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Consequences of acute stress and cortisol manipulation on the physiology, behavior, and reproductive outcome of female Pacific salmon on spawning grounds

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ABSTRACT

Life-history theory predicts that stress responses should be muted to maximize reproductive fitness. Yet, the relationship between stress and reproduction for semelparous salmon is unusual because successfully spawning individuals have elevated plasma cortisol levels. To tease apart the effects of high baseline cortisol levels and stress-induced elevation of cortisol titers, we determined how varying degrees of cortisol elevation (i.e., acute and chronic) affected behavior, reproductive physiology, and reproductive success of adult female pink salmon (*Oncorhynchus gorbuscha*) relative to different states of ovulation (i.e., ripe and unripe). Exhaustive exercise and air exposure were applied as acute stressors to manipulate plasma cortisol in salmon either confined to a behavioral arena or free-swimming in a spawning channel. Cortisol (eliciting a cortisol elevation to levels similar to those in post-spawn female salmon) and metyrapone (a corticosteroid synthesis inhibitor) implants were also used to chemically manipulate plasma cortisol. Cortisol implants elevated plasma cortisol, and impaired reproductive success; cortisol-treated fish released fewer eggs and died sooner than fish in other treatment groups. In contrast, acute stressors elevated plasma cortisol and the metyrapone implant suppressed plasma cortisol, but neither treatment significantly altered reproductive success, behavior, or physiology. Our results suggest that acute stressors do not influence behavior or reproductive outcome when experienced upon arrival at spawning grounds. Thus, certain critical aspects of salmonid reproduction can become refractory to various stressful conditions on spawning grounds. However, there is a limit to the ability of these fish to tolerate elevated cortisol levels as revealed by experimental elevation of cortisol.

Introduction

Considerable evidence supports the notion that stress can impair the reproductive outcome of a wide range of vertebrates, including birds (Silverin, 1997; Wingfield, 1988), reptiles (DeNardo and Sinervo, 1994a, 1994b), mammals (Negro-Vilar, 1993; Boonstra et al., 1998), and fish (Pickering et al., 1987; Schreck et al., 2001). The acute stress response and associated elevation of glucocorticoids is believed to be adaptive, while chronic elevation of glucocorticoids can have various negative tertiary effects, including impaired immune function and fitness whenever resources are directed towards an emergency response (Barton and Iwama, 1991; Wingfield et al., 1998; Barton, 2002; Wingfield, 2003) and animals attempt to regain allostasis (Wingfield, 2003; Schreck, 2010). Yet, much of the existing work on chronic stress/glucocorticoid elevation is focused on the long-term consequences for animals during non-reproductive periods rather than immediately before or during reproduction. For example, many toxicological studies demonstrate direct long-term reproductive impairments (e.g., suppression of reproductive hormones) associated with emergency resource reallocation to maintenance and survival (e.g., reviewed in Van Der Kraak et al., 1998; see also Jardine et al., 1996; Janz et al., 1997; Bowron et al., 2009). Furthermore, most of these studies consider iteroparous species (i.e. repeat breeders), which have the life-history option of delaying a reproductive event when challenged.

In contrast, semelparous species usually cannot delay the reproductive event because they invest in reproduction only once in a lifetime. For semelparous fishes such as Pacific salmonids (*Oncorhynchus*

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spp.), some argue that the spawning date is genetically fixed, which implies that it cannot be altered by external stressors (Quinn et al., 2000). Curiously, virtually nothing is known about whether exposing semelparous Pacific salmonids to stress on spawning grounds influences their behavior and reproductive success. Yet, these fish routinely encounter many stressors that trigger a cortisol response as they approach their spawning date, suggesting that the acute stress response remains active during the reproductive period. For example, plasma cortisol rises when fish encounter hydraulic challenges and elevated water temperature during the spawning migration (Hinch et al., 2006; Mathes et al., 2010). Furthermore, a progressive increase in baseline plasma cortisol levels of unknown etiology occurs as salmon swim to the spawning grounds (Robertson and Wexler, 1959; McBride et al., 1986; Tierney et al., 2009; Hruska et al., 2010). Plasma cortisol concentrations rise from ~25 ng ml^{-1} in pink salmon (*O. gorbuscha*) at river entry (McBride et al., 1986), to ~350 ng ml^{-1} on arrival at the spawning ground (female sockeye salmon [*O. nerka*]; Hruska et al., 2010), and ~1287 ng ml^{-1} when the fish become moribund (female sockeye salmon; Hruska et al., 2010). Thus, an acute stressor can elevate plasma cortisol against a background of progressively increasing plasma cortisol levels during the spawning migration.

A stressed state should generally be incompatible with reproduction and, based on life-history theory, one could postulate that the cortisol stress response of semelparous salmon should be muted, or physiologically irrelevant, during this period (Wingfield and Sapolsky, 2003) to mitigate any potential negative effects of cortisol elevation above the (high) baseline levels on spawning grounds. Thus, we postulate that reproductive drive in a semelparous salmon species will outweigh any cortisol-mediated mating inhibition. Acute, stress-related increases in plasma cortisol suppress the normal increases in plasma sex hormone concentrations for Pacific salmon during early phases of upriver migration (Dye et al., 1986). However, increases in plasma cortisol during migration are regarded as adaptive and necessary for salmon to be able to return to their natal streams and spawn (Carruth et al., 2002). Complicating matters is the fact that spawning Pacific salmon also undergo senescence, which alters many physiological processes, including hormone regulation (Morbey et al., 2005; Hruska et al., 2007, 2010). To address these issues, we experimentally determined how short-term changes in and experimental manipulation of plasma cortisol influenced the reproductive physiology, behavior, and spawning outcome of wild female pink salmon (*O. gorbuscha*). We administered cortisol implants and predicted that plasma cortisol elevation, lasting between 2 and 5 days, would negatively affect reproductive behavior (e.g., less time spent guarding eggs or fighting for a mate), physiology (i.e., suppression of reproductive hormones), and outcome (i.e., number of eggs released). We also predicted that the response to acute stressors (i.e., exhaustive exercise or air exposure) would be muted in semelparous salmon and would not alter these same responses. Conversely, an intraperitoneal (IP) implant of metyrapone, which blocks the last step of glucocorticoid synthesis, was expected to lower plasma cortisol levels (Doyon et al., 2006) and retard reproduction and senescence. To our knowledge, hormone manipulations of this type had not before been performed on senescing Pacific salmon.

