Effects of Experimental Manipulations of Salinity and Maturation Status on the Physiological Condition and Mortality of Homing Adult Sockeye Salmon Held in a Laboratory

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ABSTRACT

Relatively little is known about the physiological response and mortality consequences of the return of anadromous fish to freshwater (FW). We explored the consequences of the return to FW by collecting maturing sockeye salmon from the marine waters off the mouth of the Fraser River and holding ~50 sockeye in each of five treatments: saltwater (SW; salinity = 28 ppt), iso-osmotic water (ISO; 13 ppt), FW (0 ppt), SW + gonadotropin-releasing hormone (SW + GnRH), and FW + GnRH. Exogenous GnRH treatments were intended to accelerate maturation. Results demonstrate that gill Na^+,K^+ ATPase activity, sex steroid concentrations, and cortisol levels were highly responsive to experimental manipulations and followed predicted trajectories (i.e., FW + GnRH sockeye were the most mature and FW adapted). There were few among-treatment differences in hematocrit and plasma concentrations of lactate, glucose, Na^+, Cl^-, and plasma osmolality among sockeye that survived to the end of treatments, indicating that sockeye rigorously maintain internal homeostatic conditions while alive. There were large among-treatment differences in mortality (SW + GnRH > SW > FW + GnRH > FW = ISO), and each treatment experienced a notable increase in mortality rate around the fifth day of treatment. Our results indicate that salinity represented a modestly larger challenge to the experimental sockeye than did the artificially accelerated sexual maturation. Our results also suggest that maturing sockeye either successfully acclimate to FW within 5 d of exposure or perish. These findings are consistent with the predictions of the theory of anadromy, in suggesting that the return of adults to FW can be physiologically challenging and can represent a period of significant natural mortality.

Introduction

Anadromous adult salmon return to natal rivers to reproduce (Ueda and Yamauchi 1995; Høgåsen 1998). This migration and the associated sexual maturation are under the control of the hypothalmus-pituitary-gonadal (HPG) axis (Fukaya et al. 1998; Kitahashi et al. 1998*a*; Mylonas and Zohar 2001). Activation of the HPG is likely tied to photoperiod (Hasler and Scholtz 1983; Hinch et al. 2006) and occurs well before salmon leave the open ocean. Maturation involves the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which triggers increases in circulating concentrations of the sex steroids testosterone, 11-keto testosterone (males), and estradiol (females). Exogenous application of GnRH can trigger HPG activation and accelerate maturation and the timing of freshwater (FW) entry and upstream movement in many species of

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salmonids (Amano et al. 1997; Fukaya et al. 1998; Kitahashi et al. 1998*b*; Mylonas and Zohar 2001).

While in open ocean, adult anadromous salmon alter their ionoregulatory physiology in preparation for their return to FW. Stimulation of the hypothalmus-pituitary-interrenal axis produces an increase in plasma cortisol concentrations, along with changes in other compounds associated with FW entry (Clarke and Hirano 1995; McCormick 2001; Mancera and Fuentes 2006; Norris and Hobbs 2006; Makino et al. 2007). Included among the physiological responses are a shift in the number, distribution, and function of gill chloride cells (Uchida et al. 1997) and the downregulation of gill Na^+,K^+ ATPase activity (Shrimpton et al. 2005). Gill Na^+,K^+ ATPase activity in Fraser River sockeye decreased approximately 35% during movement from 800 to 300 km offshore, followed by an additional 50% decline on FW entry (Shrimpton et al. 2005). Cortisol release in adult salmonids functions in multiple additional pathways during the return migration, including maturation (Carruth et al. 2000; Wingfield and Sapolsky 2003), the transition from anabolic to catabolic metabolism (Dickhoff et al. 1989; Norris and Hobbs 2006), and olfaction-based natal homing (Ueda et al. 1996; Carruth et al. 2002). Maturation and the associated ionoregulatory changes result in the progressive loss of saltwater (SW) tolerance in maturing salmon (Ueda and Yamauchi 1995; Makino et al. 2007), and forced holding of maturing salmon in SW can retard maturation (Sower et al. 1982), reduce gamete quality (Wertheimer 1984), and cause mortality (Hirano et al. 1990; Slater et al. 1995).

The movement of diadromous fishes between hyper- and hypo-osmotic environments is a challenge to homeostasis (Wendelaar Bonga 1997; Wikelski and Cooke 2006). The FW to SW transition for Pacific salmon (*Oncorhynchus* spp.) smolts has been intensively studied (Folmar and Dickhoff 1980; McCormick and Saunders 1987; Thorpe 1994; Høgåsen 1998; Bjornsson and Bradley 2007) and is known to be a time of high mortality, likely associated with the combined effects of physiological stress and behavioral alterations, leading to increased susceptibility to predators (Hendry et al. 2004). In contrast, comparatively little is known about the physiological response of adult salmon returning to FW to spawn (Clarke and Hirano 1995; Høgåsen 1998; Hinch et al. 2006), and even less is known about the survival implications of this transition.

Herein, we describe results of an experiment designed to characterize the physiological responses and mortality associated with the return of maturing salmon to FW, part of a larger interdisciplinary research program focused on understanding the migration biology of Pacific salmon (Cooke et al. 2008). Our experiment involved collecting maturing sockeye salmon from the marine waters off the mouth of the Fraser River and holding the sockeye in tanks of different salinity levels for 1 wk (FW = 0 ppt, iso-osmotic (ISO) = 13 ppt, SW = 28 ppt), with subgroups of the FW and SW fish being treated with exogenous GnRH (SW + GnRH, FW + GnRH). We predicted that SW + GnRH and FW would be the most stressful treatments (i.e., highest mortality rate and stress metabolites, plasma ions, and osmolality values), as a result of the loss of SW tolerance caused by advanced sexual maturation in the former case and the rapid SW to FW transfer occurring before preparations for FW entry were complete in the latter case. We also predicted that the SW and FW + GnRH treatments would be the least stressful because the former represented continued holding in the same environment from which the sockeye were collected (i.e., the experimental control), while the latter provided accelerated maturation, which should equate with accelerated FW adaptation. We discuss our results in the context of how maturation level at the time of the SW to FW transition affects response to the ionoregulatory challenge and how physiological condition can be used to predict and evaluate the survivorship of homing anadromous salmonids during the SW to FW transition.

Methods

Sockeye Collection and Preparation

We chartered a commercial purse seine fishing vessel for collecting sockeye salmon from the Strait of Georgia (SOG) near the mouth of the Fraser River (Fig. 1). Fish were collected daily from August 29 to August 31, 2006. On each day, we collected both baseline and experimental specimens. The former were the first 15–30 sockeye landed each day, and these were killed immediately for physiological biopsy, which included all variables measured on experimental fish plus an extensive sequence of additional sampling (Patterson et al. 2007). The latter were placed directly into the seiner's live well within 5 min of pulling the seine net alongside the vessel. The live well was filled with circulating water drawn from approximately 1 m below sea surface, and oxygen levels were maintained at near saturation via aerators providing compressed oxygen. Each day's fishing operation ceased when 100–150 live sockeye were onboard, at which time the boat traveled approximately 1 h to the site of the experiment at the Fisheries and Oceans Canada Centre for Aquaculture and Environmental Research (CAER).

