to detect global changes in gene expression, and real-time quantitative PCR (RT-qPCR) of selected genes more rigorously quantified these changes. While many studies have examined piscine transcriptome responses to temperature acclimation and acute temperature stress

(Podrabsky & Somero, 2004; Buckley *et al.*, 2006; Healy *et al.*, 2010; Logan & Somero, 2010; Logan & Somero, 2011; Quinn *et al.*, 2011; Jeffries *et al.*, 2012; Long *et al.*, 2012), very few have focussed on cardiac tissues (Gracey *et al.*, 2004; Vornanen *et al.*, 2005) despite the emerging importance of the heart in temperature responses and tolerance. Also missing from previous studies is a consideration of the combined stress effects of exercise and warm temperature, which are challenges that adult *O. nerka* encounter in nature while migrating upstream at or above their aerobic limits (Hinch & Bratty, 2000). Therefore, the current study examined the combined effect of temperature and swimming challenges, as well as the effect of a temperature challenge alone, on cardiac mRNA responses. In addition, this study posed the question: Does the cardiac cellular responses vary intraspecifically between populations having different thermal tolerances? Thermal tolerances were based on Eliason *et al.* (2011), which showed strikingly different T_{opt} windows (12·9–20·7° C *v.* 14·5–19·0° C) and survival (100% *v.* 19% survival after a 4 day exposure to 21° C) for Chilko and Nechako populations, respectively.

MATERIALS AND METHODS

TISSUE SAMPLING

All the procedures were approved by the University of British Columbia's Animal Care Committee, in accordance with the Canadian Council on Animal Care (A06-0328 and A08-0388). Cardiac tissues were sampled in 2008 and 2009 as part of a previously reported study (Eliason *et al.*, 2011) in which full details of fish collection, fish identification, handling and testing are provided. Briefly, wild upstream migrating adult *O. nerka* were collected with beach seines from the lower Fraser River and transported to the Department of Fisheries and Oceans Cultus Lake Salmon Research Laboratory (Cultus Lake, BC, Canada) where experiments were conducted. The fish were collected within 1–3 days of entering the Fraser River and prior to encountering certainly the most demanding portion of the upriver conditions. Fish were given a passive integrated transponder (PIT) tag, a scale was removed and adipose fin was clipped for population identification by DNA analysis (Beacham *et al.*, 2005). In 2008, *O. nerka* from Chilko population were used, while in 2009, both Chilko and Nechako populations were used.

Swimming experiments were performed in 2008 and 2009 using only the Chilko population at either mean ± S.D. 12·3 ± 0·2° C or 18·0 ± 0·6° C, temperatures close to the historic minimum and maximum for the Fraser River migration. Fish were held for 1–4 weeks at 12° C in outdoor circular aquaria (8000–12 000 l) supplied with fresh water under seasonal photoperiod (49° N). The cold swimming group ($n = 6$) was maintained at the ambient water temperature. The warm swimming group ($n = 6$) was placed in 1400 l circular aquaria 3 days before the swimming test and water temperature was increased by ≤ 5° C per day to the test temperature. Fish were instrumented with a Transonic flowprobe (Transonic Systems; www.transonic.com) around the ventral aorta and catheters were placed in the dorsal aorta and the sinus venosus. The results of these instrumentations are presented elsewhere (Eliason *et al.*, 2011, 2013*a*, *b*). Fish recovered overnight at their test temperature in a swim tunnel respirometer and cardiorespiratory variables were measured before, during and after a critical swimming speed (U_{crit}) test (Jain *et al.*, 1997; Lee *et al.,* 2003*a*). A 45–60 min recovery period preceded a repeated U_{crit} test to the same level of performance (Eliason *et al.*, 2013*b*). Following recovery from the second U_{crit} swim test (>1 h), the fish were euthanized by cervical dislocation. The ventricle was quickly removed, halved and freeze-clamped in liquid nitrogen. The ventricle samples were stored at −80° C before analysis. Thus, the swimming groups were considered representative of post-exhaustive exercise. The tissue samples were used both for microarray ($n = 4$ for each temperature group) and for RT-qPCR analyses ($n = 6$ for each temperature group).

In 2009, *O. nerka* from Chilko and Nechako populations were transferred to 1400 l circular aquaria at an ambient temperature of 13° C. Cold and warm resting groups were established by

either keeping fish at the ambient 13° C temperature ($n = 6$ in both populations) or warming the aquarium to 19° C over a period of 24 h ($n = 6$ in both populations). Thus, the warming differed slightly between years being ≤ 5° C per day in 2008 and 6° C per day in 2009. In 2009, fish were maintained at the treatment temperatures for 4 days before they were quickly netted and euthanized by cervical dislocation before freeze-clamping and storing the ventricle as described above. This group of fish was considered representative of resting fish for comparative purposes and was used only in RT-qPCR determinations.