Materials and methods

Metyrapone validation

All fish were handled in accordance with the guidelines of the Canadian Council on Animal Care (Carleton University, B09-12; University of Ottawa, BL-228). A pilot laboratory experiment was carried out to determine the effectiveness of metyrapone (2-methyl-1, 2-di-3-pyridyl-1-propanone; Sigma 85625, Sigma-Aldrich) at blocking cortisol synthesis when delivered in a cocoa butter implant. Metyrapone successfully blocks cortisol synthesis in fish in the short-term (<24 h: e.g., Hopkins et al., 1995; Milligan, 2003; Rodela et al., 2009), but has rarely been used with a cocoa butter carrier (but see Doyon et al., 2006). Rainbow trout (*O. mykiss*), a congeneric of pink salmon, weighing approximately 150 g were anesthetized with benzocaine (0.05 mg ml^{-1} water; p-aminobenzoic acid ethyl ester; Sigma E1501, Sigma-Aldrich) and given an IP injection of metyrapone mixed in heated liquid cocoa butter (200 mg kg^{-1} fish in l ml cocoa butter kg^{-1} fish); upon injection into the fish, the cocoa butter rapidly cools to a thick paste, providing a slow-release metyrapone implant. After 1 and 5 days, fish were subjected to 1 min of air exposure as an acute stressor, and a blood sample was withdrawn by caudal puncture 30 min later for assessment of plasma cortisol levels. The expectation was that this 30-min delay would be sufficient for the maximum or near maximum rise in plasma cortisol level to be manifested (Gilmour et al., 2005).

Weaver Creek spawning channel

All field experiments were conducted at the Weaver Creek spawning channel located in British Columbia, Canada (see Hruska et al., 2010 for detailed information). Each experiment involved groups of naive fish (i.e., fish were not reused among experiments). The artificial channel, 2.93 km long and 6.1 m wide, is composed of a cobble (1.2–7.6 cm) substrate and has a consistent water depth of 25–30 cm. Fish densities and flow conditions were monitored throughout the spawning period and manually operated gates were used to regulate fish movements into the spawning channel (Hruska et al., 2010). Experiments were timed to coincide with peak pink salmon spawning activity in early October 2009.

Reproductive physiology on arrival

On arrival at the spawning channel in early October, female pink salmon were individually removed from the raceway via dip net and immediately placed in a trough supplied with flow-through water from the raceway. Fish were categorized as either “unripe” (N = 52, unovulated, where eggs are still confined to intact ovaries) or “ripe” (N = 60, ovulated, where eggs have been released into the body cavity and gentle abdominal pressure near the vent easily expels eggs) and a blood sample was collected via caudal puncture (2 ml blood sample; collected using 3 ml vacutainer and 1.5 in., 18 ga needle, lithium heparin; Becton Dickson, NJ) within 30 s (Cooke et al., 2006). Within 3 min the fish were released back into the spawning channel. Blood samples were stored in an ice-water slurry and centrifuged (5 min at 10,000 *g*) within 45 min, after which the plasma was frozen in liquid nitrogen immediately. Samples were subsequently stored at −80 °C until further analysis.

In addition, subsets of ripe (N = 6) and unripe (N = 12) salmon were given an intraperitoneal (IP) injection of either cortisol (hydrocortisone 21-hemisuccinate; Sigma H4881, Sigma-Aldrich; 110 mg kg^{-1} fish in 50 ml melted cocoa butter kg^{-1} fish; Dibattista et al., 2005) to elevate cortisol levels for a short period (i.e., 2 to 5 days), or metyrapone (200 mg kg^{-1} fish; 1 ml cocoa butter kg^{-1} fish) to block glucocorticoid synthesis (Mommsen et al., 1999), before being placed in individual, opaque, experimental chambers (~50 l) situated on the bank of the channel and equipped with flow-through water. Fish were left undisturbed for approximately 24 h, after which they were individually removed and blood was sampled immediately via caudal puncture.

Longevity and reproductive status study

On October 6th and 7th 2009, 120 unripe pink salmon that had voluntarily entered the raceway were marked with unique individual Peterson disk tags placed in the dorsal musculature. The tags could be read on free-swimming fish with binoculars, which allowed the fish

to be observed without any disturbances. Fish were randomly assigned to one of six treatment groups (N = 20 per treatment group): a) control fish (only tagged); b) sham injection-controls (tagged and given an IP injection of 50 ml kg^{-1} melted cocoa butter); c) cortisol-treated (as described above); d) metyrapone-treated (as described above); e) chased (acutely stressed by 3-min of being "chased" by hand around a circular tank supplied with flow-through channel water); and f) air-exposed (as in (e), followed by 1 min of air exposure to increase the severity of the acute stressor). Afterwards, fish were immediately released into the spawning channel and closely monitored during daylight hours so that moribund or dead fish could be collected daily.

Longevity in the spawning channel following release (i.e. time until death after arrival) was calculated using the methods outlined in Hruska et al. (2010). Fork length, total mass, gonad mass, epidermal coverage by fungus, and general condition also were documented. Reproductive status was reported as the percentage (%) of eggs released by each individual. The relationship between percentage of eggs remaining relative to percentage of eggs initially expected was determined following the methods of Hruska et al. (2010). Briefly, the anticipated initial gonad mass was determined from a known relationship between body mass and gonad mass established for a separate group of mature, unripe pink salmon sampled from the spawning channel ($N = 21$; gonad mass $= 10.1 \cdot$ body mass $- 297.9$, $R^2 = 0.80$, $P = 0.005$). Eggs were weighed and counted in whole ovaries and a linear body mass to fork length relationship, together with a linear fork length to gonad mass relationship, was used to interpolate the expected egg mass before ovulation for the experimental fish. Many fish had spawned all of their eggs (100% success), but any eggs remaining were weighed first as five groups of 10 eggs, with any eggs remaining thereafter being weighed collectively. Individual egg mass is known to be uniform within an individual (D. Patterson, personal communication), and so this method provided an accurate estimate of the number of eggs retained by each fish without having to count every egg.