At the CAER, fish were off-loaded to truck-mounted oxygenated live wells in small batches and driven approximately 200 m to biopsy stations located next to experimental tanks. Fish were individually dipnetted from live wells and placed into one of two V-shaped, foam-lined biopsy troughs equipped with a continuous flow of SOG water (water temperature 11°C) directed into the mouth and across the gills. While in the trough, each fish had an external cinch tag, containing a unique identifying number, inserted through the dorsal musculature anterior to the dorsal fin, and we collected an adipose fin punch for DNA-based stock identification. The DNA approach has 96% accuracy to the individual stock level (Beacham et al. 2004). Fish were sequentially assigned to one of five treatments and as applicable were injected with one 150-μg implant tablet of Syndel Laboratory's (Qualicum Beach, British Columbia) Ovaplant, a commercially available and widely used salmon analogue GnRH, into the dorsal musculature approximately 4 cm behind the dorsal fin. We did not use sham injections as a

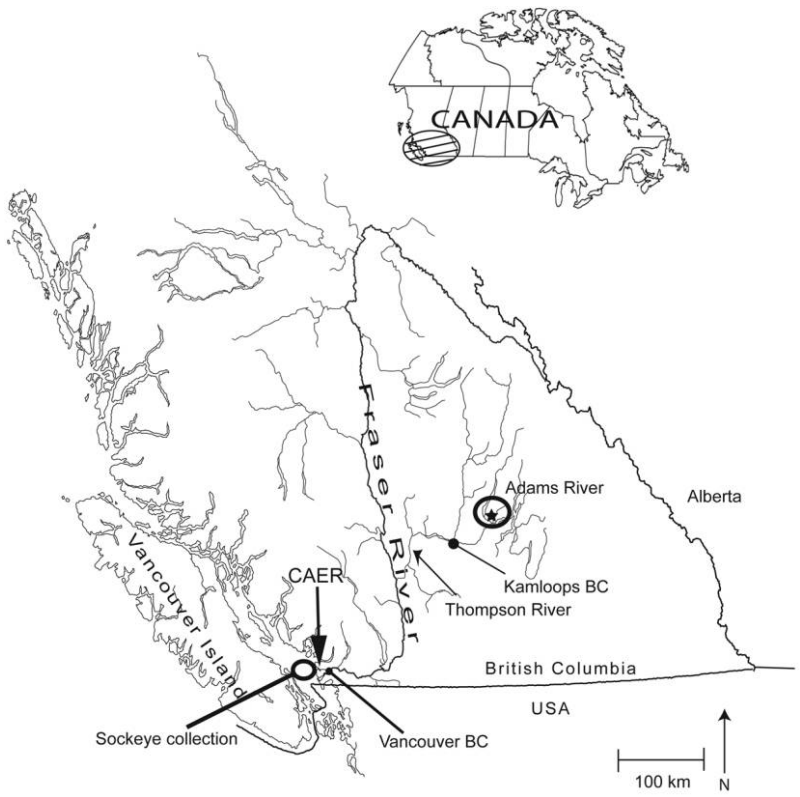

Figure 1. Lower third of British Columbia, Canada, incorporating the Fraser River watershed, Vancouver Island, and the Strait of Georgia. Labeled elements include the site of sockeye collection within the Strait of Georgia, the city of Vancouver at the mouth of the Fraser River, the site of the experiment at the Centre for Aquaculture and Environmental Research (*CAER*), the city of Kamloops at the confluence of the North and South Thompson rivers, and the spawning grounds for Adams River sockeye.

control because of potential consequences of the physical trauma of the GnRH injections. Justifications for this decision include that Ovaplant is widely used in research and commercial aquaculture applications and there has been no evidence of an adverse effect of injection (J. Powell, Snydel, unpublished proprietary data), the wound associated with cinch tagging is large compared with that caused by GnRH injection and therefore all fish were assumed to have a comparable level of wound response, and the conditions of our University of British Columbia Animal Welfare and Care permit mandated that we limit harm and stress to fish whenever possible. Effects of exogenous GnRH on circulating hormone concentrations can become manifest within hours of treatment (Mylonas and Zohar 2001). On completion of preparatory efforts, fish were placed into the assigned experiment tank. Moribund sockeye and those with visible physical wounds or sea lice infestation were excluded before entering the tanks ($n = 6$ of 351). Typical processing time for an individual fish was less than 2 min from removal from the transport truck to placement into the experimental tank, with less than 20 s of air exposure.

Stocking density within each tank was one sockeye per 0.033 m^3 water, equating to 50 fish in each of four 3-m-diameter tanks and 73 fish in the one 3.7-m-diameter tank (treatment iii, SW; see below). Treatment tanks were located outdoors and equipped with mesh covers allowing for a natural photoperiod, and tanks were circular, with water inflows directed in such a manner as to generate modest current to allow fish to orient into flow. Because the experiment used the entire large-fish-rearing capacity and the entire available SW and FW supplies of the CAER, the only facility within the region with the infrastructure to support an experiment of this magnitude, there was no ability to expand the scope of work to include replicate treatment tanks. During the 3-d fish-collection and tank-stocking period, all experimental tanks were filled with SOG water (salinity 28 ppt, temperature 11.0°C), with a flow-through rate of 150 L min^{-1}. Food was not provided because sockeye at this stage of their maturation process ceased feeding approximately 3 wk before collection. Mortalities occurring during the first or second day of the stocking sequence were recorded, removed, and replaced with fish collected on the third day. In total, we landed 351 sockeye during our 3-d sampling period, of which 71 were immediately killed as baseline samples and 273 were included in experimental treatments.

Experimental Treatments and Posttreatment Biopsy

Full stocking of the experimental tanks occurred by noon on August 31. On the morning of September 1, water inflows to

tanks were adjusted to attain treatment salinities at 48 h, and we defined this as the first day of the treatment period. For the duration of the experiment, mortalities were removed from experimental tanks without replacement. The five experimental treatments were (i) FW (salinity = 0 ppt), (ii) ISO (salinity = 13 ppt; derived from a mix of FW and SOG water), (iii) SW (salinity = 28 ppt), (iv) FW + GnRH, and (v) SW + GnRH. FW supply was from Cypress Creek, which is adjacent to the CAER, and SW was SOG water available at CAER. All tanks were maintained at 150-L-min^{-1} flow-through rates. We used an optical salinity meter and digital thermometer/dissolved oxygen probe to monitor water quality at the outflow of each tank three times per day and made minor adjustments to inflows as needed to maintain desired salinity concentrations and dissolved oxygen levels at >75% saturation. Water temperatures within treatment tanks ranged from 10.7° to 13.9°C, with SW treatments over the duration of the experiment having mean temperatures (11.7°C) lower than those of FW treatments (12.7°C). These temperatures are routinely experienced by Fraser River sockeye at this time of the year during their SW to FW transition (Patterson et al. 2007).

