Species- and sex-specific responses and recovery of wild, mature pacific salmon to an exhaustive exercise and air exposure stressor

Michael R. Donaldson [a],*, Scott G. Hinch [a], Ken M. Jeffries [a], David A. Patterson [b], Steven J. Cooke [c], Anthony P. Farrell [d], Kristina M. Miller [e]

[a] Pacific Salmon Ecology and Conservation Laboratory, Centre for Applied Conservation Research and Department of Forest Sciences, University of British Columbia, Vancouver, British Columbia, Canada
[b] Fisheries and Oceans Canada, Freshwater Ecosystems Section, Cooperative Resource Management Institute, School of Resource and Environmental Management, Simon Fraser University, Burnaby, British Columbia, Canada
[c] Fish Ecology and Conservation Physiology Laboratory, Ottawa-Carleton Institute of Biology, Carleton University, Ottawa, Ontario, Canada
[d] Department of Zoology and Faculty of Land and Food Systems, University of British Columbia, Vancouver, British Columbia, Canada
[e] Fisheries and Oceans Canada, Molecular Genetics Section, Pacific Biological Station, Nanaimo, British Columbia, Canada

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ABSTRACT

Despite the common mechanisms that underlie vertebrate responses to exhaustive exercise stress, the magnitude and the timecourse of recovery can be context-specific. Here, we examine how wild, adult male and female pink (Oncorhynchus gorbuscha) and sockeye (Oncorhynchus nerka) salmon respond to and recover from an exhaustive exercise and air exposure stressor, designed to simulate fisheries capture and handling. We follow gill tissue gene expression for genes active in cellular stress, cell maintenance, and apoptosis as well as plasma osmoregulatory, stress, and reproductive indices. The stressor initiated a major stress response as indicated by increased normalised expression of two stress-responsive genes, Transcription Factor JUNB and cytochrome C (pink salmon only). The stressor resulted in increased plasma ion cortisol, lactate, and depressed estradiol (sockeye salmon only). Gene expression and plasma variables showed a general recovery by 24 h post-stressor. Species- and sex-specific patterns were observed in stress response and recovery, with pink salmon mounting a higher magnitude stress response for plasma variables and sockeye salmon exhibiting a higher and more variable gene expression profile. These results highlight species- and sex-specific responses of migrating Pacific salmon to simulated fisheries encounters, which contribute new knowledge towards understanding the consequences of fisheries capture-and-release.

1. Introduction

Maintaining homeostasis when faced with a stressor involves an intersection of physiology, behaviour and life history that is fundamental to the persistence of animal populations (Ricklefs and Wikelski, 2002). Stressors can displace an organism's homeostatic state (Selye, 1936) and typically elicit an adaptive set of molecular, cellular, hormonal, metabolic, and behavioural responses (Romero, 2004). The responses of fish to exhaustive exercise stressors have been well characterized at the organismal level (Black et al., 1962; Wood, 1991; Milligan, 1996; Kieffer, 2000). Typically, a stressor that evokes strenuous activity and burst swimming, such as escape from predators or fisheries capture-and-release (Milligan, 1996), initiates an aerobic response

with the rapid release of catecholamines (i.e., adrenaline and noradrenaline) into circulation, along with a corticosteroid release (i.e., plasma cortisol peaks within 1–2 h; Barton, 2002). As tissue oxygen demands are rapidly exceeded and energy stores are depleted from muscle tissue, the response to the stressor can become anaerobic (Wood, 1991). Consequently, lactate accumulation in muscle and blood can alter blood pH and osmoregulatory balance (Wood, 1991; Milligan, 1996; Kieffer, 2000). Exhaustive exercise stress combined with air exposure, a common consequence of capture-and-release fisheries, can result in additional physiological disturbances (Ferguson and Tufts, 1992).

Regaining homeostasis following exhaustive exercise stress requires oxygen in excess of basal metabolic requirements and sufficient time to recover (Wood, 1991). To meet tissue oxygen demands during recovery, cardio-respiratory activity increases and, for some species, can take hours to return to routine levels (Lee et al., 2003; Donaldson et al., 2010). Recovery of ionic, metabolic and stress variables that are typically associated with exhaustive exercise stress can likewise take hours (Wood, 1991). The magnitude of the stress response and timecourse of recovery are important, as these factors can be linked to

* Corresponding author at: Centre for Applied Conservation Research, Department of Forest and Conservation Sciences, 2424 Main Mall, University of British Columbia, Vancouver, BC V6T 1Z4, Canada. Tel.: +1 604 822 1969.
E-mail address: Michael.r.donaldson@gmail.com (M.R. Donaldson).

long-term changes in whole-organism behaviour, performance, and disease resistance (Mazeaud et al., 1977). Delayed recovery can govern the frequency of maximal performance (Milligan, 1996) and can result in mortality in extreme cases (Black, 1958; Wood et al., 1983). Thus, while responding to a stressor is aimed at protecting the animal and restoring homeostasis, it also sequesters energy away from anabolic processes and maintenance functions such as reproduction to catabolic activities that mobilize energy to respond to the stressor (Wendelaar Bonga, 1997).

Despite decades of research on the physiological responses of fish to exhaustive exercise stress at the organismal and tissue levels, cellular and molecular responses have received less attention until recently. Acting in concert with the responses that occur at higher levels of biological organization, the cellular suite of stress responses helps to temporarily tolerate a variety of stressors or remove damaged cells by apoptosis (Kültz, 2005). Thus, changes in gene expression (i.e., quantitative, qualitative, and changes in reaction coefficients) can be linked with a range of stressors (Krasnov et al., 2005). In fish, a number of genes have been investigated as potential biomarkers for various stressors. For example, following various stressors, Heat Shock Protein (e.g., HSP90) expression increases to maintain cellular homeostasis (Iwama et al., 2004). Genes linked to cell apoptosis, such as cytochrome c (herein abbreviated as cyt c) and Transcription Factor JUNB (herein abbreviated as JUNB), are upregulated in response to temperature stress in sockeye salmon (Oncorhynchus nerka; Jeffries et al., 2012a). JUNB was likewise upregulated following low-water and air exposure stressors in rainbow trout (Oncorhynchus mykiss; Momoda et al., 2007). Transcription factor NUPR1, which is involved in the regulation of cell growth and apoptosis (Mallo et al., 1997), is stress responsive in rainbow trout and can remain upregulated several hours post-stressor (Momoda et al., 2007). A complicating factor in understanding the behaviour of these genes in the stress response is the fact that studies conducted on fish to date often focus on different timecourses (Krasnov et al., 2005), species, tissues (Kassahn et al., 2009), techniques (Prunet et al., 2008), and genes of interest. The role of gene expression in the recovery process is likewise poorly understood. Thus, there is a need to understand the mechanisms of response and recovery to exhaustive exercise stress and air exposure at the molecular level in relation to the responses of standard plasma indices of stress.