TOTAL RNA ISOLATION AND LABELLING FOR MICROARRAY

For the 2008 swimming experiments with Chilko *O. nerka* ($n = 4$ for each temperature group), the microarray procedures on ventricle samples were performed as described in Miller *et al.* (2011). Briefly, a small piece of ventricle (*c.* 10 mg) was cut from the frozen ventricle under liquid nitrogen and homogenized with stainless steel beads in TRI reagent (Ambion Inc.; www.lifetechnologies.com) on a M301 mixer mill (Retsch Inc.; www.retsch.com). Total RNA was purified from 100 µl aliquots of homogenates using Magmax-96 for Microarray Kits (Ambion) with a Biomek NXP (Beckman-Coulter; www.beckmancoulter.com) and eluted with RNAase-DNAase-free water. Purity was verified by measuring A_{260}:A_{280} of eluate and RNA concentration calculated with A_{260} values. Eluates were stored at −80° C until used for cDNA synthesis.

The total RNA (500–5 µg) was amplified using MessageAmpII-96 kit (Ambion). An Invitrogen indirect labelling kit (www.lifetechnologies.com), with modifications, was used to reverse transcribe amplified RNA (5 µg) to cDNA and label with Alexa dye. The cDNA was purified using Zymo-25 clean-up columns (Zymo Research; www.zymoreserach.com) and eluted using the 2× coupling buffer (Invitrogen). Samples were processed by resuspending the Alexa dye with dimethyl sulphoxide (DMSO) and then adding the cDNA before incubating for 1 h at room temperature. A reference control for the microarray study contained cDNA from the ventricle samples of all the fish and was hybridized onto each microarray slide as a control. The samples were labelled with Alexa 555 and the reference control with Alexa 647 (Abcam; www.abcam.com). One hundred and fifty microlitres of DNA binding buffer (Zymo) were added to each Alexa tube in order to clean the samples and reference. Sample and references were combined into Zymo-25 clean-up columns and the unbound portion of the labelled cDNA was removed by centrifugation at 12 000 *g*. The labelled cDNA was washed three times with DNA wash buffer (Zymo) and eluted in 9 µl of 1× Tris-EDTA buffer. Two micro litres of poly dA were added to the targets and denatured for 10 min at 80° C. A volume of 125 µl of pre-warmed SlideHybe3 buffer (Ambion) was added before loading into the hybridization chamber in Tecan-HS4800 pro-hybridization station (TecanTrading AG, www.tecan.com).

MICROARRAY

The 16 006 feature microarray produced by Genomics Research on Atlantic Salmon Project (GRASP; www.uvic.ca/cbr/grasp) was used in this study. The array was prepared for hybridization by washing once (30 s) in 0·014% sodium dodecyl sulphate (SDS) and twice (30 s) in sterile distilled water (sdH_2O) at 23° C and then denatured for 3 min at 85° C with agitation followed with 1 min wash at 5× sodium citrate sodium chloride (SSC) 0·01% SDS and 0·2% bovine serum albumin (BSA) at 43° C. Slides were prehybridized for 1 h at 43° C with medium agitation, washed twice (90 s) in sdH_2O and once (60 s) in 8× SSC, 0·1% SDS and 4× Denhardt's (Sigma-Aldrich; www.sigma-aldrich.com) at 43° C. Samples were injected at 43° C with agitation and hybridized for 16 h at 43° C in high-viscosity mode with low-frequency agitation. After hybridization, slides were washed twice (30 s) in 2× SSC, 0·01% SDS at 43° C and twice (30 s) in 0·2× SSC at 23° C. Slides were dried at 23° C for 2·5 min.

The fluorescent image was acquired by scanning the slide using a Perkin Elmer ScanArray Express (www.perkinelmer.com), adjusting the photomultiplier gain for optimized visualization. Imagene (BioDiscovery, www.biodiscovery.com) was used to quantify the image, and spots (features) with poor quality or no signal (<2 S.D. from background) at both wavelengths were marked. In order to remove intensity-dependent dye bias (Yang *et al.*, 2002), raw microarray intensity data were normalized in GeneSight 4.1, (BioDiscovery; www.biodiscovery.com) using

the local intensity-dependent Locally Estimated Scatterplot Smoothing (LOESS) normalization. Marked spots were replaced by the experimental means for each gene passing quality assessment (14 835 genes). The data were $\log_2$ transformed and an intensity ratio was computed by calculating the differences in $\log_2$-transformed intensities between the sample and the reference control. These $\log_2$-transformed intensity ratios were used in further analyses. Microarray data were expressed in terms of mean normalized (background corrected) $\log_2$ ratios between each fish and the reference control. The microarray data were deposited within Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE50054 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE50054).