Aside from carcass removal and supplemental SW + GnRH treatment, experimental tanks and fish were left undisturbed for the duration of the weeklong treatment period. In order to supplement small sample sizes resulting from high mortality of SW + GnRH sockeye, on September 4 we selected 17 sockeye from the SW treatment (the 3.7-m tank stocked with 73 sockeye) for GnRH injection. However, 15 of the 17 died within 96 h of injection, and we exclude these 17 supplemental SW + GnRH fish from subsequent analyses. It is important to note that because experimental fish that survived the treatment period were to be fitted with telemetry transmitters and released to the SOG as part of a companion study (Hinch et al. 2008), at 1 d before the end of the treatment period, salinities within the FW and ISO tanks were ramped to full-strength SOG water (28 ppt) at a rate of 2-ppt salinity increase per hour, with 28 ppt attained approximately 12 h before physiological samples were collected. This salinity exposure history generates sockeye representative of river entry because telemetry tracking studies demonstrate that late-run sockeye routinely move between the FW of the Fraser River and the full-strength salinity of the SOG in the days before committing to the upriver migration (Cooke et al. 2006*a*).

The ISO, SW, and SW + GnRH treatments ended September 7, and the FW and FW + GnRH treatments ended September 8. At the end of each treatment, each surviving fish was subjected to nonlethal physiological biopsy (details in Cooke et al. 2005). Briefly, individual salmon were placed in a padded V-shaped trough provided with a continuous supply of SOG water from a tube near the salmon's mouth. We measured fork length, used a handheld microwave energy meter to determine gross somatic energy (GSE; MJ kg^{-1}; Crossin and Hinch 2005), and collected a 1.5-mL blood sample and a <4-mm clip of gill filament tips (~0.03 g; McCormick 1993). Blood samples were held in an ice water slurry for no more than 10 min and then centrifuged to separate plasma from formed elements. We used a handheld ruler to determine hematocrit value as the ratio of plasma to formed elements (expressed as the percentage of total sample volume as formed elements) and then pipetted the plasma fraction into cryovials for storage. Plasma and gill samples were placed on dry ice until transferred to a −80°C freezer. Handling times for the entire biopsy procedure for each fish were typically less than 2 min. All biopsies occurred between 10 a.m. and 3 p.m. During the course of the treatment period, we attempted to collect gill and blood samples from fish that died during the treatment period, but because of rapid blood clotting within carcasses, these samples were rarely of sufficient quality (i.e., only 27 of 109 treatment period mortalities yielded blood samples, and only nine of these processed correctly; no gill samples for Na^+,K^+ ATPase processed correctly), and these data are not presented here. Except where noted, all physiological data reported herein are from samples collected from fish that survived to the biopsy event at the end of the weeklong treatment period.

In the laboratory, we used procedures described by Farrell et al. (2001) to determine plasma osmolality and the concentration of Na^+ and Cl^- ions and the concentration of plasma metabolites known to alter in response to stress (glucose, lactate, and cortisol). We used a kinetic assay to determine gill Na^+,K^+-ATPase activity (McCormick 1993), and we used radioimmunoassay (McMaster et al. 1992) to determine plasma concentrations of testosterone and 17β-estradiol (E2). We used E2 data to assign sex to experimental fish because sexual dimorphism was not yet fully expressed at the time of biopsy, with males having nondetect E2 values. Plasma E2 concentrations of baseline sockeye of known sex (via gonadectomy) correctly sexed 70 of 71 individuals (D. Patterson, unpublished data). In total, each biopsied fish was evaluated for hematocrit (HCT), plasma lactate (LAC), glucose (GLU), cortisol (CORT), sodium (Na^+), chloride (Cl^-), osmolality (OSMOL), testosterone (T), estradiol (E2), and gill Na^+,K^+ ATPase activity (ATPase).

Data Analysis Overview

We focused all analyses just on fish from the Adams River and Little River–Little Shuswap populations. These populations are part of the Fraser River late-run stock complex and are the dominant component of the samples we collected (51 baseline and 235 experimental fish were from this group). These populations reproduce in tributaries of the Thompson River (a Fraser River tributary; Fig. 1) and have similar life histories and reproduction schedules (D. Patterson, unpublished data). For simplicity, we refer to these as Adams sockeye. Because DNA-derived stock ID for each individual included in the experiment was not available until after experimental treatments had begun, we could not ensure equal numbers of Adams sockeye within each treatment, but each treatment began with >30 Adams sockeye. Except as otherwise noted, physiological variables used in statistical tests presented herein were $\log_{10}$ trans-

formed, except for HCT, which was arcsin transformed. All statistical tests were evaluated at $\alpha = 0.05$, and we used Wilks's lamba as the test statistic for all MANOVAs.

Preliminary analyses (i.e., ANOVA analyses, all P values >0.100) indicated that male and female baseline sockeye did not differ in measured physiological variables (excluding sex steroid concentrations) nor were there differences between the sexes in end-of-treatment-period physiology within any treatment. Physiology of baseline fish did not differ between collection dates (Aug. 29, 30, or 31; M. S. Cooperman, unpublished data); whether experimental fish spent 1, 2, or 3 d in experimental tanks filled with SOG water during the pretreatment tank-stocking period was not related to either within-treatment mortality or the physiology of experimental fish at the end of the treatment period. Time an individual sockeye spent onboard the fishing vessel (i.e., the order of off-loading and placement into tanks) was also not related to mortality or end-of-treatment physiology (Hinch et al. 2008; M. S. Cooperman, unpublished data). A χ^2 test with Yates's correction indicated that females died at greater rates than males in two of the five treatments, but there was no effect of sex on mortality in the other three treatments (see Table 1). On the basis of these preliminary results, we treated experimental fish as equivalent regardless of capture date or sex in all subsequent analyses, except as noted for the role of sex on GSE.

Analysis of Treatment Effects on Mortality and Physiology

We calculated within-treatment cumulative mortality at a daily time step for each of the five treatment groups as cumulative no. dead on day $X_{(x=1-8)}$/no. at start of treatment. We then plotted cumulative mortality as a function of day of treatment, and we visually assessed the mortality plots to evaluate among-treatment differences and the relative contribution of salinity and GnRH treatments to observed mortality. We used MANOVA to test for among-treatment differences in physiology. The grouping variable was treatment (SW + GnRH, SW, ISO, FW, FW + GnRH), and model affects were LAC, GLU, CORT, Cl^-, Na^+, OSMOL, and ATPase. HCT was excluded because of 10 of 122 cases missing data values, and T and E2 were excluded because these values were directly manipulated via GnRH treatment. We did not include length as a covariate in statistical tests involving experimental fish because fish were randomly assigned to treatments and lengths did not differ among treatments (one-way ANOVA of fork length among treatments: $P = 0.597$). Similarly, we did not use sex as a covariate because of lack of a significant sex effect on physiology (see "Results"). We followed the MANOVA with a sequence of one-way ANOVAs to describe univariate among-treatment differences in each of our physiological variables. Each ANOVA included the baseline fish and the five treatments. When ANOVA indicated a significant difference, we used Fisher's least significant difference multiple range test to evaluate which groups accounted for observed differences. Cases missing data values for any variable were excluded from ANOVA. We constructed side-by-side box plots for each physiological variable used in the ANOVAs to provide for visual comparisons. Because cortisol serves in multiple physiological pathways, including osmoregulation and sexual maturation, we executed a multifactor ANOVA with Type III sum of squares (i.e., the effect of one factor is evaluated after removing the effect of the other), with treatment as the grouping variable (baseline samples excluded), CORT as the main effect, and T as the covariate, to distinguish whether CORT concentrations varied as a function of maturation or salinity conditions.