Migratory behaviour includes a suite of physiological adaptations to stress-related selective pressures that operate on the individual, and have outcomes at the population level (Ramenofsky and Wingfield, 2007). Pink salmon (Oncorhynchus gorbuscha) and sockeye salmon both undergo semelparous and anadromous reproductive migrations, but differ in their respective life history, performance, behaviour, thermal tolerance, abundance, and conservation status. Sockeye salmon typically grow for one year in freshwater before migrating to sea, and generally return to spawn at age four. In contrast, pink salmon migrate directly to sea after emergence from redds, returning to spawn at two years of age. As a consequence, pink salmon are the smallest (in both length and weight) of the Pacific salmonids at maturity (Heard, 1991), which results in a lower absolute prolonged swimming performance relative to sockeye salmon, although length-adjusted swimming performance is not different with that of sockeye salmon (Williams and Brett, 1987; MacNutt et al., 2006) and pink salmon also behaviourally reduce transport and activity costs compared to sockeye salmon (Standen et al., 2002) by seeking out optimal microhabitats of least migratory resistance.

Throughout their spawning migrations, Pacific salmon face many stressors, including threat of predation and fisheries. Pink and sockeye salmon often migrate through the same locations during overlapping time periods. As a consequence, fisheries can catch non-target species which are subsequently released as bycatch to resume their migrations. There is a growing understanding that post-release survival of salmon is influenced by fisheries capture method (e.g. Donaldson et al., 2011) and that the duration of recovery depends on the duration of the stressor

(e.g., Donaldson et al., 2010). A sex-specific trend is also emerging where female salmon tend to have lower survival than males following fisheries-related (Robinson et al., 2013) and temperature-related stressors (Jeffries et al., 2012b; Martins et al., 2012).

Despite the rich body of literature documenting exhaustive exercise stress in fish (Kieffer, 2010), only a few studies have focused on wild, adult salmonids (e.g., Farrell et al., 2001a,b) and fewer still have compared species- and sex-specific patterns following exercise (e.g., Pottinger, 2010). Likewise, although knowledge is being accumulated on the genes involved in the stress response, less is known about gene expression during the recovery process. To address these knowledge gaps, this study first sought to characterize the response and recovery of wild, maturing, adult Pacific salmon to fisheries-related exhaustive exercise stress and air exposure by examining a series of plasma stress, osmoregulatory and reproductive indices and the expression of genes active in cellular stress, cell maintenance, and apoptosis. This study then tested the hypothesis that the physiological recovery processes are species- and sex-specific by comparing the recovery patterns of male and female sockeye and pink salmon.

2. Materials and methods

2.1. Study site and animals

Experiments were conducted at Fisheries and Oceans Canada's Weaver Creek Spawning Channel, near Agassiz, British Columbia, Canada. Water temperatures throughout the course of the study were 11–12 °C, measured using a permanent temperature probe operated by Fisheries and Oceans Canada staff.

A total of 112 wild, adult sockeye salmon and 88 pink salmon were used in the experiment and were distributed across two treatment groups (control or exercise and air exposure treatment) and five sampling intervals (0, 0.5, 1, 4, 24 h). Individual fish were sampled once to collect gill tissue and a blood sample to avoid repeated handling and sampling. Equal proportions of male and female pink and sockeye salmon allowed sex-specific differences to be examined (e.g., plasma cortisol; Donaldson et al., 2010). Pink salmon experiments were conducted October 1st–7th, 2009 and sockeye salmon experiments were conducted October 8th–14th, 2009. In 2009, peak spawning was October 19–24th for pink salmon and October 15–19th for sockeye salmon.

2.2. Study design

Fish were captured by dip net from a raceway downstream of the spawning channel entrance. Individuals were rapidly transferred (1–2 s to minimize air exposure) to a holding tote on a research vehicle, and transported ~300 m (2–3 min transport time) to the experimental area. Individuals in the exercise and air exposure treatments were transferred into a donut shaped exercise tank (diameter 150 cm, inner diameter 50 cm, water depth 40 cm) supplied with fresh, flowing water pumped from the spawning channel. Fish were manually chased for 3 min, then collected by dip net and exposed to air for 1 min as previously described (Donaldson et al., 2010). Briefly, fish placed in the tank were manually coaxed to continually burst swim (Milligan, 1996) by three experimenters positioned around the exercise tank, which resulted in fish being visibly exhausted (no longer able to burst swim, unresponsive to tactile stimuli and some individuals unable to maintain equilibrium; Wood, 1991). The procedure was intended to simulate an exhaustive fisheries capture and release event (e.g., angling and release or rapid release from a net fishery). The air exposure was intended to simulate air contact while being removed from a hook or net.

Fish were randomly assigned a sampling interval of 0, 0.5, 1, 4, and 24 h. Control fish were neither exercised nor air-exposed and were transferred directly into holding boxes. Control fish were sampled 24 h

later to allow for full recovery from minor handling. Exercised and air exposed fish were immediately placed into individual, dark plastic holding boxes with secure lids (L × W × D = 93.7 × 54.0 × 47.3 cm). Each box received freshwater pumped at 0.63 L·s^{-1} using an electric submersible pump placed in the spawning channel. The outflow was positioned at the back of the box. The inflow was centred in the lower third of the box to direct water at the fish's mouth. The boxes were large enough to enable fish to orient into the water flow with periodic tail beats to change or maintain position, however when collected for sampling, fish were often not observed to be oriented directly into the flow. We speculate that the sampling boxes provided an environment similar to fish in the wild holding in low flow riverine areas behind large rocks with lower direct velocity than methods designed to facilitate fish recovery using high velocity ram ventilation (e.g., Milligan et al., 2000; Farrell et al., 2001b). Our results indicate that the sampling boxes did not impose additional holding stress since plasma stress indices for the control group were similar to studies where pink and sockeye salmon were rapidly dip netted and blood sampled from the same location (see Discussion; Hruska et al., 2010; McConnachie et al., 2012).