REAL-TIME QUANTITATIVE PCR

Six genes of interest were selected from the microarray results (from cold and warm-treated swimming Chilko *O. nerka*) to be investigated with RT-qPCR protocol. Small ventricle samples (*c*. 20 mg) from all the fish (both swimming and resting, both populations and both treatment temperatures) were cut under liquid nitrogen. Total RNA was extracted using the guanidine isothiocyanate method (Chomczynski & Sacchi, 1987) with sample homogenization in 700 µl of TRIzol Reagent (Ambion). After isolation, total RNA was purified with DNA-free Kit (Ambion). The success and purity of isolation was confirmed with electrophoresis and spectrophotometry. A mass of 1 µg of RNA was reverse transcribed to cDNA with iScript cDNA Synthesis Kit (Bio-Rad; www.bio-rad.com) according to the manufacturer's instructions. Gene expression was obtained with RT-qPCR with an ABI Prism 7000 sequence analysis system (Applied Biosystems; www.lifetechnologies.com). The primers for heat shock protein (*hsp*) *90β* and *hsp30* were obtained from Ojimaa *et al.* (2005) and Tymchuk *et al.* (2009). The primer pairs for *hsp90α*, interferon-inducible protein and for 60S ribosomal protein (*rp*)*l27* were designed from Gene bank EST sequences (CB493960 and CB503707; CB500466.1; CA037570) and the primers for cytochrome c oxidase (*cox*) subunit III and NADH dehydrogenase (*nadh-d*) subunit 1 from *O. nerka* mitochondrion complete genome sequence (accession number EF055889.1) using Primer Express 2.0.0 (Applied Biosystems). The primer sequences and RT-qPCR reaction parameters are reported in Table I. RT-qPCR was performed for duplicate 21 µl reaction mixtures containing 1 µl of cDNA, 0·2 µM of each forward and reverse primer and 10 µl SYBR Green Master Mix (Applied Biosystems). Following heating, cooling regime for reactions was 1 cycle of 50° C for 2 min, 1 cycle of 94° C for 10 min, 40 cycles of 95° C for 15 s and 60° C for 1 min. PCR products were subjected to melt down curve analysis after reactions in order to validate presence of single amplicon. A mixture of cDNA from all the samples was used for generating a standard curve to relate cDNA amount to threshold cycle number for all primer sets to assess assay efficiency. Threshold cycle (C_T) values for individual samples were normalized to housekeeping gene *60S rpl27* that had highly similar expression levels in all the samples in microarray analyses (across multiple studies). All data are presented as gene C_T value divided by housekeeping gene C_T value using delta-delta C_T formula.

STATISTICAL ANALYSIS

Comparison of gene expression from the microarray study between cold and warm-treated swimming Chilko *O. nerka* was performed with a Mann–Whitney *U*-test. Given the small sample sizes available for analysis, the goal for the microarray study was to identify the most highly differentially expressed genes that would then be validated on more samples via RT-qPCR; hence, a *P*-value cut-off of 0·01 was applied. The database for annotation, visualization and integrated discovery programme, DAVID (6.7; http://david.abcc.ncifcrf.gov/) was used to discover enriched functional-related gene groups within significantly differentially regulated genes between cold- and warm-treated swimming Chilko *O. nerka*.

The RT-qPCR data were compared with a multi-way ANOVA using temperature, population and swimming test as factors, followed by a Tukey's *post hoc* test. The *hsp90α*, *hsp30* and interferon-inducible protein (*iip*) expression data were $\log_{10}$ transformed in order to meet the assumptions of multi-way ANOVA. The data were normally distributed and groups had equal variances. The d.f. was 1 for the factors temperature, population and swimming test as

TABLE I. Primers used in quantitative real-time PCR

Gene	Forward primer	Reverse primer	Standard curve slope	Standard curve r^2
60S ribosomal protein (*rp)l27*	GGACCTCTTGGCAACCTTCTT	CGCTACCCCCCGCAAAGT	3·12	0·993
Heat-shock protein (*hsp)90α*	TGGGCTACATGGCTGCCAAG	TCCAAGGTGAACCCAGAGGAC	3·68	0·991
hsp90β	AATGGGTAACCTGGTCAGTG	CTGAATACAGACAGGTCTGA	3·70	0·986
hsp30	TGGCAGGTAAGCAAGTGATAGGA	TCCAGGCTCCAAAAGTGGTT	3·31	0·996
Interferon-inducible protein (*iip*)	GAGGCTGCAAAGTGACCAGAT	CAGCCACAGCCCGTTGTAC	3·37	0·997
NADH dehydrogenase I (*nadh-dI*)	CCGAAAAGGGCCCAACA	TCCGCGATGGGTTGTAGTAGT	3·78	0·992
Cytochrome oxidase (*cox*) III	TCGGCCACACCTTCACAA	CTCAGGTTTAGCTTTTTTCAGTGCTT	3·33	0·990

well as for the interactions between temperature and population and temperature and swimming test. When significant interactions were observed between factors, the data were split and the effects of single factors were tested separately with one-way ANOVA. The comparison of swimming capacity between cold and warm-treated Chilko *O. nerka* was analysed with an independent samples *t*-test. The analyses were performed for the first U_{crit} value, second U_{crit} value and average U_{crit} value from both swimming tests. Values are presented as mean ± S.E. unless stated otherwise. Statistical significance for ANOVA, *post hoc* and *t*-test analyses was set at $\alpha = 0{\cdot}05$. The statistical comparisons were performed with SigmaPlot 12.5 (Systat Software, Inc.; www.sigmaplot.com).