We used Type III sum-of-squares ANOVA with Duncan's multiple range test to evaluate among-treatment differences in GSE (baseline sockeye not included). Gender was not included in the ANOVA because Crossin et al. (2007) demonstrated no between-sex differences in energy density within several stocks of Fraser River sockeye. Although treatment-specific means and standard errors and results of the statistical test are presented herein, we exercise caution in interpreting results, given that ISO, FW, and FW + GnRH fish were reverted to SW 12 h before energy density values were measured. The resulting osmotic acclimation to the salinity change may lead to short-term alteration in the water content of reverted individuals, which would confound energy values because the energy probe works via microwave readings of internal water content, which varies inversely to fat content (i.e., energy). Comparisons

Table 1: Total number of male and female Adams sockeye present at the start of each treatment and the numbers surviving to the end of each treatment

Treatment	No. at Start (Males : Females)	No. Survivors (Males : Females)	χ^2 Test	
			Test Statistic	*P* Value
Baseline	51 (23 : 28)	NA	NA	NA
SW + GnRH	36 (18 : 18)	7 (1 : 6)	2.84	.092
SW	41 (25 : 16)	24 (19 : 5)	6.31	.012
ISO	37 (30 : 7)	33 (29 : 4)	5.55	.018
FW	34 (21 : 13)	31 (21 : 10)	2.83	.092
FW + GnRH	36 (19 : 17)	23 (13 : 10)	.06	.802

Note. Also presented are results of a χ^2 test with Yates's correction to assess gender differences in mortality within each treatment, with P values ≤0.05 indicating a significant effect of gender. SW = saltwater; GnRH = gonadotropin-releasing hormone; ISO = iso-osmotic water; FW = freshwater.

among treatments of similar salinity exposures are nonetheless appropriate to make (i.e., FW vs. FW + GnRH) because prior research has not identified a maturation × osmoregulation interaction, except during late senescence (i.e., immediately before and after spawning; Crossin et al. 2007).

We executed a nonmetric multidimensional scaling ordination (NMS; PC-Ord, ver. 5.0) to illustrate among-treatment differences in multivariate physiology. We used the nonparametric NMS because it is well suited to multivariate data where variables are measured on dissimilar scales and, because NMS is based on ranked similarities between groups, it relaxes the assumption of a linear relationship between independent and dependent variables and preserves between-group differences (Clarke 1993; McCune and Grace 2002). Variables included in the ordination were HCT, LAC, GLU, CORT, OSMOL, ATPase, and T. In order to avoid negative values in the data matrix, we used $\log_{10}+1$ transformation of LAC, GLU, ATPase, and T. We did not include E2 data in the ordination because most males (62 of 83) had nondetect values, and we excluded Cl^- and Na^+ because of high colinearity with each other and OSMOL (correlation coefficients: Cl^- : Na^+, 0.70; Cl^- : OSMOL, 0.93; Na^+ : OSMOL, 0.73; all correlation P values <0.001). In cases where individual data values were missing, we substituted treatment-specific mean value, and no treatment had more than one missing value within a variable. We did not include baseline fish in the ordination in order to ensure maximal among-treatment separation in the solution. Before ordination, we applied a general relativization by column totals, and we tested for outliers (>2 SD from the multivariate mean). No cases were flagged as outliers, so all 122 fish that survived to the end of the treatment period were included. We used Euclidean distance and the "slow and through" autopilot setting with 250 runs with real data and random start configurations and 250 runs per tested dimension Monte Carlo simulation with randomized data to determine the number of dimensions and final configuration to use in the solution. We applied orthogonal varimax rotation to the solution to maximize the correlation of ATPase along the first axis.

Results

The ISO and FW treatments experienced the lowest total mortality over the duration of the treatment period (15.0% and 15.4%, respectively), FW + GnRH experienced 35% mortality, SW had 40% mortality, and SW + GnRH yielded the highest total mortality (80%; Fig. 2). SW treatment contributed to approximately 2.5 times greater mortality than did FW (15% mortality in FW vs. 40% in SW = 2.7 times difference; 35% mortality in FW + GnRH vs. 80% in SW + GnRH = 2.3 times difference; mean contribution of salinity = 2.5). Meanwhile, GnRH treatment contributed to approximately 2.15 times greater mortality rate than did salinity-only treatments (15% mortality in FW vs. 35% in FW + GnRH = 2.3 times difference; 40% mortality in SW vs. 80% in SW + GnRH = 2.0 times difference; mean contribution of GnRH = 2.2).

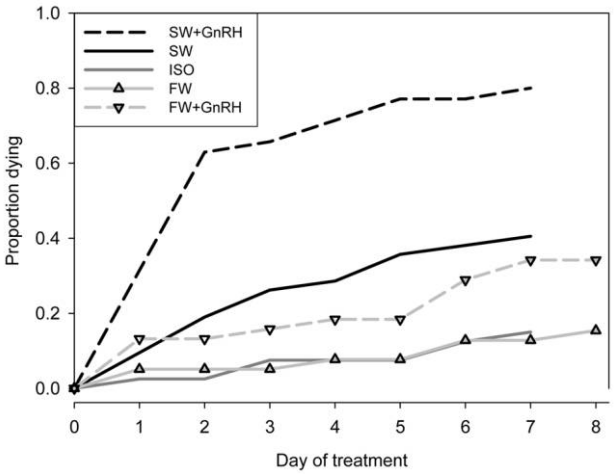

Figure 2. Cumulative mortality plots for Adams sockeye used in each of the five experimental treatments. Number of sockeye present at the start of each treatment is presented in Table 1. Line color denotes salinity (*black* = saltwater [SW], *dark gray* = iso-osmotic water [ISO], *light gray* = freshwater [FW]), while dashed lines are treatments including exogenous gonadotropin-releasing hormone (GnRH). Salinity transitions from SW to ISO or FW began on day 0 and were at target concentrations by day 2, and the return to full SW started on day 7 and was completed by day 8.

In four of the five treatments, the highest daily mortality rates occurred at the start of the treatment period, as SW + GnRH, SW, FW, and FW + GnRH each experienced their peak daily mortality rate on the first day of treatment (31.5%, 9.5%, 5.1%, and 13.2%, respectively). SW and SW + GnRH had similar mortality on the second day of treatment, whereas FW and FW + GnRH mortality fell to 0 on the second treatment day and did not experience second-highest daily mortality rates (5% in both cases) until the sixth day of treatment. In contrast, ISO experienced its highest daily mortality rate (5%) on the third day of treatment, and this level also occurred on the sixth treatment day.