For tissue sampling, individuals were rapidly removed from their box and placed supine in a water-filled V-shaped foam-padded sampling trough (Cooke et al., 2005) for immediate biopsy and length measurements. The biopsy, which lasted <2 min, collected a 2.5 mL blood sample by caudal puncture using a sterile 3.8 cm, 21-gauge needle and a heparinised vacutainer (lithium heparin, 3 mL, Becton-Dickson, NJ, USA), which was then stored in ice-chilled water for <1 h until subsequent processing. Also, ~3 mm gill filament tips from the first gill arch were collected using sharpened end-cutter pliers, sterilized with 95% ethanol and rinsed with distilled water (Cooke et al., 2005). Gill samples were transferred using sterile forceps to cryovials, flash frozen in liquid nitrogen, and subsequently transferred to −80 °C freezers for subsequent analyses. The rapid caudal puncture technique has been found in adult Pacific salmon to yield statistically similar plasma values compared to cannulation techniques (Clark et al., 2011a).

2.3. Plasma assays

The chilled ~2.5 mL blood sample was centrifuged at 7000 g for 3 min and plasma was stored in liquid nitrogen prior to being frozen at −80 °C until analysis. Plasma was subsequently analysed for the following: cortisol, testosterone, and 17 B-estradiol using commercial ELISA kits (ELISA nos. 402710, 402110, 402510, with Molecular Devices Spectramax 240pc plate reader; Neogen Inc., Lansing, MI, USA); lactate and glucose (YSI 2300 stat plus analyser, YSI Inc., Yellow Springs, OH, USA); osmolality (Advanced Instruments 3320 freezing point osmometer, Advanced Instruments Inc., Norwood, MA, USA); chloride (digital chloridometer; Haake Buchler Instruments, Inc., Saddle Brook, NJ, USA); and sodium and potassium (Cole-Parmer model 410 single

channel flame photometer; Cole-Parmer Inc., Montreal, Canada; Farrell et al., 2001b).

2.4. Quantitative real-time PCR (qRT-PCR) methods

Gill tissue was collected from non-lethal biopsies to quantify gene expression via qRT-PCR (Table 1). We examined four genes of interest and two reference genes (Table 1). Primers for Heat Shock Protein 90 (HSP90AB1), cytochrome c (cyt c), and two reference genes, Si:dkey-78d16.1 protein (78d16.1) and Bone Morphogenetic Protein 4 (BMP4), were designed in-house, with primers developed to equally match contigs of rainbow trout and Atlantic salmon (Jeffries et al., 2012a). Transcription Factor Jun B (JUNB) and Nuclear Protein 1 (NUPR1) primers were published in Momoda et al. (2007). RNA was extracted following the protocols described in Miller et al. (2009). To prepare samples for qRT-PCR, an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to synthesize cDNA from 1 μg of total RNA. An ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) was used to perform Relative Quantification (RQ) assays. The cDNA template was diluted 1:2.5 and assays were conducted in 384-well plates using 20 μL reaction volumes [10 μL Kapa SYBR fast qPCR Master Mix (2×) (Kapa Biosystems, Inc., Woburn, MA, USA); 0.4 μL of a mixture of 0.2 μM forward and reverse primers; 2 μL of diluted cDNA; 7.6 μL of RNase/DNase-free water]. The cycling conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s, and a dissociation stage (95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s) was added at each each RQ run to ensure that only one gene product was amplified for each primer set. All samples were run in duplicate and with non-template controls included. Target gene expression was normalised to two reference genes that were not responsive to the experimental factors, as determined by the reference gene association function of the DataAssist v 3.0 software suite (Applied Biosystems Inc., Foster City, CA, USA).

2.5. Statistical methods

Normalised expression of target genes was determined using the comparative C_T method (Livak & Schmittgen, 2001) using DataAssist v 3.0 (Applied Biosystems Inc., Foster City, CA, USA). Statistical analyses were conducted on the expression of the genes of interest relative to two reference genes. A Principal Component Analysis (PCA) with a Varimax factor rotation was used, which conducts an orthogonal transformation of factors with eigenvalues ≥1.0, a technique that partitions correlated variables into factors for subsequent analyses (Wagner and Congleton, 2004). This approach is used to identify general trends among similarly responding variables in the data set. Variables with loading factors ≥±0.5 were considered to contribute to the factor. All response variables were log_{10}-transformed prior to PCA. Plasma

Table 1
Primer sequences for reference genes and genes of interest used in qRT-PCR.

Gene type	Gene name	Gene symbol	EST number	Primer sequence (5′–3′)
Reference genes	Bone Morphogenetic Protein 4	BMP4	CA056395	F-TTGCCCATAGTCAGTGTTAGCG R-GTGCCATCTCCATGCTCTACC
	Si:dkey-78d16.1 protein	78d16.1	CA056739	F-AAAGGTCCCACGCTCCAAAC R-ACACACCCATCTGTCTCATCACC
Genes of interest	Cytochrome C	cyt c	CB494539	F-CGAGCGTGCAGATCTTATAGC R-CTTCTCCGCTGAACAGTTGATG
	Heat Shock Protein 90	HSP90AB1	CB493960	F-TGGGCTACATGGCTGCCAAG R-TCCAAGGTGAACCCAGAGGAC
	Transcription Factor JUNB	JUNB	N/A[a]	F-CTACACGCACAGCGATATTCG R-TCGTCGCTGCTCTGCATGT
	Nuclear Protein 1	NUPR1	N/A[a]	F-GACAAATCGGACGGCTAATCCT R-CTGCCTGCCCATTTGGTTTT

[a] From rainbow trout (*Oncorhynchus mykiss*; Momoda et al., 2007).

estradiol was excluded from the PCA since it was only measured in females.

MANOVAs and ANOVAs were conducted on the rotated factors to test for the effects of time, sex, species, and their factorial interactions. Where noted, ANOVAs with Tukey's post-hoc tests were conducted and dissimilar letters represent statistical differences in figures. All statistical analyses were conducted using JMP 9.0 (SAS Institute Inc., Cary, NC, USA). For MANOVAs, significance was tested at $\alpha = 0.013$ (Bonferroni correction based on 4 rotated factors). Fork length was variable with species and sex (61.70 ± 0.43 cm and 66.40 ± 0.88 cm for female and male sockeye salmon, respectively, and 49.89 ± 0.36 cm and 51.37 ± 0.42 cm for female and male pink salmon, respectively), but MANOVA did not reveal any significant effects of fork length on physiological response variables for pink (Wilk's lambda = 2.902; $F_{18} = 0.806$; $P = 0.669$) or sockeye salmon (Wilk's lambda = 0.614; $F_{18} = 0.614$; $P = 0.845$). Thus, fork length was not included as a covariate in analyses.

3. Results

No mortality was observed during the study. The PCA resulted in four rotated factors (abbreviated RF; Table 2; Fig. 1). The four gill gene variables loaded on RF1. Osmoregulatory variables (i.e., plasma chloride, sodium, and osmolality) loaded on RF2. Stress variables (i.e., plasma cortisol and lactate) loaded on RF3. Hormones (e.g., plasma cortisol and testosterone) loaded on RF4.