RESULTS

SWIMMING CAPACITY

These fish were part of a much larger study where U_{crit} values are reported for the larger group of *O. nerka* (Eliason *et al.*, 2013*b*). Here, Chilko *O. nerka* swam equally well at the two test temperatures. For the first swim test, U_{crit} was $2{\cdot}1 \pm 0{\cdot}1$ and $2{\cdot}0 \pm 0{\cdot}1$ body lengths (L_B) s^{-1} for cold-treated and warm-treated fish (*t*-test, $P > 0{\cdot}05$) and $1{\cdot}9 \pm 0{\cdot}1$ and $2{\cdot}1 \pm 0{\cdot}1$ L_B s^{-1} (*t*-test, $P > 0{\cdot}05$) for second swim test. These results are consistent with a T_{opt} window of 12·9–20·7° C, which is reported for the Chilko population (Eliason *et al.*, 2011).

MICROARRAY

Microarray analysis on Chilko *O. nerka* sampled after exhaustive exercise revealed 347 genes with a differential expression between the cold and warm-treated groups (*U*-test, $P < 0{\cdot}01$). The 10 genes with the greatest difference in expression levels are presented in Table II and the rest of the genes are in Table SI (Supporting Information).

Pathway enrichment analysis performed in DAVID and based on the significant gene list from the *U*-test revealed a significant elevation of genes related to the stress response in the warm group (GO:0006950; $P < 0{\cdot}05$), a cluster that included heat shock proteins. Indeed, among the genes with the greatest difference between temperature groups were *hsp30*, *hsp90α* and *hsp90β* (fold changes are presented in Table II).

In addition to the stress response, DAVID analysis also revealed that the warm group increased their mRNA response to fungus (GO:0009620; $P < 0{\cdot}01$) as well as the expression of antiviral protective proteins, such as *iip*, which was also one of the most significantly different mRNA expression levels between groups as analysed with the *U*-test. Expression of other immune-related genes including precerebellin-like protein (*plp*) and major histocompatibility complex (*mhc*) *class I b region* (II) was also increased after the warm treatment.

On the other hand, the warm treatment down-regulated the expression of 187 genes (Table SI) and among these were genes involved in energy metabolism such as oxidative phosphorylation (GO:0006119; $P < 0{\cdot}01$) and electron transport chain (GO:0022900; $P < 0{\cdot}001$). Within those clusters, nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase (*nadh-uo*) (mitochondrion complex I) and *coxIII* (mitochondrion complex IV) were the most significant ones (Table II).

Given the microarray results, *hsp90α*, *hsp90β*, *hsp30*, *iip*, *coxIII* and *nadh-d* subunit 1 were selected for RT-qPCR analyses.

TABLE II. Genes having the most significantly different expression levels in swimming Chilko *Oncorhynchus nerka* as analysed from the microarray with a *U*-test. Positive values indicate that expression of the gene is higher in the warm-treated group and negative values indicate that the expression is higher in the cold-treated group

Gene	Accession ID	Fold change
Heat shock protein (*hsp*)*30*	CB498291	3·7
hsp90α	CB498021	3·1
Interferon inducible protein (*iip*)	CB500466	2·6
hsp90β	CB493619	2·1
mhc *class I b region*	CA059874	0·8
Parvalbumin, thymic	CB496913	−2·9
NADH-ubiquinone oxidoreductase chain 2	CN442556	−1·0
40S ribosomal protein S27-like protein	CA045933	−1·4
FXYD domain containing ion transport regulator 5	CB491094	−1·6
NADH-ubiquinone oxidoreductase chain 1	CA046928	−2·1

REAL-TIME QUANTITATIVE PCR

When expression levels were compared for temperature, swimming, population and their interacting effects (multi-way ANOVA; see *F*-values of ANOVA in Table III), temperature had the most significant effect on mRNA expression levels, with swimming and population having secondary influences. There were also significant interactions between temperature and swimming treatments and temperature and population for some of the measured variables (Table III).

The powerful effect of temperature was revealed for the *hsp* group. Compared with cold-treated *O. nerka*, warm-treated *O. nerka* had significantly elevated *hsp90α*, *hsp90β* and *hsp30* expression for both populations at rest and with

TABLE III. The *F* and *P* values for the effect of temperature treatment, swimming test, population and their interactions on expression of *hsp90α*, *hsp90β*, *hsp30*, interferon-inducible protein (*iip*), NADH dehydrogenase (*nadh-d*) and *coxIII* in ventricles of *Oncorhynchus nerka*