Spawning behavior in enclosures

Behaviors were studied in unripe and ripe salmon held in enclosures that had been constructed within the spawning channel. A blood sample (as described above) was withdrawn from 30 salmon (6 treatment groups as above; N = 5 for each treatment group) in the raceway before placing them in a holding tank for transfer to a section of the spawning channel that housed a net-pen (2 m wide by 15 m long; constructed out of Vexar rigid mesh fencing; Masternet, Mississauga, Ontario). Fish were treated according to their experimental group before being placed into the enclosure. Twenty "ripe" male pink salmon (i.e. males that released sperm when squeezed gently near the vent) had been placed into the net-pen 12 h earlier. Fewer males were placed in the pen than females to facilitate competition among females. Two trials were completed for unripe salmon in early October 2009, and two trials were completed for ripe fish in late October 2009.

Behavioral observations were carried out for 10 min daily on four consecutive days. The order of observing each fish was randomized daily. Reproductive behaviors of pink salmon are well known, and are similar to behaviors displayed by other semelparous Pacific salmon (Heard, 1991; Mehranvar et al., 2004). Females prepare their nesting area, fend off intruders from their territory through aggressive action, and spend time with males to ensure fertilization occurs. We recorded on what day fish established a territory, how much time the fish spent holding that territory (represented as a percent, averaged over days on territory), what percentage of their time females spent with males (averaged across days on an established territory), the number of nest construction digging behaviors that occurred (averaged across days spent on a territory), how many times a fish made an aggressive display towards a conspecific, and how many times that fish was on the receiving end of an aggressive act (both were summed and divided by total observation minutes, and aggression received was subtracted from aggression given to yield an overall aggression score). The daily duration of behavioral observations on each fish (i.e., 10 min) was consistent with other studies (Cook et al., 2011) and is believed to be representative of longer time periods given the reasonable predictability and stability of behavioral repertoires for this species. After 4 days, the fish were collectively culled in a process lasting<10 min; fish were killed by cerebral percussion. After immediate blood sampling, the percentage of eggs released was estimated (as described above).

Plasma analysis

Plasma glucose and cortisol concentrations were measured as indicators of stress (Farrell et al., 2001a, 2001b). Briefly, plasma glucose values were determined using a YSI 2300 STAT Plus glucose analyzer (YSI Inc., Yellow Springs, Ohio). Plasma cortisol levels were measured using a commercial ELISA kit (Neogen Corporation # 402710, Lexington KY). For cortisol, the assay has 47% cross-reactivity with the drug prednisolone, which would not be present in the samples.

The assay also has ~15% cross-reactivity with cortisone and 11-deoxycortisol. The analytical sensitivity (B/B_0, 80%) for the cortisol assay was at 0.04 ng ml^{-1}. Testosterone and 17β-estradiol are both major reproductive hormones and plasma concentrations of these hormones also were measured by ELISA kits (Neogen Corporation, www.neogen.com, catalog numbers: 402110, 402510). Testosterone and 17β-estradiol were extracted from plasma samples using ethyl ether according to the kit manufacturer's protocols. The assay manufacturer states that the estradiol assay does not cross-react with any other estrogens. Analytical sensitivity (B/B_0, 80%) was at 0.03 ng ml^{-1}. According to the manufacturer, the testosterone assay is 100% cross-reactive with dihydrotestosterone and the analytical sensitivity (B/B_0, 80%) was at 0.006 ng ml^{-1}. Cortisol, glucose, testosterone, and 17β-estradiol were assayed in duplicate at appropriate dilutions. Inter- and intra-assay variability was <10% for all assays. More detailed descriptions of the analytical techniques can be found in Farrell et al. (2001a, 2001b).

Statistical analysis

Results from the metyrapone pilot study were analyzed using a two-way analysis of variance (ANOVA) to determine whether cortisol values varied by treatment and time. Results from the cortisol and metyrapone validation study before and after 24 h were compared using two-way repeated measures ANOVA models with time and treatment as effects. For the channel experiment, longevity among treatment groups was compared using a log-rank survival analysis to 50% mortality. The percentage of eggs released by each fish was averaged within groups and compared using a one-way ANOVA. For the enclosure experiments, all hormone and blood physiology values and behavioral metrics were compared before and after 4 days using two-way repeated measures ANOVA models with time and treatment being the independent variables. Time until territory establishment was determined using log-rank survival analyses. The percentage of eggs released by each fish was averaged for each treatment group and compared using a one-way ANOVA. Tukey's post-hoc tests were employed following significant one-way ANOVAs to determine differences among groups (where $p<0.05$). The assumptions of equality of variances and normal distribution were tested for all analyses and relevant transformations applied where assumptions could not be met. Percentage data were arcsine transformed prior to analysis. Where transformation of the data was not possible or effective, non-parametric analyses were performed. All analyses were conducted using JMP, version 8.0.2 (SAS Institute Inc., Cary, NC). The level of significance (α) for all

tests was assessed at 0.05. All data are presented as mean ± standard error unless otherwise noted.

Results

Effectiveness of metyrapone

Metyrapone-treated rainbow trout subjected to an acute stressor exhibited significantly lower plasma cortisol concentrations than sham-treated fish 1 day following treatment (two-way ANOVA Time effect: $F=7.8$, $df=1$, $p=0.02$; Fig. 1), but not after 5 days (Treatment effect: $F=3.1$, $df=1$, $p=0.1$; Interaction: $F=4.7$, $df=3$, $p=0.03$; Fig. 1). Therefore, we assumed that pink salmon would experience a short-term depression of plasma cortisol during acute stress (i.e., for at least 24 h but not as long as 5 days) and used cocoa butter as a vehicle for metyrapone delivery.