A MANOVA of the rotated factors (RF1, RF2, RF3, and RF4) revealed a significant whole model (Wilk's lambda = 0.006; $F_{108,13.765} = 13.765$; $P < 0.001$) with significant effects for time (Wilk's lambda = 0.057; $F_{24,500.08} = 26.517$; $P < 0.001$), sex (Wilk's lambda = 2.215; $F_{4,143} = 79.201$; $P < 0.001$) and species (Wilk's lambda = 1.222; $F_{4,143} = 43.673$; $P < 0.001$), as well as the species * time interaction (Wilk's lambda = 0.462; $F_{24,500.08} = 1.221$; $P < 0.001$) and the species * sex interaction (Wilk's lambda = 0.101; $F_{4,143} = 2.215$; $P < 0.001$), but not the time * sex (Wilk's lambda = 0.816; $F_{24,500.08} = 1.250$; $P = 0.192$) or species * time * sex interactions (Wilk's lambda = 0.849; $F_{24,500.08} = 0.996$; $P = 0.470$). Only significant terms and interactions were included in subsequent ANOVAs testing the effects of time, sex, species, species * time interaction and species * sex interaction, which found significant whole models for RF1 (SS = 59.065; $F_{15} = 6.494$, $P < 0.001$), RF2 (SS = 42.886; $F_{15} = 24.221$, $P < 0.001$), RF3 (SS = 121.348; $F_{15} = 44.949$, $P < 0.001$), and RF4 (SS = 100.874; $F_{15} = 26.127$, $P < 0.001$).

RF1 was only influenced by time and species (Table 3). RF2 was influenced by time, sex, species, and the species * time interaction. RF3 was influenced by time, sex, species, species * time and species * sex interactions. RF4 was influenced by time, sex, and their interaction.

The general pattern of physiological response was similar for both species, yet pink salmon mounted a higher magnitude response for many of the plasma variables measured, whereas sockeye tended to have higher and more variable gene expression.

3.1. RF1: cellular stress variables

Gill cyt c expression, which was significantly increased at 2 h, was highest when measured at 4 h, and recovered to control values at 24 h post-treatment for pink salmon (SS = 0.163; $F_6 = 4.179$; $P = 0.001$; Fig. 2). Gill JUNB was highest when measured at 4 h and returned to control values at 24 h for both pink (SS = 0.201; $F_6 = 5.789$; $P < 0.001$) and sockeye salmon (SS = 0.116; $F_6 = 2.521$; $P = 0.026$). Gill HSP90AB1 and NUPR1 expression remained unchanged over time for both species. Gene expression did not differ by sex for either species.

3.2. RF2: osmoregulatory variables

Indices of osmoregulatory status generally increased post-treatment (Fig. 3). Plasma sodium increased over time and recovered by 24 h in both species (pink females SS = 0.015; $F_6 = 8.208$; $P < 0.001$; pink males SS = 0.0246; $F_6 = 9.444$; $P < 0.001$; sockeye females SS = 0.213; $F_6 = 8.008$; $P < 0.001$; sockeye males SS = 0.029; $F_6 = 4.301$; $P = 0.002$). Plasma potassium was highest when measured at 2 or 4 h post-treatment for pink females (SS = 0.782; $F_6 = 3.323$; $P = 0.011$) and sockeye males (SS = 2.583; $F_6 = 12.490$; $P < 0.001$). Plasma osmolality increased over time and recovered by 24 h for pink salmon (pink females SS = 0.021; $F_6 = 32.527$; $P < 0.001$; pink males SS = 0.0167; $F_6 = 12.344$; $P < 0.001$) and was variable for sockeye salmon males (SS = 0.023; $F_6 = 3.246$; $P = 0.009$) and not significant for sockeye females. Plasma chloride decreased post-treatment for both sexes of pink salmon (pink females SS = 0.023; $F_6 = 30.948$; $P < 0.001$; pink males SS = 0.0183; $F_6 = 11.607$; $P < 0.001$) but remained variable for sockeye males (SS = 0.019; $F_6 = 2.785$; $P = 0.021$) and did not change for sockeye females.

3.3. RF3: organismal stress variables

For both species, plasma stress variables that responded to the stressor had recovered to control values by 24 h post-exercise (Fig. 4).

Table 2

Rotated factor loadings and final communalities for factor analysis of log$_{10}$-transformed plasma and gill gene response variables for adult pink and sockeye salmon. Variables with loading factors ≥±0.5 are marked in bold.

Response variable	Rotated factor #				Final communality estimates
	1	2	3	4	
Eigenvalue	3.198	2.688	1.485	1.291	
Gill cyt c	**0.918**	−0.039	0.150	−0.032	0.869
Gill HSP90AB1	**0.881**	−0.163	−0.018	−0.094	0.811
Gill JUNB	**0.635**	0.091	0.320	−0.071	0.519
Gill NUPR1	**0.584**	−0.141	−0.123	0.118	0.391
Plasma glucose (mmol·L^{-1})	0.132	0.297	0.182	−0.195	0.177
Plasma lactate (mmol·L^{-1})	0.088	0.319	**0.812**	−0.163	0.794
Plasma chloride (mmol·L^{-1})	−0.182	**0.914**	−0.017	0.197	0.908
Plasma sodium (mmol·L^{-1})	−0.220	**0.644**	0.155	−0.170	0.516
Plasma potassium (mmol·L^{-1})	−0.193	−0.224	0.186	0.061	0.126
Plasma osmolality (mOsM·kg^{-1})	−0.060	**0.955**	0.139	0.143	0.956
Plasma cortisol (ng·mL^{-1})	0.147	−0.041	**0.734**	**0.662**	0.999
Plasma testosterone (ng·mL^{-1})	−0.033	0.052	−0.042	**0.638**	0.412
Cumulative variance explained (%)	26.647	49.045	61.417	72.174	

Note: Variables with factor loadings ≥±0.5 are shown in bold. Response variables were log$_{10}$ transformed prior to Principal Component Analysis.