Gene	Temperature	Swimming test	Population	Temperature: population	Temperature: swimming
hsp90α	$F = 274{\cdot}3$	$F = 36{\cdot}9$	$F = 2{\cdot}6$	$F = 0{\cdot}09$	$F = 6{\cdot}0$
	$P < 0{\cdot}001$	$P < 0{\cdot}001$	$P > 0{\cdot}05$	$P > 0{\cdot}05$	$P < 0{\cdot}05$
hsp90β	$F = 18{\cdot}1$	$F = 1{\cdot}0$	$F = 6{\cdot}6$	$F = 3{\cdot}1$	$F = 0{\cdot}1$
	$P < 0{\cdot}001$	$P > 0{\cdot}05$	$P < 0{\cdot}02$	$P > 0{\cdot}05$	$P > 0{\cdot}05$
hsp30	$F = 169{\cdot}9$	$F = 63{\cdot}6$	$F = 2{\cdot}5$	$F = 0{\cdot}03$	$F = 6{\cdot}5$
	$P < 0{\cdot}001$	$P < 0{\cdot}001$	$P > 0{\cdot}05$	$P > 0{\cdot}05$	$P < 0{\cdot}05$
iip	$F = 41{\cdot}0$	$F = 51{\cdot}6$	$F = 7{\cdot}8$	$F = 0{\cdot}2$	$F = 2{\cdot}9$
	$P < 0{\cdot}001$	$P < 0{\cdot}001$	$P < 0{\cdot}01$	$P > 0{\cdot}05$	$P > 0{\cdot}05$
nadh-d	$F = 70{\cdot}6$	$F = 1{\cdot}1$	$F = 35{\cdot}1$	$F = 4{\cdot}9$	$F = 0{\cdot}02$
	$P < 0{\cdot}001$	$P > 0{\cdot}05$	$P < 0{\cdot}001$	$P < 0{\cdot}05$	$P > 0{\cdot}05$
coxIII	$F = 18{\cdot}6$	$F = 2{\cdot}3$	$F = 0{\cdot}3$	$F = 1{\cdot}7$	$F = 3{\cdot}1$
	$P < 0{\cdot}001$	$P > 0{\cdot}05$	$P > 0{\cdot}05$	$P > 0{\cdot}05$	$P > 0{\cdot}05$

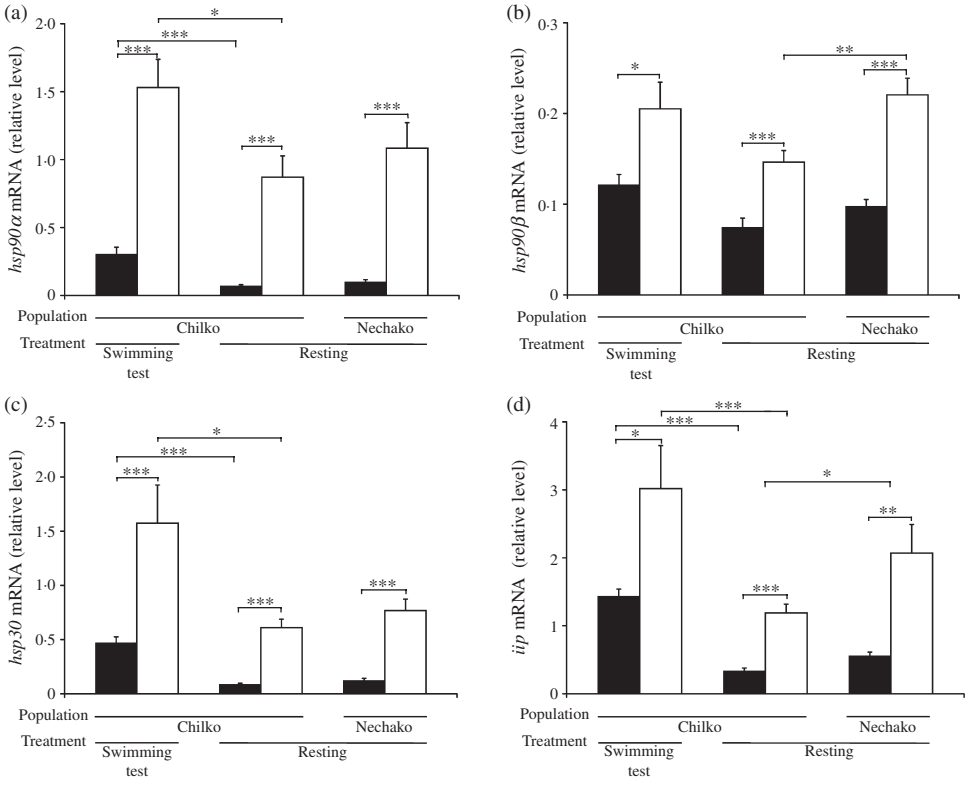

FIG. 1. The relative expression of heat shock proteins (a) *hsp90α* mRNA, (b) *hsp90β* mRNA, (c) *hsp30* mRNA and (d) interferon-inducible protein (*iip*) mRNA for cold- (12–13° C; ■) and warm- (18–19° C; □) treated *Oncorhynchus nerka* populations from Chilko and Nechako. Gene expression was tested from fish that had either experienced a swimming challenge or were killed at resting state. Values are mean + s.e. ($n = 6$ for each group). Significant differences between treatments (*, $P \leq 0{\cdot}05$; **, $P \leq 0{\cdot}01$; ***, $P \leq 0{\cdot}001$).

swimming in the Chilko population (Fig. 1), a finding that confirmed the microarray results. Indeed, the warm treatment tripled the expression of both *hsp90α* and *hsp30*.