Raceway blood physiology and hormone validations

Reproductive hormone titers were indicative of whether pink salmon in the spawning channel were ripe or unripe (Dye et al., 1986; Table 1). Plasma estradiol and testosterone were both significantly lower in ripe fish (estradiol: $F=70$, $df=1$, $p<0.001$; testosterone: $F=25$, $df=1$, $p<0.001$; Table 1). However, plasma cortisol concentrations were similar (one-way ANOVA, $F=0.31$, $df=1$, $p=0.6$; Table 1) and plasma glucose concentrations were higher in ripe fish (one-way ANOVA, $F=13$, $df=1$, $p<0.001$; Table 1) for arriving pink salmon.

For unripe fish held in isolation chambers, cortisol implants significantly elevated plasma cortisol by 10-fold, but metyrapone implants had no effect on circulating cortisol levels after 24 h (two-way repeated-measures ANOVA: Treatment effect: $F=55$, $df=1$, $p<0.001$; Time: $F=70$, $df=1$, $p<0.001$; Interaction: $F=15$, $df=3$, $p<0.001$; Fig. 2A). Plasma glucose was unchanged 24 h after either treatment (Treatment effect: $F=0.69$, $df=1$, $p=0.4$; Time: $F=0.90$, $df=1$, $p=0.4$; Interaction: $F=0.39$, $df=3$, $p=0.9$; Fig. 2B). Plasma concentrations of both estradiol (Treatment effect: $F=0.8$, $df=1$, $p=0.8$; Time: $F=8.5$, $df=1$, $p=0.02$; Interaction: $F=1.5$, $df=3$, $p=0.3$; Fig. 2C) and testosterone (Treatment effect: $F=0.13$, $df=1$, $p=0.7$; Time: $F=5.7$, $df=1$, $p=0.04$; Interaction: $F=1.7$, $df=3$, $p=0.2$; Fig. 2D) decreased 24 h after either treatment.

For ripe fish held in isolation chambers, the cortisol implant again increased plasma cortisol values, but the response was attenuated compared with that of unripe fish (Fig. 2E). Plasma cortisol concentration was not affected by the metyrapone implant (two-way repeated-measures ANOVA: Treatment effect: $F=1.0$, $df=1$, $p<0.001$; Time: $F=34$, $df=1$, $p<0.001$; Interaction: $F=5.4$, $df=3$, $p<0.001$; Fig. 2E). Plasma glucose values increased 24 h after either treatment (Treatment effect: $F=5.1$, $df=1$, $p=0.3$; Time: $F=6.8$, $df=1$, $p=0.02$; Interaction: $F=2.2$, $df=3$, $p=0.03$; Fig. 2F). Estradiol was unaffected by either treatment (Treatment effect: $F=0.69$, $df=1$, $p=0.4$; Time: $F=0.90$, $df=1$,

Table 1
Initial blood hormone and glucose values of ripe and unripe pink salmon (*Oncorhynchus gorbuscha*) removed from the Weaver Creek raceway in October, 2009, presented as mean (±SE). $N=52$ for unripe fish and $N=60$ for ripe fish. All data were analyzed using the Wilcoxon Rank-Sum Test, except for cortisol (*), which was analyzed using log-transformed data in a one-way ANOVA.

Variable	Unripe	Ripe	Statistics	
			Statistic	P-value
Glucose (mmol l^{-1})	$4.6^{B}\pm0.18$	$5.7^{A}\pm0.17$	13.0	<0.001
Cortisol (ng ml^{-1})*	338 ± 21	297 ± 21	0.310	0.579
Estradiol (ng ml^{-1})	$4.5^{A}\pm0.3$	$0.28^{B}\pm0.3$	70.4	<0.001
Testosterone (ng ml^{-1})	$150^{A}\pm13$	$63^{B}\pm12$	24.8	<0.001

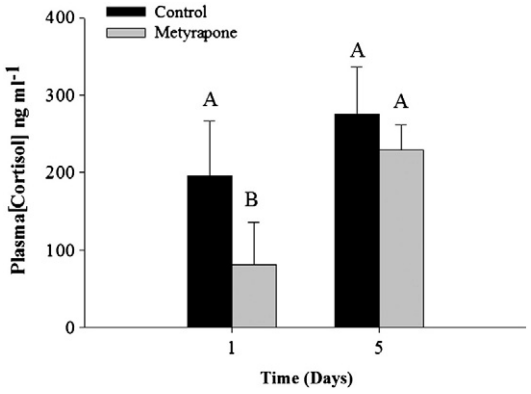

Fig. 1. Mean (±SE) cortisol values for control and metyrapone-treated rainbow trout (*Oncorhynchus mykiss*) subjected to an air-exposure stressor either 1 or 5 days after treatment with metyrapone. Data were log-transformed and analyzed using a two-way ANOVA. Dissimilar letters denote a significant difference between treatment groups and/or time periods (Tukey–Kramer HSD test, $p<0.05$). Sample sizes are as follows: 1 day: control = 2, metyrapone = 5. 5 days: control = 4, metyrapone = 4.