Plasma lactate was highest between 0.5 and 1 h (pink females SS = 8.233; F_6 = 62.666; P < 0.001; pink males SS = 8.781; F_6 = 20.360; P < 0.001; sockeye females SS = 6.843; F_6 = 28.949; P < 0.001; sockeye males SS = 11.041; F_6 = 64.836; P < 0.001). Plasma cortisol was highest when measured between 1 and 2 h for both species (pink females SS = 3.488; F_6 = 22.469; P < 0.001; pink males SS = 2.773; F_6 = 9.315; P < 0.001; sockeye females SS = 1.019; F_6 = 11.958; P < 0.001; sockeye males SS = 0.559; F_6 = 5.007; P < 0.001).
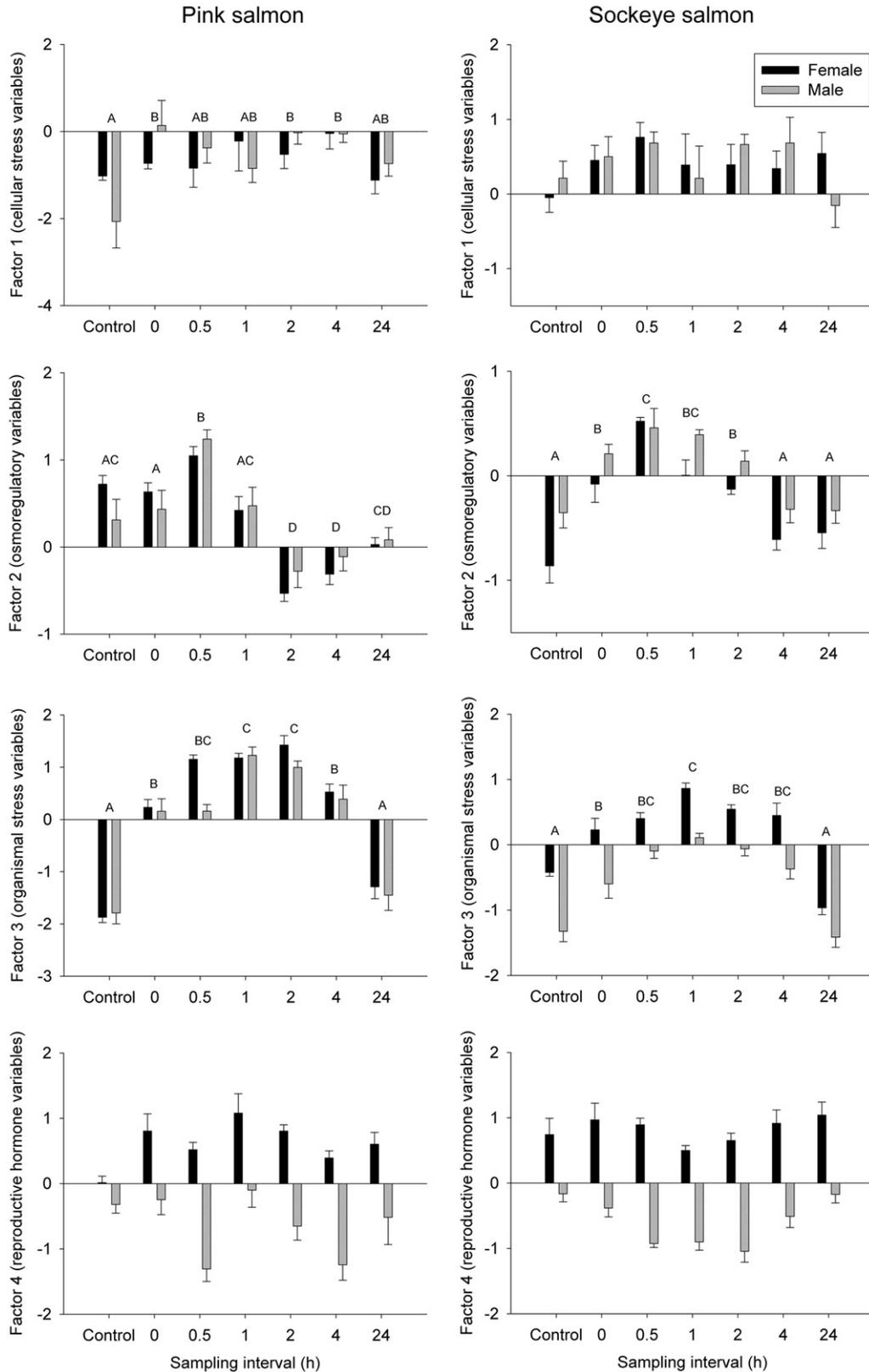
Fig. 1. Means ± SE for rotated factor loadings of log_{10}-transformed plasma and gill gene response variables for adult pink and sockeye salmon. PCAs based on plasma and gill samples. Dissimilar letters denote statistical differences from Tukey's post-hoc tests following ANOVAs.

Table 3

ANOVA effects tests that were significant for the whole model comparing rotated factor loadings of $\log_{10}$-transformed plasma and gill gene response variables for adult pink and sockeye salmon.

Rotated factor #	Rotated factor name	Source	DF	SS	F ratio	P-value
1	Cellular stress variables	Time	**6**	**16.843**	**4.629**	**<0.001**
		Sex	1	0.491	0.810	0.370
		Species	**1**	**46.264**	**76.294**	**<0.001**
		Species * time	6	6.141	1.688	0.127
		Species * sex	1	0.366	0.604	0.438
2	Osmoregulatory variables	Time	**6**	**26.650**	**37.628**	**<0.001**
		Sex	**1**	**1.300**	**11.012**	**0.001**
		Species	**1**	**6.069**	**51.412**	**<0.001**
		Species * time	**6**	**6.067**	**8.567**	**<0.001**
		Species * sex	1	0.366	3.102	0.080
3	Organismal stress variables	Time	**6**	**100.026**	**92.627**	**<0.001**
		Sex	**1**	**10.340**	**57.449**	**<0.001**
		Species	**1**	**2.971**	**16.506**	**<0.001**
		Species * time	**6**	**10.160**	**9.408**	**<0.001**
		Species * sex	**1**	**1.658**	**9.215**	**0.003**
4	Reproductive hormone variables	Time	**6**	**5.885**	**3.811**	**0.001**
		Sex	**1**	**80.511**	**312.793**	**<0.001**
		Species	1	0.281	1.093	0.298
		Species * time	**6**	**7.688**	**4.978**	**<0.001**
		Species*sex	1	0.018	0.069	0.793

Note: Bold values denote significant effects of tests at $\alpha = 0.013$. Response variables were $\log_{10}$ transformed prior to Principal Component Analysis.