There were large among-treatment differences in end-of-treatment physiology (MANOVA, $F = 7.71$, $P < 0.0001$). ANOVAs revealed that among-treatment differences existed in each of the 10 measured variables (all ANOVAs, $P < 0.0004$; Fig. 3). Further, within each variable, treatments typically yielded sockeye that were physiologically different from baseline condition. GnRH-treated fish had greatly elevated levels of T and E2 compared with those in salinity-only treatments, but GnRH treatment did not elevate hormone levels above baseline concentrations. In contrast, salinity-only treatments had hormone concentrations below those observed in baseline fish (Fig. 3*A*, 3*B*; Table 2). CORT increased as a function of declining salinity (Fig. 3*C*) as experimental fish tended to have CORT concentrations higher than those of baseline fish, but the differences were statistically significant only in fish exposed to ISO or FW treatments (Fig. 3*C*). There was no clear affect of GnRH on CORT values (Fig. 3*C*). Multifactor ANOVA of CORT and T indicated that among-treatment differences in CORT were

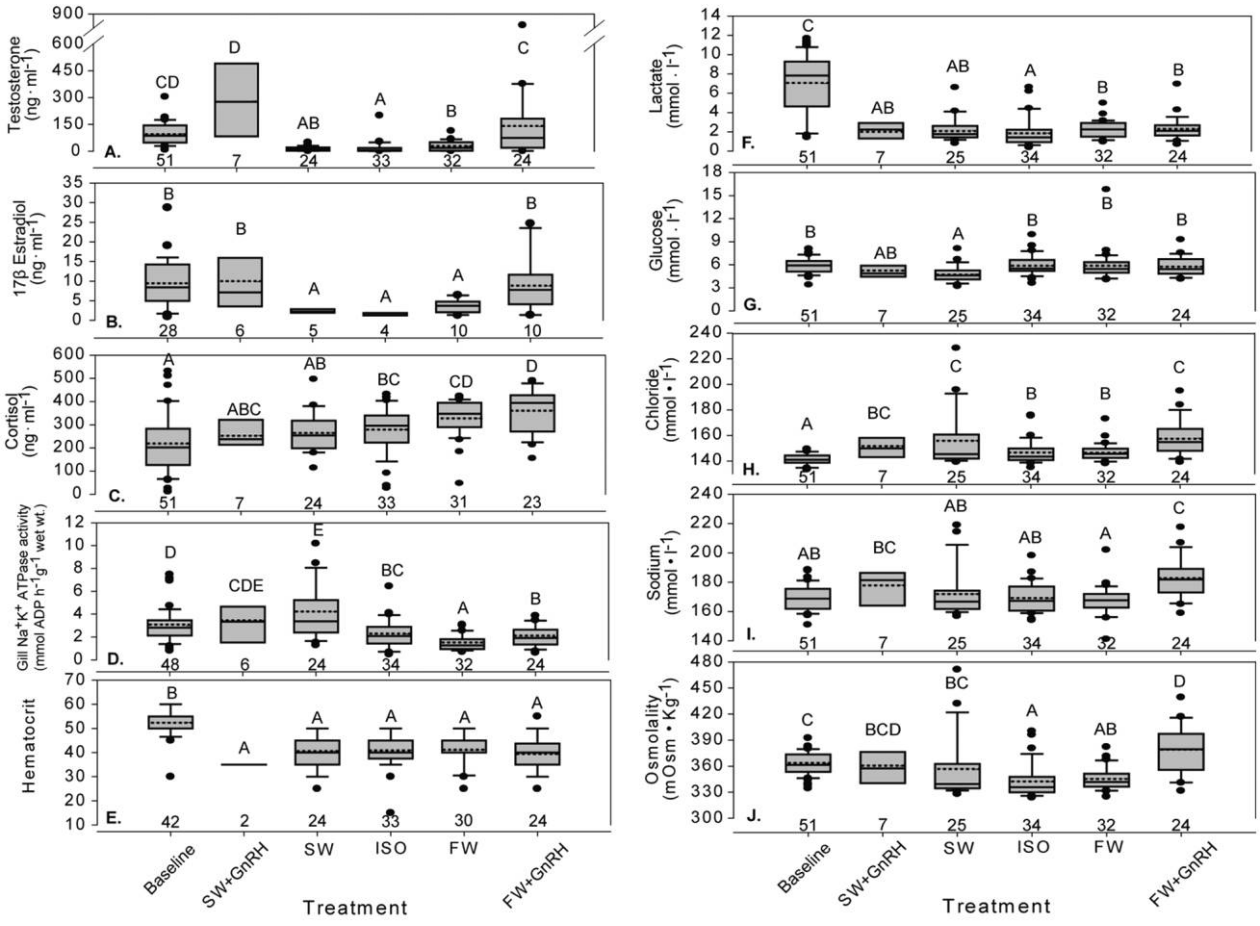

Figure 3. Side-by-side box plots of measured physiological variables of baseline sockeye and experimental sockeye that survived to end-of-treatment biopsy. Boxes are the interquartile range, upper and lower whiskers are the ninetieth and tenth percentiles, respectively, and data points are outlying values. Solid lines in boxes are sample medians, and dashed lines are sample means. Numbers along *X*-axes are sample sizes, and letters above boxes present results of Fisher's least significant difference multiple range test ($\alpha = 0.05$), where groups sharing common letters within a variable did not differ; 17β-estradiol data are for females only, while all other variables used all available data. *SW* = saltwater; *GnRH* = gonadotropin-releasing hormone; *ISO* = iso-osmotic water; *FW* = freshwater.

independent of T values (multifactor ANOVA: main effect CORT, $F = 7.94$, $P < 0.000$; covariate T, $F = 0.37$, $P = 0.541$). ATPase activity declined with decreasing salinity as only fish held in FW or ISO had ATPase values lower than those of baseline fish (Fig. 3*D*). GnRH treatment was associated with modest increases in ATPase activity (Fig. 3*D*). HCT values did not differ among treatment groups, but all treatments had lower values than did baseline samples (Fig. 3*E*). For stress metabolites, LAC values were notably lower in experimental fish relative to baseline, but there were no large among-treatment differences, whereas GLU concentrations were largely similar among baseline and treatment groups (Fig. 3*F*, 3*G*). Among-treatment differences in plasma ion and OSMOL values were minimal, but ISO and FW treatments yielded lower than baseline OSMOL, while GnRH treatment of FW-held fish was associated with an increase in the plasma ions Cl^- and Na^+ (Fig. 3*H*–3*J*).

GSE differed among the five treatment groups (ANOVA: $F = 3.07$, $P = 0.020$). SW fish had a higher mean energy density than did both the SW + GnRH and FW + GnRH groups, with ISO and FW values intermediate (Table 3). GnRH treatment was associated with a decline in GSE because mean energy levels in SW + GnRH were 6.1% lower than in SW and because the FW + GnRH mean was 2.2% lower than in FW, although only the SW : SW + GnRH comparison significantly differed (Table 3).