Population differences in expression of heat shock protein mRNA were less pronounced than the effects of temperature. Population had a significant influence on the *hsp90β* response (Table III) and expression of *hsp90β* in warm-treated Nechako *O. nerka* was significantly higher than that in warm-treated Chilko population, a result consistent with the differences in upper temperature tolerance of the two populations (Eliason *et al.*, 2011).

Exhaustive swimming significantly increased expression of *hsp90α* and *hsp30* (Table II) when compared with resting fish (Fig. 1). Also, a significant interaction between temperature and swimming treatment (Table III) revealed that the warm treatment exacerbated the effect of exhaustive swimming on the expression of *hsp*s (Fig. 1).

Warm treatment and exhaustive exercise also had significant effects on the expression of *iip* without any interaction between these two factors (Table III). In exhaustively

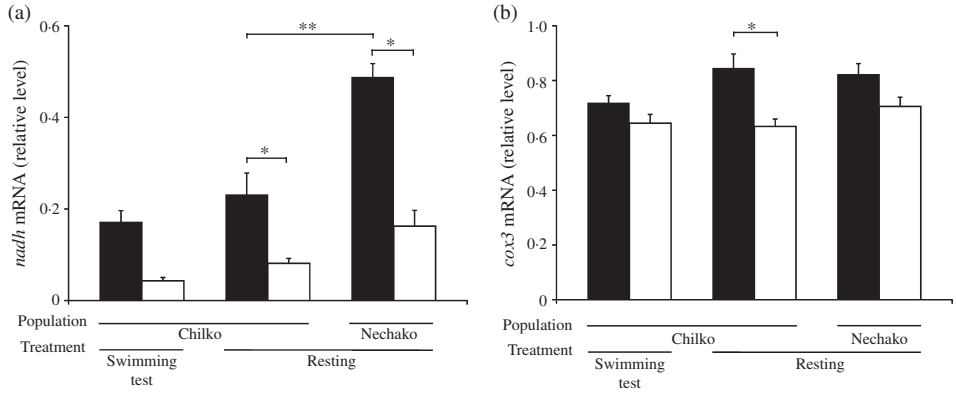

FIG. 2. The relative expression of (a) NADH dehydrogenase I (*nadh*) and (b) *coxIII* in cold- (12–13° C; ■) and warm- (18–19° C; □) treated *Oncorhynchus nerka* populations from Chilko and Nechako. Gene expression was tested from fish that had either experienced a swimming challenge or were killed at resting state. Values are mean + s.e. ($n = 6$ for each group). Significant differences between treatments (*, $P \leq 0{\cdot}05$; **, $P < 0.01$).

swum fish, the expression of *iip* was more than twice that of resting fish. Likewise, the warm treatment significantly increased the interferon-inducible protein expression.

Population had a significant effect on expression of *nadh-d* along with temperature treatment. The expression of *nadh-d* was significantly higher in cold-treated resting *O. nerka* compared with the warm-treated groups (in both Chilko and Nechako) (Fig. 2). Moreover, cold-treated resting Nechako fish had significantly higher expression of *nadh-d* compared with the Chilko population. There was also a significant interaction between population and temperature treatment (Table III). On the other hand, *coxIII* expression levels were almost similar in all the groups investigated. The temperature treatment alone had a significant effect on expression of *coxIII* (Table III), the levels being higher in cold-treated Chilko *O. nerka* when compared with warm-treated fish from the same population (Fig. 2).

DISCUSSION

The current study provides convincing microarray and RT-qPCR evidence that adult *O. nerka* cardiac ventricle tissue mounts a major stress defence (in particular, increased expression of *hsp90α* and *hsp30*) when the fish are exposed to a warm temperature (18–19 ° C). This water temperature is now commonly encountered during upriver migration and lies at the upper end of their T_{opt} window. Moreover, exhaustive exercise considerably enhanced this expression and there was significant interaction between temperature and swimming. Thus, the hearts of adult *O. nerka* in nature probably undergo major cellular stress while the fish are migrating upstream to spawn.

It is well established that increased expression of *hsp*s is a critical response to protect and recover from many types of stress, including temperature stress (Currie, 2011). Increased expression in fishes is well documented for heat shock, anoxia or hypoxia and microbial infection (Healy *et al.*, 2010; Methling *et al.*, 2010; Roberts *et al.*, 2010; Stensløkken *et al.,* 2010; Logan & Somero, 2011; Quinn *et al.*, 2011).