3.4. R4: reproductive hormone variables

Plasma testosterone was highly variable but did not change significantly over time for males or females of either species. Plasma estradiol, which was not included in PCAs since it was measured only in females, decreased immediately following stress in sockeye salmon (SS = 0.192; F_6 = 3.104; P = 0.018), but not pink salmon (SS = 1.568; F_6 = 0.669; P = 0.675; Fig. 5).
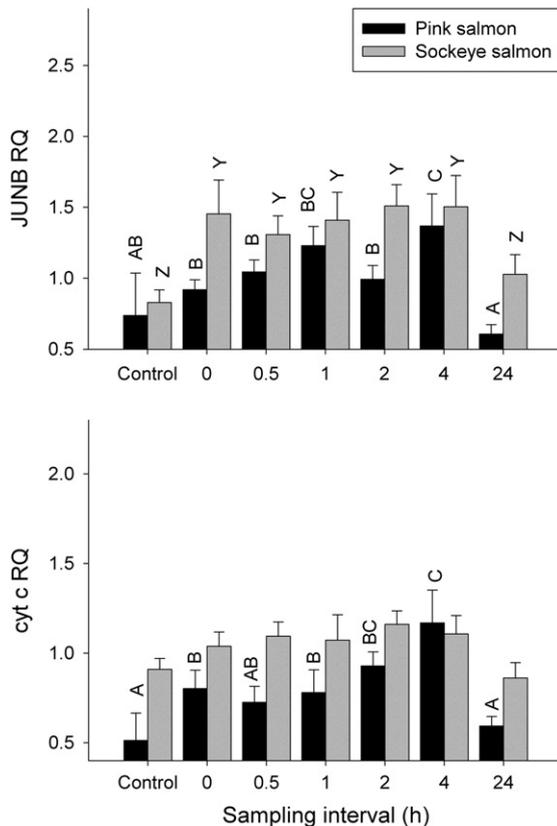

Fig. 2. Relative Quantification (RQ) of cellular stress variables gill JUNB and cyt c for adult pink and sockeye salmon. Sexes pooled. Dissimilar letters denote statistical differences from Tukey's post-hoc tests following ANOVAs.

4. Discussion

4.1. Cellular stress response and recovery

JUNB provides an important link between the endocrine stress responses and downstream transcriptional processes (Vamvakopoulos and Chrousos, 1994). JUNB forms the transcription factor activator protein 1 transcription complex, which is linked to cellular proliferation, apoptosis and stress response (Piechaczyk and Farràs, 2008). In fish, JUNB is responsive to a range of stressors, including low-water and confinement (Momoda et al., 2007), acute temperature (Lewis et al., 2010), and chronic temperature (Jeffries et al., 2012a). Here, JUNB expression was highest when measured at 2 and 4 h in pink salmon and between 0.5 and 4 h in sockeye salmon after the stressor, yet had returned to control levels by 24 h, much like the plasma stress variables. Similarly, JUNB expression in liver peaked at 3 h and recovered by 24 h when juvenile rainbow trout were stressed by a 0.5-h exposure to low water and a 30-s air exposure (Momoda et al., 2007). Likewise, red blood cell expression of this gene increased in rainbow trout at 4 h and 24 h following acute heat stress (Lewis et al., 2010). The JUNB timecourse, where peak expression can occur several hours after the stressor, points to this gene's role in the recovery process as well as other maintenance functions including arrested cell growth, apoptosis, or even cell proliferation (Piechaczyk and Farràs, 2008).

Like JUNB, cyt c expression was responsive to exercise stress, with highest values measured at 4 h for pink salmon, potentially due to the release from the mitochondria into the cytoplasm of cells undergoing apoptosis (Loeffler and Kroemer, 2000). Interestingly, Jeffries et al. (2012a) found the up-regulation of cyt c as well as JUNB in moribund sockeye salmon following chronic high temperature exposure. The finding that pink salmon showed increased expression of both cyt c and JUNB over time, but that this expression returned to control values by 24 h, suggests that while these genes may respond strongly to stress and can signal mortality, they may also recover following the stressor, indicating the role of these genes in cellular maintenance and recovery for Pacific salmon. Gene expression was not sex-specific, despite an earlier report to this effect for JUNB in immature rainbow trout (Momoda et al., 2007).

HSP90AB1 remained largely unchanged over time, perhaps suggesting that the stressor used here may not have been sufficient to induce such a response (Iwama et al., 2004). NUPR1 which is a stress-responsive transcription factor found in several tissues that responds to a range of stressors (Chowdhury and Samant, 2009; Cano and
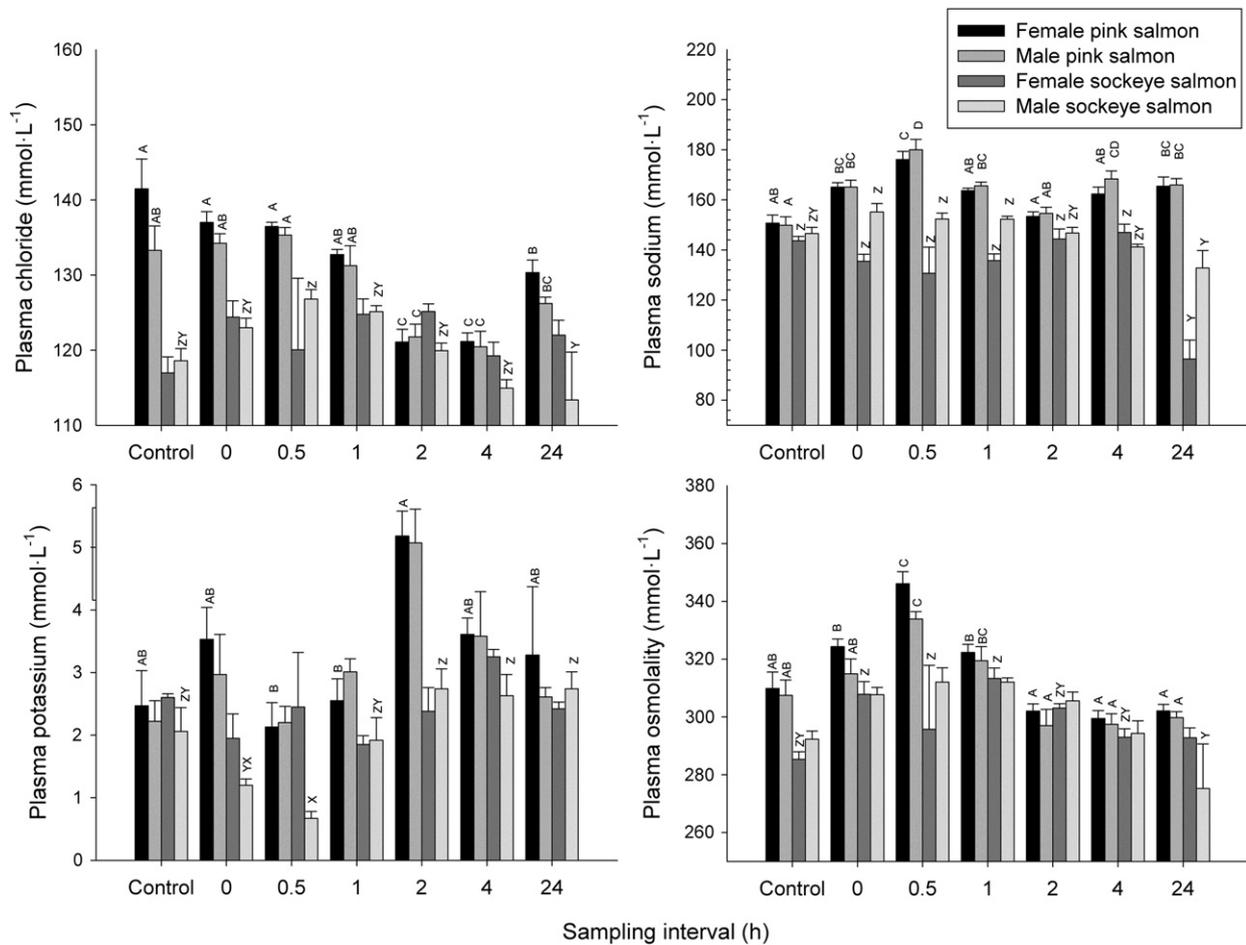
Fig. 3. Means ± SE for osmoregulatory variables plasma chloride, sodium, potassium, and osmolality for adult pink and sockeye salmon. Dissimilar letters denote statistical differences from Tukey's post-hoc tests following ANOVAs.