NMS ordination required 91 iterations to produce a stable three-dimensional solution with a final stress of 6.78 and an instability of >0.000 as the best fit to the available data (Fig. 4). Within NMS, stress is a measure of the suitability of the solution because it indicates how well the solution reflects the structure of the original data set following the reduction in dimensionality. Instability is a measure of the magnitude of fluctuations in stress over the last 10 iterations of the ordination. The final stress and instability of our solution are indicative of a stable solution with low risk of drawing false inferences. Monte Carlo simulation with 250 runs of randomized data indicates that our solution had lower stress than ex-

Table 2: End-of-treatment mean (±1 SD) testosterone and 17β-estradiol concentration data (ng mL^{-1}) for Adams sockeye

	Male		Female		
Treatment	*n*	Testosterone	*n*	Testosterone	17β-estradiol
Baseline	23	61.3 (45.1)	28	127.8 (52.7)	9.5 (6.2)
SW + GnRH	1	276.2 (…)	6	281.5 (226.2)	9.9 (8.5)
SW	19	12.3 (9.9)	5	19.0 (18.7)	2.4 (.5)
ISO	29	16.3 (37.8)	4	28.1 (21.6)	1.5 (.4)
FW	21	15.9 (18.4)	10	51.0 (29.7)	3.6 (1.7)
FW + GnRH	13	57.2 (55.1)	10	242.3 (234.1)	8.8 (6.7)

Note. Male estradiol data are not presented because 62 of 83 males had nondetect values (detection limit = 0.4). SW = saltwater; GnRH = gonadotropin-releasing hormone; ISO = iso-osmotic water; FW = freshwater.

pected by chance (mean stress of Monte Carlo simulation, 9.80; test of difference between Monte Carlo and real data, $P = 0.0040$). Cumulative variance explained by the solution was 96.8%, with axis 1 explaining 24.2%, axis 2 20.5%, and axis 3 52.1%. ATPase was the only variable of the seven used in the analysis to load on axis 1 with correlation $\geq \pm 0.5$, and axis 1 presents a gradient of FW to SW treatment, although there is a modest amount of salinity overlap along the axis. Axis 2 appears to represent stress level because LAC and CORT were the only variables to load at $\geq \pm 0.5$, but there is no clear separation among treatment groups along this axis. The sex steroid T was the only variable to load at $\geq \pm 0.5$ on axis 3, and there is strong separation between GnRH- and non-GnRH-treated fish along the axis. In total, fish from the five treatments are well dispersed across the three dimensions of the solution, indicating large amounts of among-treatment overlap in resulting physiology, although certain patterns are evident. Within the axis 1 × 2 plot, specimens tend to be arraigned from the lower left (quadrant 3) to the upper right (quadrant 2), with a relative scarcity of individuals in the upper left (quadrant 1) and lower right (quadrant 4), suggesting that high ATPase values (a SW-adaptive condition) correspond to low LAC and CORT values, while low ATPase (the FW-adaptive condition) corresponds to high LAC and CORT. Similarly, the relative paucity of specimens in quadrant 2 of the axis 1 × 3 plot (i.e., high T and high ATPase values) may be indicative of the incompatibility of advanced maturation and SW tolerance. Of additional interest is that four FW + GnRH fish had exceptionally low T levels, suggesting that some individuals were not responsive to GnRH treatment.

Discussion

We consider ATPase, T, and E2 as first-order responses because they are physiological systems directly manipulated by treatment conditions. Among-treatment differences in these variables indicate that our five experimental treatments successfully generated distinctly different challenges to the experimental sockeye and yielded end-of-treatment sockeye of a broad spectrum of salinity adaptation and maturation levels. The three salinity-only treatments yielded ATPase values of SW > ISO > FW (treatment means: SW = 4.20, ISO = 2.27, FW = 1.49), confirming that experimental sockeye regulated ATPase activity in response to encountered salinities. Similarly, sex steroid concentrations indicate that treated sockeye were responsive to exogenous GnRH because these fish had notably higher T and E2 concentrations than did their salinity-only counterparts. The effect of exogenous GnRH was also evident in the ATPase data because FW + GnRH ATPase activity (mean = 2.03) was higher than in the FW treatment, consistent with the knowledge that ATPase activity increases during the final stages of maturation and spawning (Shrimpton et al. 2005). A comparable GnRH-induced ATPase increase in SW + GnRH (mean = 3.48) over SW was not evident or expected. Despite its obvious effect, GnRH treatment generally did not elevate sex steroid concentrations above baseline conditions, and T and E2 levels in salinity-only treatments were three to four times lower than those observed in baseline fish, suggesting that hormone suppression occurred during the treatment period. Physiological stress is known to disrupt the functioning of the HPG axis and inhibit sex steroid production (Wingfield and Sapolsky 2003; Norris and Hobbs 2006) and is a likely mechanism to explain the low concentrations. However, lactate and glucose concen-

Table 3: Gross somatic energy (GSE) concentrations (kJ kg^{-1}) within each of the five treatments and results of Duncan's multiple range test (MRT)

Treatment	*n*	Mean GSE (SEM)	Duncan's MRT
SW + GnRH	7	7.40 (.21)	A
SW	23	7.88 (.19)	B
ISO	32	7.69 (.11)	AB
FW	30	7.51 (.11)	AB
FW + GnRH	23	7.35 (.11)	A

Note. Treatments that do not share common letters are statistically different at $\alpha = 0.05$. SW = saltwater; GnRH = gonadotropin-releasing hormone; ISO = iso-osmotic water; FW = freshwater.

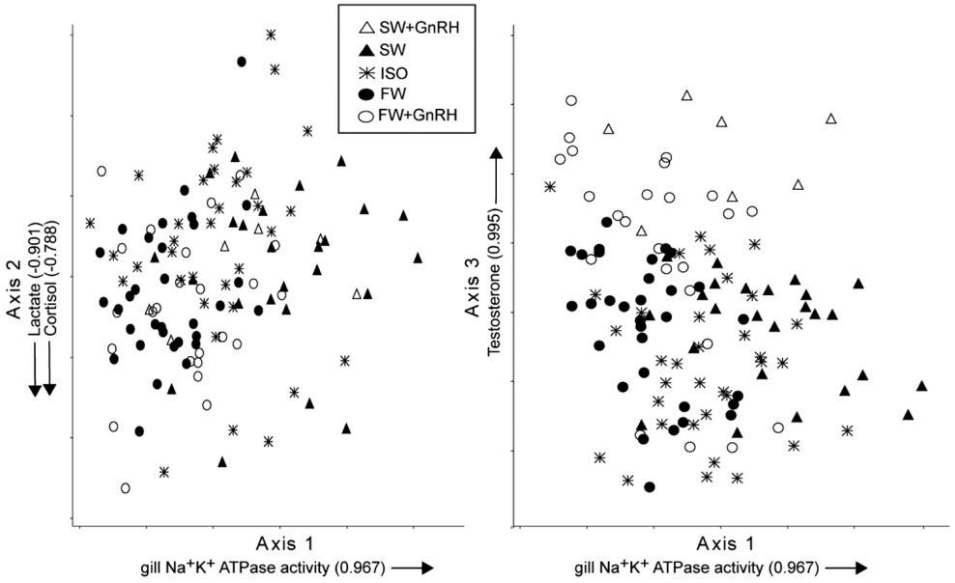

Figure 4. Three-dimensional nonmetric multidimensional scaling ordination of posttreatment physiology of sockeye ($n = 122$) that survived to the end of the treatment period. Axis 1 explains 24.2% of the variance in the data, axis 2 20.5%, and axis 3 52.1%. Axes are labeled with variables that loaded with correlation scores $\geq \pm 0.50$, and arrows point in the direction of increasing value. *SW* = saltwater; *GnRH* = gonadotropin-releasing hormone; *ISO* = iso-osmotic water; *FW* = freshwater.