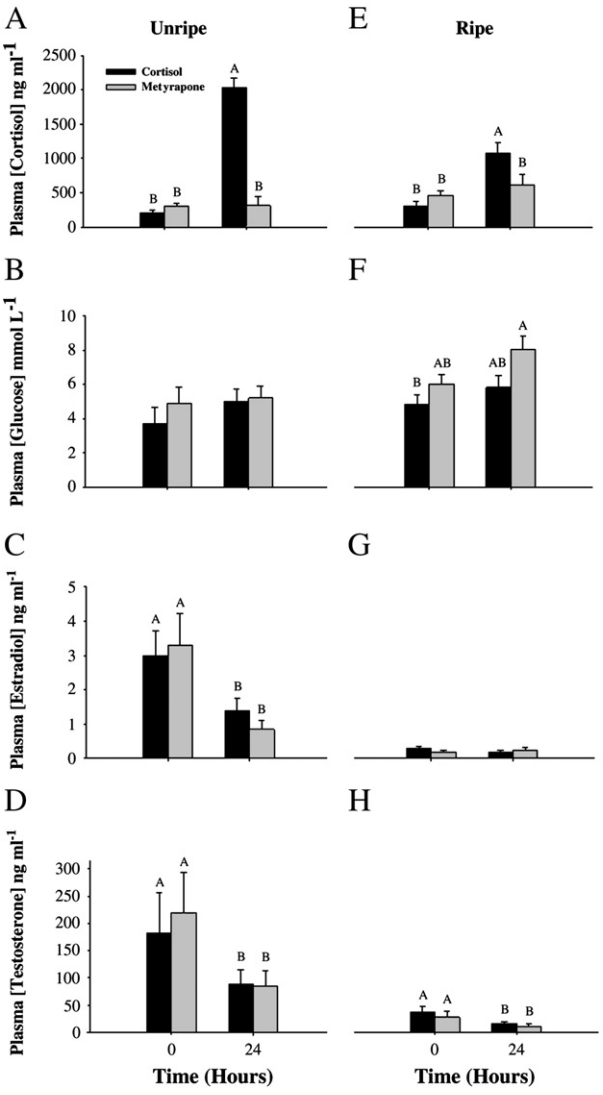

Fig. 2. A–H. Summary of pink salmon (*Oncorhynchus gorbuscha*) plasma hormone and glucose values for unripe (A–D) and ripe (E–H) fish both before and 24 h after treatment with cortisol or metyrapone. Values are stated as mean (±SE). Dissimilar letters denote significant differences among treatment groups and time periods (Tukey–Kramer HSD test, $p<0.05$). $N=6$ for each treatment for unripe fish; $N=12$ for ripe fish. All Ranked Sum data were analyzed using a two-way repeated-measures ANOVA, with time and treatment as independent variables.

p = 0.4; Interaction: F = 1.2, df = 3, p = 0.3; Fig. 2G). Plasma testosterone was decreased 24 h after both treatments (Treatment effect: F = 0.83, df = 1, p = 0.4; Time: F = 21, df = 1, p < 0.001; Interaction: F = 0.27, df = 3, p = 0.6; Fig. 2H).

Longevity and reproductive study

Pink salmon treated with cortisol exhibited reduced longevity relative to all other treatment groups (log-rank survival time to 50% mortality; $\lambda^2 = 13.1$, df = 5, p = 0.02; Fig. 3). Cortisol-treated fish also released fewer eggs during their time in the channel compared with all other treatment groups except the sham group [47% for cortisol-injected; 69% for sham-treated; >85% for all other groups (one-way ANOVA, F = 13, df = 5, p < 0.001; Fig. 4)].

Enclosure experiment: reproductive status

Treatment with cortisol, metyrapone or acute stress did not influence the extent to which fish ripened during the experiment (Fig. 5A). For those fish that did ripen during the enclosure experiment, differences in egg release (%) were observed (Wilcoxon Rank Sum; $\lambda^2 = 11.2$, df = 5, p = 0.04; Fig. 5B). Control and chased fish released more than 80% of their eggs, chase + 1 min air exposure and cortisol-treated fish released approximately 50% of their eggs, and metyrapone and sham-treated fish released the fewest eggs (<10%). For ripe fish, there were no statistically significant differences in egg release among treatment groups (data not shown). However, cortisol-treated fish released ~50% of their eggs, whereas all other treatment groups released >70% of their eggs.

Enclosure experiment: hormone profiles

Among unripe fish, cortisol-treated fish exhibited elevated cortisol concentrations 4 days following treatment (two-way repeated-measures ANOVA: Treatment effect: F = 4.6, df = 1, p = 0.002; Time: F = 0.4, df = 1, p = 0.9; Interaction: F = 2.5, df = 5, p = 0.04; Fig. 6A). Plasma glucose concentration increased in all fish during the 4 day experiment (Treatment effect: F = 0.7, df = 1, p = 0.6; Time: F = 15.6, df = 1, p < 0.001; Interaction: F = 0.6, df = 5, p = 0.7; Fig. 6B). Plasma estradiol levels decreased (Treatment effect: F = 0.5, df = 1, p = 0.8; Time: F = 72, df = 1, p < 0.001; Interaction: F = 0.6, df = 5, p = 0.7; Fig. 6C), whereas plasma testosterone concentrations remained unchanged over the 4 day experimentation period (Treatment effect: F = 1.1, df = 1, p = 0.4; Time: F = 3.3, df = 1, p = 0.08; Interaction: F = 0.3, df = 5, p = 0.9; Fig. 6D).

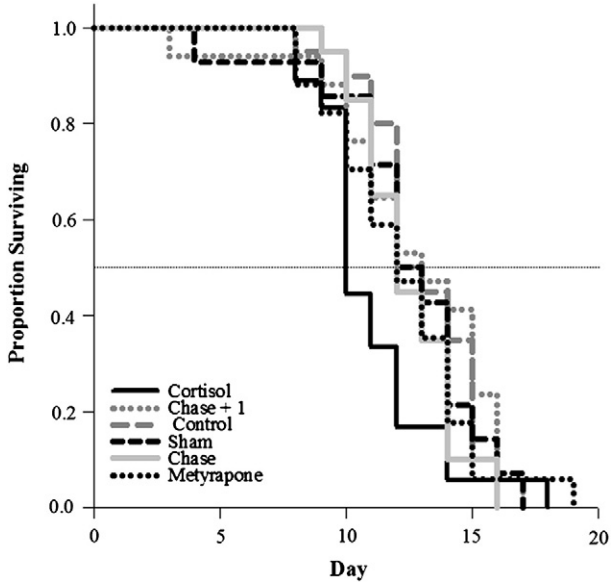

Fig. 3. Log-rank survival analysis to 50% mortality in each treatment group (see text for details of treatment groups), comparing longevity among pink salmon (*Oncorhynchus gorbuscha*) in the Weaver Creek spawning channel. Sample sizes were as follows: chase and control = 20, cortisol = 18, chase + 1 and metyrapone = 17 and sham = 14.