Hamidi, 2011), including the glucocorticoid response for rainbow trout liver (Momoda et al., 2007) and mouse pancreas (Path et al., 2004), was not reflected in PCAs. However, a lack of gene expression does not necessarily mean that there were no changes in protein expression. Likewise, there remains the possibility that these genes could be expressed differently in other tissues, since microarray studies have identified a broad suite of energy metabolism genes upregulated in rainbow trout liver following exercise/handling-related stressors (Momoda et al., 2007; Wiseman et al., 2007; Cairns et al., 2008).

4.2. Response and recovery of osmoregulatory variables

The temporary increases in plasma ions observed during the first 4 h of recovery are consistent with previous observations in freshwater fish (Wood, 1991). Plasma osmolality followed the increases in plasma solutes (lactate, sodium, and chloride), which resulted in each of these variables loading with osmolality on PCA RF2. Increased plasma ions generally paralleled the increase in plasma lactate, which is generated by glycolysis and is known to decrease muscle and blood pH (Wang et al., 1994), disrupting ion-osmoregulatory balance and shifting water from blood to muscle tissue. In both species, plasma potassium decreased at 30 min post-stress, a finding which corroborates previous work on sockeye salmon (Gale et al., 2011). Decreased plassi-um following stress could be due to the temporary re-uptake of potassi-um ions from the extracellular space (Nielsen et al., 1992). The general increase in plasma potassium over the longer term likely occurred as a result of potassium ions being gradually lost from muscle and accumu-lating in plasma (Sejersted and Sjøgaard, 2000).

4.3. Organismal stress response and recovery

Plasma lactate values from control groups for both species (~1 mmol·L⁻¹) are comparable to values expected for resting salmo-nids (Wood et al., 1983; Milligan, 1996; Barton, 2002) and are nearly identical to the control values obtained for adult coho salmon (Donaldson et al., 2010). Our results indicate that the sampling boxes did not provide any additional stress, and in fact may have provided an equivalent or even less stressful environment than the spawning channel itself, since the plasma stress values obtained from both pink and sockeye salmon were either equivalent or lower relative to samples collected from fish that were dip netted directly from the channel and sampled immediately, potentially indicating that the plasma values ob-tained here approximate the baseline for these species at this stage of maturation. For example, Hruska et al. (2010) identified that female and male sockeye salmon that were sampled from the same spawning channel as the present study (but a different year) had mean plasma cortisol concentrations of ~350 and ~91 ng/mL respectively, whereas the control female and male sockeye salmon in our study had ~207 and ~71 ng/mL, respectively. Likewise, in the same year and location as the present study, for female pink salmon, the mean plasma cortisol was ~300 ng/mL for fish captured and sampled directly from the spawning channel compared to ~84.5 ng/mL for the control group in the present study. Plasma stress variables had recovered to control values by 24 h post-stress, as expected (Wood, 1991).

The immediate response upon capture (i.e., at time zero) of plasma lactate, but not cortisol, was similar to sockeye salmon caught by either rapid angling or beach seine (~3 min) and sampled immediately

(Donaldson et al., 2011). The timing of the highest measured plasma cortisol and lactate values is consistent with literature values which typically show a peak between 0.5 h and 2 h before recovering (Wood et al., 1983; Milligan, 1996; Barton, 2002). Plasma cortisol values here compared with those observed following a similar exhaustive exercise-related stressor in sockeye salmon (Donaldson et al., 2011) but were comparatively higher relative to plasma cortisol values from other salmonid species following a confinement stressor (Pottinger, 2010), which may reflect either a higher level of stress following the experimental treatment here or a different reproductive state (see below).

Plasma glucose typically increases in response to acute stressors in a range of salmonid species (e.g., Pottinger, 2010), but has been found to remain relatively high yet stable over time in migrating Pacific salmon (Donaldson et al., 2010). The finding that female pink salmon had elevated plasma glucose following the stressor was thus somewhat atypical from other recent Pacific salmon studies. Some species-specific variability in the response and recovery of many of the variables investigated here was anticipated since identical stressors can elicit variable responses within the same family (Black, 1955; Pottinger, 2010). However, the dramatically higher magnitude of the difference

in plasma lactate and cortisol response for pink salmon was unexpected, although general increases in blood lactate have been observed previously throughout the spawning period for this species (Congleton, 1972).

4.4. Reproductive hormones and species- and sex-specific differences

Previous studies have had difficulty disentangling the influence of body size and reproductive maturity on species-specific physiological responses to stress (Pottinger, 2010). Body size can influence the physiological stress response and recovery of some species (Gingerich and Suski, 2012), but analyses did not reveal an effect of body size on response variables in the present study. Similarly, plasma indices of stress were independent of body mass for mature adult Chinook salmon (Clark and Farrell, 2011).

In Pacific salmon, reproductive hormones tend to increase progressively during maturation before rapidly decreasing immediately before spawning (Williams et al., 1986; Jeffries et al., 2011). Pink salmon did have comparatively higher plasma estradiol (females) relative to sockeye salmon, suggesting a slightly earlier state of maturation. Indeed, pink salmon were sampled 12–23 days prior to peak spawning and sockeye salmon were sampled only 1–11 days prior to peak spawning. However, since species-specific differences did not emerge as a significant factor in the reproductive hormone ANOVA, it remains unclear if maturation state alone (rather than genetic differences, for example) explains sex-specific differences in the response and recovery of the other plasma variables. Regardless, the heightened response to exhaustive exercise stress of less mature pink salmon is consistent with a pattern described for rainbow trout, where maturity and higher sex hormone concentrations moderate the stress response (Pottinger et al., 1995, 1996).