In terms of the response to elevated temperature, many studies have focused on short-term (acute) exposures to temperature extremes close to critical temperatures (a temperature shock) (Fangue *et al.*, 2006; Todgham *et al.*, 2006; Healy *et al.*, 2010; Quinn *et al.*, 2011). Nonetheless, a chronic (1–2 week) temperature stress well below such thermal extremes can also increase *hsp* expression, especially *hsp70* and *hsp90* (liver; Podrabsky & Somero, 2004), a response that takes several weeks to subside (4 weeks in Logan & Somero, 2010). Similarly, *hsp30* and *hsp70* expression has been shown to increase in juvenile Atlantic salmon *Salmo salar* L. 1758 at 23° C (whole body; Lund *et al.*, 2002), which is well below their critical temperature of 32° C (Elliott & Elliott, 1995). In adult *O. nerka*, increased *hsp* expression has been demonstrated after a seven-day acclimation to 19° C (gills, Jeffries *et al.*, 2012) and during the transition from the marine to a freshwater environment, which can involve a temperature change, among other factors, from *c.* 10 to *c.* 17° C (Evans *et al.*, 2011). Similarly, a mild increase (3·7° C) from ambient temperature induces expression of *hsp*s in corals (Barshis *et al.*, 2013) and, perhaps even more importantly, a number of studies have recently hypothesized that high basal levels of *hsp* expression may be thermally protective. Barshis *et al.* (2013) showed that basal expression of *hsp* at ambient temperatures was higher in thermally resilient corals compared with thermally sensitive corals, which elevated their *hsp* expression only under thermal stress. Wu *et al.* (2012) also observed that over-expression of *hsp*s in grass carp *Ctenopharyngodon idella* (Valenciennes 1844) increased cellular thermoresistance. Here, the Chilko population did not show a higher basal level of *hsp* expression compared with the Nechako population, despite the known higher resilience of the Chilko population to high temperature (Eliason *et al.*, 2011). Also, *hsp*s did not increase as much in the Chilko population as in the Nechako population with warm treatment, suggesting that warm treatment was also more stressful at the cellular level for the Nechako population. Interestingly, in the current study, the expression of *hsp90α* and *hsp30* was increased more than the expression of *hsp70* in microarray analyses of warm-treated fish, although the expression of *hsp70* also increased with warm treatment (fold change 1·18, $P < 0{\cdot}01$, CA053225 in Supporting Information). *Hsp70* is often considered a hallmark for thermal stress (Currie, 2011); thus, it is curious that expression of *hsp70* did not change as much as *hsp90α* and *hsp30*. One explanation could be that the detection capacity of GRASP microarray for *hsp70* is low. Indeed, in common killifish *Fundulus heteroclitus* (L. 1766), microarray analyses revealed that *hsp30* and *hsp90* had greater changes after warm treatment than *hsp70*, while the opposite was true using RT-qPCR analyses (Healy *et al.*, 2010). Regardless, the present results suggest that differential mRNA expression can be induced at a water temperature no more than 4° C above the population-specific T_{opt} and within the historic river migration temperatures. Whether or not the response seen here with *O. nerka* is a more general response in fishes awaits further research. Of course, only mRNA responses were examined here and further studies are needed to determine the accompanying stress responses at a protein level as previous studies of thermal stress have observed increases in both mRNA expression and protein levels of *hsp*s (Currie, 2011). The overriding concern with *O. nerka* is whether or not the cellular defence will continue to be effective given that the Fraser River is projected to continue warming (Morrison *et al.*, 2002; Ferrari *et al.*, 2007; Patterson *et al.*, 2007).

Interestingly, a significant population difference existed in the expression of *hsp90β*, with expression being greater for the Nechako compared with the Chilko population. The *hsp90β* gene is considered to be the constitutive form of *hsp90* (Hermesz *et al.*, 2001) and is not considered thermally responsive (Newton *et al.*, 2012). Although expression of *hsp90α* increased more than expression of *hsp90β* after warm treatment, *hsp90β* also increased significantly. The population differences could indicate an adaptive response to high temperature. Indeed, these populations also differ in the number of protective *β*-adrenoreceptors associated with the cardiac cell membrane being higher in the Chilko population along with their higher temperature tolerance compared with the Nechako population (Eliason *et al.*, 2011). This study adds further evidence to the suggestion that functional, cellular and molecular intraspecific differences exist for the cardiorespiratory physiology of the Fraser River *O. nerka* populations (Lee *et al.*, 2003*b*; Farrell *et al.*, 2008; Eliason *et al.*, 2011).

In addition to thermal stress, exhaustive swimming also elicited *hsp* responses. Notably, when *O. nerka* were swum to exhaustion at a warm temperature, the heat shock response became enhanced with a significant interaction between temperature treatment and swimming test. Thus, independent and interactive effects of exhaustive exercise were clearly apparent. Exhaustive exercise increases epinephrine levels in blood (Opdyke *et al.*, 1982; Butler *et al.*, 1986), and Currie *et al.* (2008) found that the *hsp* mRNA and protein response to heat stress in red blood cells was similarly exacerbated by epinephrine in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792). Thus, humoral or neural sympathetic mediation could be involved in the *hsp* response of the *O. nerka* ventricle. Clarkson *et al.* (2005), however, have reported that exhaustive exercise at 12–15° C, *i.e.* at temperatures around T_{opt} of *O. mykiss* (Jobling, 1981), did not alter *hsp* protein levels. Thus, it seems that exercise may not be sufficient to initiate the *hsp* response unless the fish are also exposed to a warm temperature.