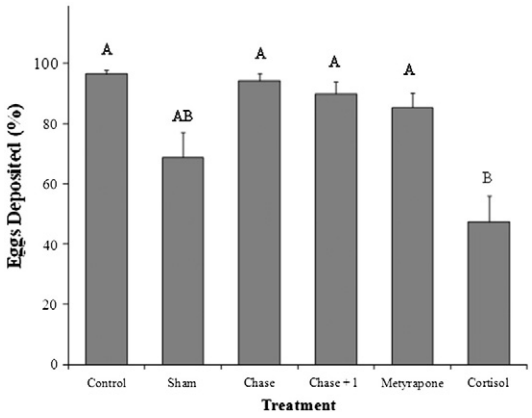

Fig. 4. A comparison across treatment groups (see text for details of treatment groups) of the percentage (%) of total possible eggs deposited by pink salmon (*Oncorhynchus gorbuscha*) in the Weaver Creek spawning channel during early October, 2009. All data were transformed into ArcSine (square root) values before analysis. Sample sizes were as follows; chase and control = 20, cortisol = 18, chase + 1 min air exposure and metyrapone = 17 and sham = 14. Dissimilar letters denote significant differences among treatment groups (Tukey–Kramer HSD test, p < 0.05).

For ripe fish, plasma cortisol levels varied across treatment groups after 4 days (Fig. 6E). Control fish exhibited the highest levels (1756 ± 274 ng ml^{-1}), and cortisol-treated fish displayed similar concentrations (averaging 1592 ± 207 ng ml^{-1}). Values were similar among sham-treated (1118 ± 211 ng ml^{-1}), chased fish (846 ± 289 ng ml^{-1}), and chased + 1 (757 ± 186 ng ml^{-1}) fish, whereas the lowest value (577 ± 193 ng ml^{-1}) was observed in metyrapone-treated fish (two-way repeated-measures ANOVA: Treatment effect: F = 2.7, df = 1, p = 0.03; Time: F = 55, df = 1, p < 0.001; Interaction: F = 3.2, df = 5, p = 0.01; Fig. 6E). Plasma glucose concentrations increased during the 4 day period, across treatments (Treatment effect: F = 1.7, df = 1, p = 0.1; Time: F = 5.4, df = 1, p = 0.02; Interaction: F = 0.93, df = 5, p = 0.5; Fig. 6F). Plasma estradiol levels were low and did not change (Treatment effect: F = 1.2, df = 1, p = 0.3; Time: F = 2.5, df = 1, p = 0.1; Interaction: F = 0.7, df = 5, p = 0.6; Fig. 6G), whereas plasma testosterone concentrations decreased after 4 days (Treatment effect: F = 0.4, df = 1, p = 0.8; Time: F = 91, df = 1, p < 0.001; Interaction: F = 0.2, df = 5, p = 0.9; Fig. 6H).

Enclosure experiment: behavior observations

Among unripe fish, treatment did not influence the rate of territory establishment (log-rank survival analysis; $\lambda^2 = 2.4$, df = 5, p = 0.8). Based on behavioral observations for fish on territories, cortisol-treated fish spent ~10% less time holding their territory compared with controls (one-way ANOVA: F = 12, df = 5, p = 0.03; Table 2). Additionally, cortisol-treated fish were less aggressive and experienced more aggressive acts by conspecifics (F = 13, df = 5, p = 0.04; Table 2). Among ripe fish, no differences were noted for territory establishment (log-rank survival analysis; $\lambda^2 = 4.0$, df = 5, p = 0.5). In addition, no behavioral differences were observed among the treatments groups (Table 3).

Discussion

By experimentally elevating plasma cortisol in unripe fish for between 2 and 5 days with a cortisol in cocoa butter implant, we negatively impacted the longevity, reproductive behavior, and reproductive

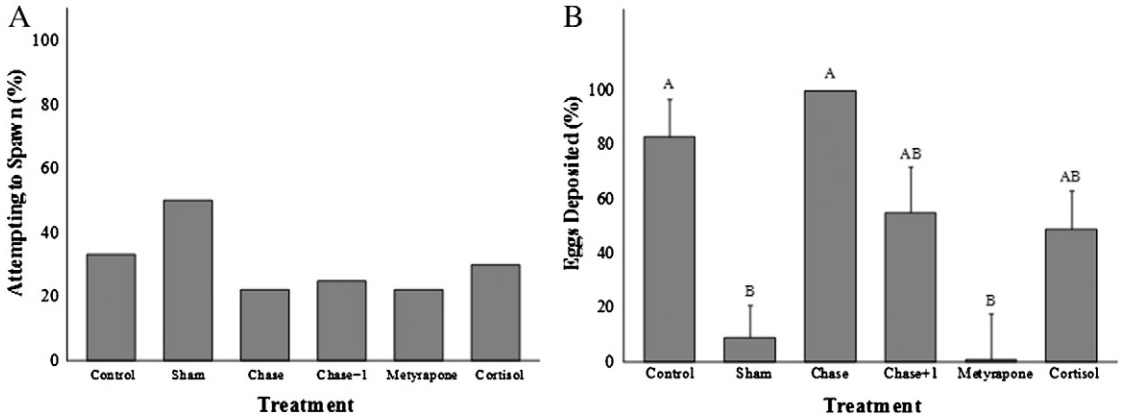

Fig. 5. (A and B). Figure A presents the percentage of pink salmon (*Oncorhynchus gorbuscha*) that became ripe during the behavior trials and thus were able to spawn during the enclosure experiment. Figure B presents the percentage of total eggs available (%) that were deposited during the 4 day trials by ripened fish across treatment groups. Sample sizes were as follows: chase = 1/9, chase + 1 = 2/8, control = 3/9, cortisol = 3/10, metyrapone = 2/9, sham = 4/8. All data were transformed into ArcSine (square root) values before being analyzed. Dissimilar letters denote significant differences among treatment groups (Tukey–Kramer HSD test, $p<0.05$).

outcome of pink salmon on their spawning grounds. Conversely, acute stressors that also presumably elevated plasma cortisol, namely exercise and air exposure, did not affect reproductive outcomes in either ripe or unripe fish. These results demonstrate that a sustained elevation of plasma cortisol carries significant reproductive costs for semelparous salmon on their spawning grounds (despite their high baseline cortisol levels), but that temporary elevations may not. In an ecologically relevant context, events that could elicit a prolonged stress response that might last 2–5 days include periods of high water temperature (Mathes et al., 2010), seasonally high (or low) river discharge (Rand et al., 2006), river obstructions or regions that are hydraulically complex (Hinch and Bratty, 2000), or disease (Wagner et al., 2005). In contrast, very short-term stressors, which might include fisheries interactions, failed predation events, and antagonistic interactions with conspecifics just prior to or during spawning may result in fewer effects on reproduction.