trations, indicators of physiological stress related to energetic demands, and cortisol, an integral component of the stress response, were not elevated to concentrations suggestive of a stressed physiology (for a discussion of normal and stress LAC, GLU, and CORT concentrations at various stages of the spawning migration, see Hinch et al. 2008) in salinity-only treatments (SW treatment mean LAC = 2.0 mmol L^{-1}; mean GLU = 5.7 mmol L^{-1}), suggesting that some other mechanism may be responsible for the observed suppression. To illustrate, Sandblom et al. (2009) report mean chronic plasma lactate and glucose concentrations of laboratory-held, cannulated (i.e., a true measure of resting physiology) prespawn Weaver Creek sockeye, another population of the Fraser River late-run sockeye complex, of 2.2 and 10.3 mmol L^{-1}, respectively.

Several recent studies have evaluated ATPase and sex steroid values in migrating Fraser River late-run sockeye as they move from the ocean to spawning grounds. Shrimpton et al. (2005) measured ATPase in Adams sockeye at multiple locations during the 2003 migration and found mean values of 3.4 at landfall at the Queen Charlotte Islands (salinity ≈ 32–34 ppt), 2.2 within Johnson Strait (a.k.a. entry to the SOG; salinity ≈ 24–30 ppt), 1.1 at river entry, and 1.2 at spawning grounds. For sex steroids, Hinch et al. (2008) found mean T concentrations of Adams sockeye in the estuarine waters of the SOG during the 2006 migration of 25.8 (males) and 40.8 (females) and mean female E2 of 8.0 (all steroid concentration units = ng mL^{-1}), Young et al. (2006) found T concentrations at entry to the Thompson River (≈275 km upstream of the Fraser River mouth and 200 km from spawning areas) during the 2003 migration of 17.5 (males) and 34.9 (females) and mean female E2 female of 6.0, and Truscott et al. (1986) reported mean T in spawning ground 1978 Adams sockeye of 366 (males) and 479 (females). On the basis of these values, our experiment appears to have produced experimental fish comparable to marine, river entry, and "close to spawning ground arrival" physiology.

End-of-treatment cortisol values also indicate a strong physiological response to experimental conditions because cortisol concentrations progressed from low values in SW and SW + GnRH to high in FW and FW + GnRH (range of within-treatment means: 251–363 ng mL^{-1}). Cortisol has multiple physiological functions in teleost fishes, including in the physiological stress response, FW adaptation, and sexual maturation (Carruth et al. 2000; McCormick and Bradshaw 2006; Norris and Hobbs 2006). The most probable explanation for the observed cortisol pattern is an interaction between ionoregulatory and maturation functions, as salinity treatments are able to explain the sequential increase in cortisol from SW to ISO to FW treatments, but only differences attributable to maturation level can explain why FW + GnRH fish had higher mean and median values than did FW fish. We consider it unlikely that handling stresses (the process of capture, tagging, and confinement) could account for the observed cortisol pattern because all fish were similarly handled. In a study of Weaver Creek sockeye (a lower Fraser River population, migration distance = 160 km from ocean), cortisol values in individually dipnetted and immediately biopsied sockeye (methods similar to those we used here) of 90–350 ng mL^{-1} on arrival to spawning grounds and 740–1290 ng mL^{-1} postspawning (Hinch et al. 2008) lend support to our conclusion that some of our treatment groups were in "close to spawning ground arrival" con-

dition. Our results suggest that monitoring cortisol concentrations of a migratory population of anadromous salmonids may provide a useful means of nondestructive, real-time monitoring of a population's physiological status (i.e., determining a population's current condition within the maturation-senescence progression). Indeed, Carruth et al. (2000) demonstrated a strong relationship between cortisol and the reproductive schedule of Kokanee salmon (*Oncorhynchus nerka kennerlyi*), the landlocked form of sockeye salmon.

In contrast to the above-noted differences, there were few among-treatment differences in the other measured physiology variables (i.e., HCT, LAC, GLU, Na^+, Cl^-, OSMOL). However, there were large among-treatment differences in mortality (SW + GnRH > SW > FW + GnRH > FW = ISO). Our results indicate that salinity represented a modestly larger survival hurdle to the experimental fish than did the GnRH treatment. Given that the routine metabolic rate of maturing sockeye is approximately 30% higher in SW than in FW (Wagner et al. 2006), the high mortality in SW may have been related to SW-treated fish having energetic demands in excess of the available scope for metabolic activity. Our finding that prolonged exposure to high salinity levels during the later stages of the maturation sequence is a significant survival challenge to anadromous fish is consistent with the findings of other research. For example, Hirano et al. (1990) noted that maturing chum salmon that died in SW had elevated plasma osmolality, and Saito et al. (2001) found elevated plasma Na^+ concentrations in chum salmon that matured in SW. It is unclear why fish that were receptive to GnRH (i.e., at a suitable stage of maturity to be responsive to acceleration of the HPG axis) would be harmed by their physiological response to exogenous GnRH. Indeed, Sato et al. (1997) found no survival differences between control and GnRH-injected fish released to FW. Metabolic scope limitation is one possible explanation because survivors of both GnRH treatments had lower mean energy density values than their salinity-only counterparts (mean energy density values: FW + GnRH 2.1% lower than FW; SW + GnRH 6.1% lower than SW).