Reproductive hormones can become depressed by stress (Pickering et al., 1987; Schreck et al., 2001). This appeared to be the case for estradiol in female sockeye salmon here, which is relevant due to the essential role that reproductive hormones play in the final stages of maturation and senescence for spawning Pacific salmon (Hruska et al., 2010; Jeffries et al., 2012b). Several studies on Pacific salmon have identified that females typically experience higher mortality compared with males (Patterson et al., 2004; Crossin et al., 2008; Jeffries et al., 2012b; Martins et al., 2012; Robinson et al., 2013) and it has been suggested that female salmon have less capacity to cope with environmental stressors (Clark et al., 2011b). Previously identified sex-specific differences in maturing Pacific salmon include higher heart rates, plasma cortisol, lactate, glucose, testosterone and estradiol in female sockeye
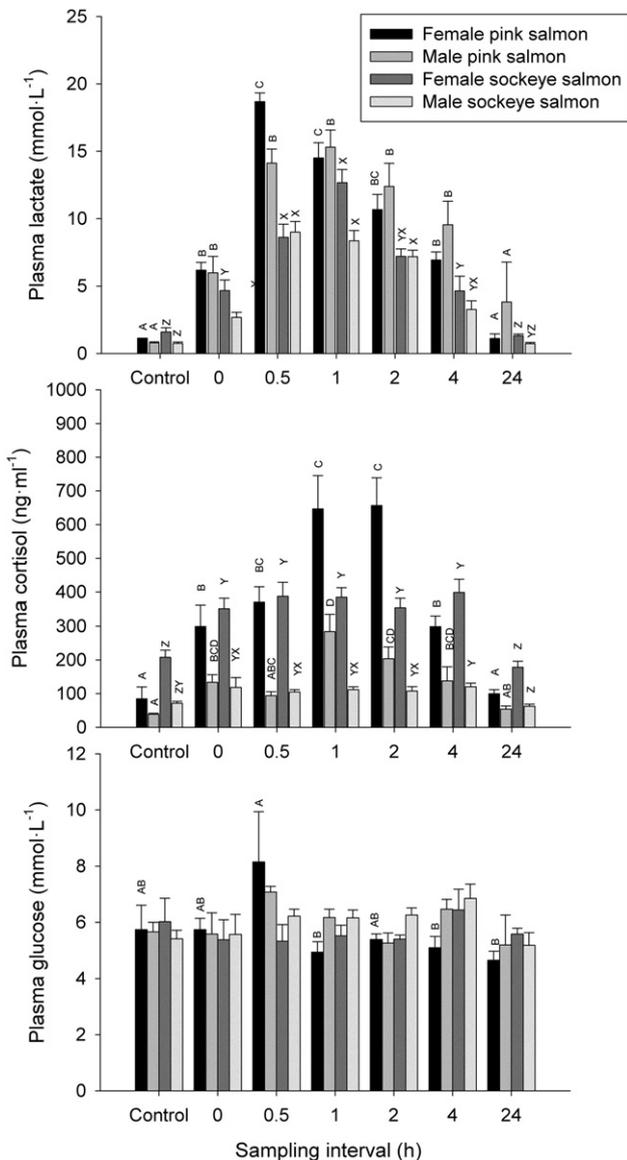

Fig. 4. Means ± SE for organismal stress variables plasma lactate, cortisol, and glucose for adult pink and sockeye salmon. Dissimilar letters denote statistical differences from Tukey's post-hoc tests following ANOVAs.
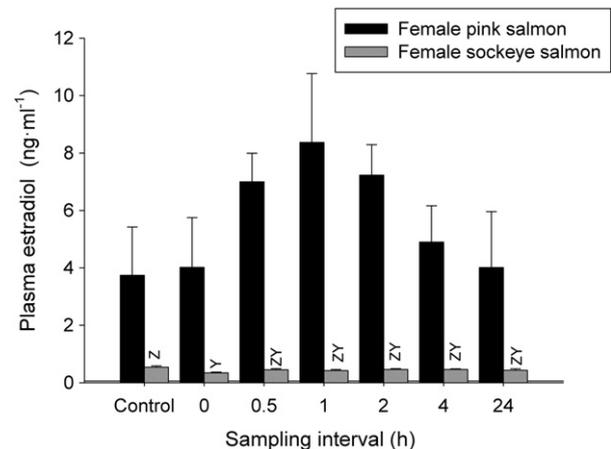

Fig. 5. Means ± SE for reproductive hormone variable plasma estradiol for adult female pink and sockeye salmon. Dissimilar letters denote statistical differences from Tukey's post-hoc tests following ANOVAs.

salmon relative to males (Sandblom et al., 2009; Jeffries et al., 2012b). Sex-specific differences for plasma cortisol are particularly well known and have been observed in acute and chronically stressed rainbow trout and Pacific salmon (Fagerlund, 1967; Pottinger et al., 1995, 1996; Sandblom et al., 2009). Sex-specific differences can even be observed under routine conditions, for example, control female and male coho salmon had ~200 and ~100 ng·mL^{-1} plasma cortisol, respectively (Donaldson et al., 2010), an approximate 2:1 ratio for plasma cortisol in females and males, which compares with control values for pink and sockeye salmon in the present study.

5. Conclusions

While there were some similarities in how pink and sockeye salmon responded and recovered to a controlled exhaustive exercise and air exposure stressor, the magnitude of response and patterns of recovery differed between species. The more rapid and stronger stress response of pink salmon, as indicated by the accentuated plasma cortisol and lactate, osmoregulatory disturbances, and the higher and more variable gene expression of sockeye salmon may be related to differences in reproductive state between species, although inherent species-specific differences remain a possibility. JUNB and cyt c (pink salmon only) responded to stress but recovered by 24 h, mimicking the general recovery pattern of plasma stress variables and providing new information on how these genes respond and recover following exhaustive exercise stress in wild fish. The magnitude of the plasma stress response was generally higher in females compared to males for both species. Collectively, these results provide new data on the species- and sex-specific nature of how wild, maturing Pacific salmon respond to and recover from fisheries-related exhaustive exercise stress and air exposure. Future research could aim to distinguish whether or not the sex- and species-specific patterns identified here relate to the survival of released fish, which could be important for enhancing Pacific salmon fisheries management.

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