Cortisol manipulation and reproductive hormones

In a variety of fish species, elevation of glucocorticoids results in decreased reproductive hormone concentrations (see review by Barton and Iwama, 1991), which in iteroparous fish can lead to a postponed reproductive event. Additionally, a stressful reproductive environment (e.g., fish exposed to bleached kraft pulp mill effluent) negatively impacts reproductive fitness in various ways (Jardine et al., 1996; Janz et al., 1997; Bowron et al., 2009). In semelparous Pacific salmon exposed to a natural hydraulic challenge during their reproductive migration up the Fraser River system in BC (at the Hell's Gate fishway, Hinch et al., 2006; in the Thompson Canyon, BC, Young et al., 2006), reproductive hormone titers (i.e., 11-ketotestosterone, estradiol and testosterone) fall dramatically while cortisol levels increase. Further upstream, where the river is less challenging and perhaps less than 1 day later in the migration, baseline values of cortisol are restored (~100 ng ml^{-1}) and reproductive hormones return to their elevated levels (Hinch et al., 2006). Yet prior to the present study, the potential interactions between cortisol and reproductive hormone oscillations had not been investigated in terms of impacts on behavior at spawning grounds and reproduction for a semelparous species. The raceway blood profiles and hormone validation data collected in the present study indicated that, even though cortisol titers in cortisol-treated fish were increased to levels observed in senescing salmon (Stein-Behrens and Sapolsky, 1992; Barry et al., 2010; Hruska et al., 2010), cortisol treatment did not alter reproductive hormone titers in either unripe or ripe fish.

It is important to recognize that the experimental elevation of cortisol titers with IP implants is not itself a stress response, but instead results in elevated cortisol that is consistent with a stress response. Nonetheless, collectively these data are consistent with the notion that semelparous salmon may be resilient to the effects of stress hormones during the final phases of reproduction (Wingfield and Sapolsky, 2003). However, in the case of Pacific salmon, it is unclear when such a transition takes place during the migration. In mainstream riverine habitats, fish mount a cortisol response to a stressor and cortisol does, indeed, result in suppression of reproductive hormone titers (Hinch et al., 2006; Young et al., 2006). Yet, our data indicate that, upon arrival at spawning grounds, reproductive hormones are not altered by either certain acute stressors that are expected to elevate plasma cortisol levels (see below) or experimental cortisol manipulation. Because we did not observe any differences between ripe and unripe fish with respect to the influence of cortisol elevation on hormone titers, the onset of resistance to elevated cortisol appears to occur prior to ovulation, a point that warrants investigation in a further study. The transition may be associated with the decline from stable levels of reproductive hormones as the fish move into an ovulated state. During ovulation, there is a critical need to increase 17α-hydroxy-20 β-dihydroprogesterone (17α, 20β-P) to complete reproduction (Dye et al., 1986) because this hormone induces sexual maturation necessary for the ovulation process, whereas estradiol and testosterone mediate maturation and ovulation (Goetz, 1983; Mishra and Joy, 2006).

Channel longevity and reproductive success

Cortisol-treated fish exhibited decreased longevity and high egg retention during the channel experiment, despite our finding that cortisol treatment did not change reproductive hormone titers. Therefore, chronic cortisol elevation on spawning grounds negatively influences reproductive function and success. Even though egg release by metyrapone- and sham-treated fish during the unripe enclosure experiment was reduced when compared to other treatment groups, overall these fish still released the majority of their eggs and longevity was comparable to control groups. As such, even if the cocoa butter implant did prevent some egg release in the sham, cortisol, and metyrapone treatments (see discussion below), the existence of differences among these treatments lends support to the notion that the driver of the differences was of a physiological nature rather than an artifact of the use of cocoa butter.

This suite of findings is particularly important because fisheries managers are concerned with the largely unexplained phenomenon of "pre-spawn mortality"—fish that die on spawning grounds either without spawning or with significant egg retention (Quinn et al., 2000). The eggs of such fishes are often still viable (Tierney et al., 2009), so it appears that other factors are inhibiting reproductive behavior and/or are advancing senescence. In a study of sockeye salmon