The SW + GnRH treatment was the one case where mortality results matched our a priori prediction, as this treatment yielded the highest mortality (80%). The high mortality is, at least in part, probably related to the loss of SW tolerance associated with advanced sexual maturation. Although specific mechanisms linking maturation to loss of SW tolerance in Pacific salmon are not clear, elevated E2 levels are known to reduce the number of gill filament chloride cells and retard gill Na^+,K^+ ATPase activity as a normal part of the programmed senescence schedule of semelparous Pacific salmon (Ueda and Yamauchi 1995; Uchida et al. 1997). This suggests that ionoregulatory failure would be the expected proximal cause of death of SW + GnRH treated fish. However, the physiological condition of SW + GnRH fish that survived to the end of the treatment period were largely indistinguishable from survivors of other treatments because only Na^+, which was only slightly elevated, differed from the other treatments. We had also predicted that the FW treatment would represent a significant challenge to sockeye, owing to the rapid transfer from the high-salinity SOG to FW before completion of ionoregulatory preparation for FW entry. In fact, FW treatment, along with ISO, yielded the lowest mortality (≈15%), indicating that sockeye can withstand the challenge of rapid FW entry. Among the secondary response variables, FW and ISO were not largely different from other treatment groups, but both of these treatments had lower chloride levels than did other treatments, and ISO had lower lactate values than did FW. However, all response variables were within the expected range for migratory sockeye (Hinch et al. 2006). We had predicted that FW + GnRH would yield low mortality, owing to exogenous hormone treatments accelerating development of FW acclimation, but FW + GnRH (40%) yielded more than twice the mortality as FW, reinforcing the conclusion that GnRH treatment and the resulting advanced maturation represented a challenge to the sockeye. FW + GnRH yielded Cl^- and Na^+ values comparable to those observed in SW treatments and higher than in FW or ISO, suggesting enhanced ion accumulation and/or retention in FW as a function of advanced maturation (consistent with the upswing in ATPase value noted in the FW + GnRH treatment). Our finding that male and female sockeye did not differ in physiology during either baseline or end-of-treatment biopsy sampling yet the sexes had different mortality rates in two out of five treatments (SW, ISO) suggests that males may be more capable of maintaining physiological homeostasis than are females during the type of challenges represented by these treatments. However, caution is warranted because there were no male-female mortality differences in three out of five treatments (FW, FW + GnRH, SW + GnRH).

The NMS ordination further illustrates that fish that survived the treatment period generally did not differ in physiological condition, particularly in the secondary response variables. The ordination also provides some insight into the potential mechanisms of death. Axis 2 is strongly associated with both CORT and LAC, suggesting that it represents a stress gradient, and individuals from four of the five treatments are well dispersed along the axis. SW + GnRH individuals are largely absent from the high-stress portion of the axis, suggesting that high-stress SW + GnRH fish died before the end of treatment, while at least some of the high-stress fish of other groups were able to survive. Hence, it appears that at least within the group that experienced the highest mortality rate (SW + GnRH), high stress levels were associated with death.

The observation that all treatment groups experienced a notable increase in mortality at or near the fourth or fifth day of the treatment period, coupled with the absence of large differences in the secondary response variables, provides evidence that sockeye rigorously maintain their physiological systems (i.e., ionic balance) within relatively narrow ranges (i.e., homeostasis) and that perhaps 4–6 d represents the outer limit of the duration at which homeostasis can be maintained in the face of the constant challenge represented by our weeklong holding experiment. Consistent with this idea, Uchida et al.

(1997) reported that plasma osmolality of maturing chum salmon (*Oncorhynchus keta*) forced to remain in SW remained at normal levels until either 3 or 5 d after treatment start, at which time a distinct increase in plasma osmolality occurred, rapidly followed by death. Other studies of ionoregulation in diadromous fishes also suggest a roughly weeklong window for restoration of homeostasis (McCormick 2001). For example, Pacific salmon smolts typically need ≈4–5 d to reach final gill Na^+,K^+ ATPase levels on entry to SW (Hoar 1988), and adult rainbow trout (*Oncorhynchus mykiss*) need 4 d to make the shift from net ion loss to uptake following movement into FW, and full ionic recovery occurred at 8 d after salinity transfer (Battram and Eddy 1990).

The water temperatures used in our experiment (means: 11.7°C SW, 12.7°C FW) reflect commonly encountered temperatures experienced by migrating Adams River sockeye during their SW to FW transition and are within the range of temperatures encountered during FW river migration and spawning. We contend that the small differences in treatment temperatures are inconsequential and would have little influence on our ability to assess the role of salinity and maturation status on mortality and physiology. We have implanted temperature-sensing iButtons into dozens of migrating Adams stock Fraser River sockeye in the ocean and recovered them on spawning grounds (S. G. Hinch and D. A. Patterson, unpublished data), and the data demonstrate that the migration route is extremely thermally dynamic and continually changing. In the SOG, which is highly influenced by tides and the Fraser River, >80% of encountered temperatures are from 9° to 13°C, but exposures range from 8° to 18°C, and sockeye can experience this entire thermal range within a few hours. In FW, the fish encounter a similar broad thermal range, with the warmest temperatures experienced in the lower Fraser River, and when transiting through lakes, they will experience temperatures ranging from 7.0° to 21.3°C (Patterson 2007; Mathes et al. 2010). Their spawning ground temperatures are 11°–12°C.

Similarly, we do not think that the different rates of salinity change at the start of the experiment (i.e., attaining desired end points at the same time when converting SW tanks at 28 ppt salinity to either ISO at 13 ppt or FW at 0 ppt) are responsible for the observed among-treatment differences because migrating sockeye typically encounter much larger and probably faster salinity transitions as they approach, enter, and move through the highly tidal SOG–lower Fraser River ecosystem. Indeed, telemetry tracking of migrating Adams sockeye clearly demonstrates that these fish routinely enter the low-salinity waters of the lower Fraser River, followed by falling back into the SOG before committing to the true upriver migration (for an example, see Cooke et al. 2008). Additionally, the physiological data presented here were collected 7 or 8 d after the start of low-salinity exposures, and the relevant literature indicates that physiological adjustments to new salinities are typically completed within 4–6 d (see "Discussion" and citations therein). As such, our reversion of the FW- and ISO-treated fish to full SW shortly before biopsy collection accurately reflects the physiology of rapid salinity transitions associated with river-entry behaviors. For a discussion of the physiological condition of adult sockeye collected from FW without experimental reversion to SW, we direct the reader to Shrimpton et al. (2005), Cooke et al. (2006*a*), Patterson et al. (2007), and citations therein.

Unfortunately, despite the continuing decline of many anadromous salmon stocks, the SW to FW transition of adult salmon has received relatively little scientific attention likely because of the expense and logistic challenges to conduct such studies and possibly because of the belief that ocean-migrating homing salmon experience low natural mortality rates; therefore, this life-history stage may be perceived of as less of a concern in conservation and management. Considerable study has focused on upriver migration mortality because this issue is easier to assess and has been documented as being very large in some years (e.g., since 1996, Fraser River late-run sockeye stocks have experienced >60% river migration mortality; Cooke et al. 2004; Hinch et al. 2008). Our experimental results confirm recent field observations on wild adult salmon migrants that the SW to FW transition undertaken by maturing anadromous adult Pacific salmon can be physiologically stressful and a time of notable mortality (Hirano et al. 1990; Ueda 1998; Cooke et al. 2006*b*; Crossin et al. 2007). Our results suggest that reducing the frequency of stress events during the first few days of FW residency, such as restricting fishing methods with a low capture/high escape rate or limiting the occurrence of suboptimal water temperatures, may reduce incidental mortality and thereby improve escapement. Given that both theory (Hendry et al. 2004) and our recent laboratory-based empirical results suggest that the return to FW can be a time of high mortality, it appears time to address the existing research shortfall with large-scale field-based observations and experiments.

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