Further evidence for an additive effect of exhaustive swimming on warm *O. nerka* was provided by the observation that exhaustive exercise at warm temperature generated an enhanced immune response as well as an increased *hsp* response. The expression of immune-related genes in exhaustively swum Chilko *O. nerka* was more than twice that of the resting fish. Warming also had a significant effect on *iip* expression. The Nechako population had higher expression compared with the Chilko population, adding further evidence for population differences at the cellular level. Because warm temperature also enhances virulence of some pathogens (Lewis *et al.*, 2010; Jeffries *et al.*, 2012), the possibility that the fish were responding to increased virulence or pathogen load rather than temperature *per se* cannot be discounted. Correspondingly, the microarray analysis revealed a greater response to fungus in the warm group compared to the cold group as well as higher expression of *mhc class 1 b region* and *precerebellin-like protein (plp). mhc class 1* and *iips* are well known to be involved in the immune defence system of animals (Rock & Goldberg, 1999; Sen, 2000). While the exact function of *plp* is unknown, its expression increases after bacterial infection in fish and it has been assumed to be a part of the acute phase response, *i.e*. a protective response to a traumatic insult (Gerwick *et al.*, 2007; Raida & Buchmann, 2009). *hsp*s can also play a role in immune system and inflammatory processes, and elevated *hsp* levels are observed after bacterial infections (Roberts *et al.*, 2010).

Cold acclimation is known to enhance mitochondrial oxidative capacity of cardiac and skeletal muscle in fishes (Driedzic, 1992; Guderley, 2004), where it is

seen as a compensatory response to the slowing of enzymatic catalytic rates (a Q_{10} effect). The present results are consistent with this idea. In general, the expression of genes involved with oxidative phosphorylation was higher in cold-treated fish when compared with warm-treated fish. Specifically, RT-qPCR analyses revealed that *nadh-d* was enhanced. Also, the Nechako population showed a higher expression than the Chilko population. Furthermore, there was also significant interaction between population and temperature treatment, with *nadh-d* having a higher response to cold treatment in the Nechako population compared to the Chilko population. Likewise, the lowest expression of *coxIII* was in warm-treated Chilko *O. nerka* at rest, with it being similarly expressed in all other groups. In a previous study, cardiac expression of both *cox* and *nadh-uo* was higher in cold-acclimated than in warm-acclimated common carp *Cyprinus carpio* L. 1758 (Gracey *et al.*, 2004). Cardiac *cox* expression decreased while *nadh-d* expression increased with cold acclimation in *O. mykiss* (Vornanen *et al.*, 2005). Moreover, while an initial decrease in the activities of aerobic enzymes preceded the increase after 2 weeks of cold acclimation in *O. mykiss* (Bouchard & Guderley, 2003), *cox* activity increased in three-spined stickleback *Gasterosteus aculeatus* L. 1758 skeletal muscle after only 3 days of cold acclimation (Orczewska *et al.*, 2010). Thus, the timing and direction of change of muscle oxidative capacity in response to temperature show variability among species and muscle types. Training and migration also increase the oxidative enzymes of fish skeletal muscle (Anttila *et al.*, 2006, 2011). Here, the greater expression of cardiac *nadh-d* in Nechako compared with Chilko *O. nerka* may be associated with longer migration distance of the former, but exhaustive swimming did not trigger increased cardiac expression of either *nadh-d* or *cox*. The full implication of these observations will have to await quantification of differences in oxidative capacity between populations of *O. nerka* at an activity–protein level, as has been previously demonstrated with cold acclimation in other fishes (Driedzic, 1992; Guderley, 2004).

In conclusion, the current study brings significant new insights into how fishes respond to temperature changes and exhaustive exercise at the mRNA level, which adds to the growing evidence of intraspecific variation in whole organism performance among *O. nerka* populations, which may influence both temperature tolerance and aerobic capacity (Lee *et al.*, 2003*b*; Eliason *et al.*, 2011; Jeffries *et al.*, 2012). Warm temperatures similar to those encountered during a natural upstream migration of *O. nerka* triggered a cytoprotective defence through enhanced heat shock and immune responses and more so in the Nechako than in the Chilko population. The enhanced responses in the Chilko population occurred just after 4 h of exhaustive swimming. Thus, the cytoprotective role played by *hsp*s may be particularly crucial to the functional capacity of the whole animal at the warm temperatures that are currently encountered by these fish during their spawning migration in the Fraser River.

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Supporting Information

Supporting Information may be found in the online version of this paper:
Table S1. Genes having significantly different expression levels in swimming Chilko *Oncorhynchus nerka* as analysed from microarray with a *U*-test. Positive value indicates that expression of gene is higher in warm-treated group and negative value indicates that expression is higher in cold-treated group.

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