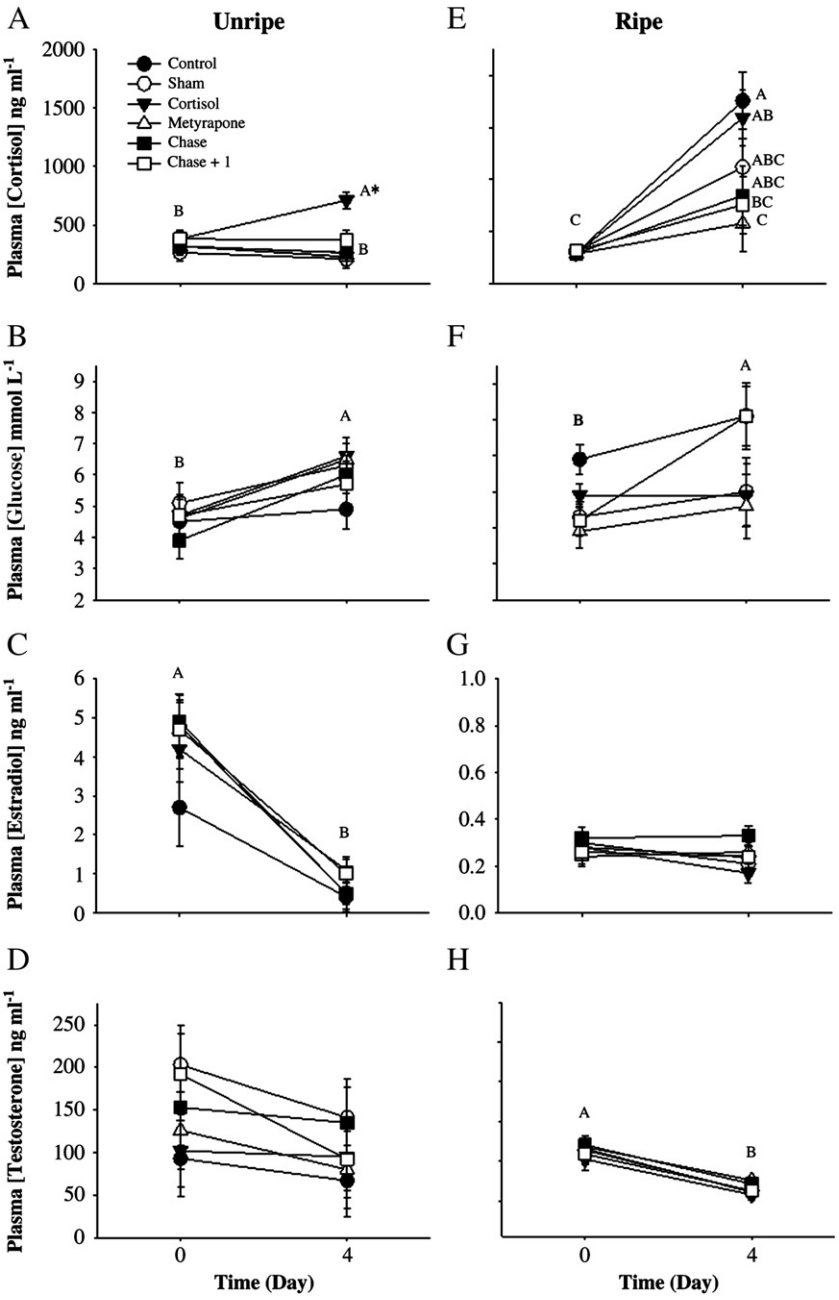

Fig. 6. (A–H). Blood hormone and glucose values of unripe (A–D) and ripe (E–H) pink salmon (*Oncorhynchus gorbuscha*) before experimentation and 4 days after treatment (see text for treatment details), stated as mean values (±SE). All Ranked Sum data were analyzed using two-way, repeated-measures ANOVAs with time and treatment as the independent variables. Dissimilar letters denote significant differences among treatment groups and time periods (Tukey–Kramer HSD test, $p<0.05$). Sample sizes were as follows for unripe fish: before; cortisol = 10, control, chase and metyrapone = 9, chase + 1 and sham = 8. After; cortisol = 10, chase and control = 9, chase + 1, sham and metyrapone = 8. Sample sizes were as follows for ripe fish: $N = 10$ for all groups except for the "after" chase group where $N = 9$.

Table 2
Pink salmon (*Oncorhynchus gorbuscha*) behavior profiles for unripe fish during 4 day trials; values are stated as mean (±SE). All data were analyzed using Wilcoxon Rank-Sum tests, and Tukey's HSD test was used to determine where differences lay when a significant effect was obtained (noted by letter scores). All data that are expressed as percentages were transformed into ArcSine (square root) values before being analyzed. Data for all variables except the aggression score were averaged over days that fish were on established territories. Aggression scores were added for all days spent on territories and divided by number of observational min. Each fish had a similar score for aggressive attacks against, and this score, divided by number of observational min, was subtracted from the previous value to obtain the overall aggression score. Sample sizes were as follows: chase = 9, control and sham = 7, chase + 1 and cortisol = 6, metyrapone = 5.

Variable	Treatment						Statistics	
	Control	Sham	Cortisol	Metyrapone	Chase	Chase + air	Stat	P-value
% Time on territory	100 ± 3^{A}	97 ± 3^{AB}	89 ± 3^{B}	94 ± 4^{AB}	98 ± 3^{AB}	90 ± 3^{AB}	12	**0.03**
% Time with male	40 ± 11	29 ± 11	38 ± 12	31 ± 13	37 ± 10	22 ± 12	7.9	0.2
Average # of digs	0.6 ± 0.7	0.7 ± 0.7	2 ± 0.8	0.2 ± 0.7	1 ± 0.7	0.2 ± 0.8	5.0	0.4
Aggression score	0.7 ± 0.2^{A}	0.3 ± 0.2^{AB}	-0.02 ± 0.2^{B}	0.4 ± 0.2^{AB}	0.2 ± 0.2^{AB}	0.1 ± 0.2^{AB}	13	**0.04**

Table 3
Pink salmon (*Oncorhynchus gorbuscha*) behavior profiles for ripe fish during 4 day trials; values are stated as mean (±SE). All data were analyzed using Wilcoxon Rank-Sum tests and Tukey's HSD test was used to determine where differences lay when a significant effect was obtained (noted by letter scores). All data that are expressed as percentages were transformed into ArcSine (square root) values before being analyzed. Data for all variables except the aggression score were averaged over days that fish were on established territories. Aggression scores were added for all days spent on territories and divided by number of observational min. Each fish had a similar score for aggressive attacks against, and this score, divided by number of observational min was subtracted from the previous value to obtain the overall aggression score. Sample sizes were as follows: metyrapone = 10, sham, cortisol, control and chase + 1 = 9 and chase = 7.

Variable	Treatment						Statistics	
	Control	Sham	Cortisol	Metyrapone	Chase	Chase + Air	Statistic	P-value
% Time on territory	97 ± 2	98 ± 2	97 ± 2	97 ± 2	99 ± 3	100 ± 2	7.8	0.2
% Time with male	45 ± 8	33 ± 8	54 ± 8	47 ± 7	36 ± 9	40 ± 8	4.1	0.5
Average # of digs	2 ± 0.7	1 ± 0.7	2 ± 0.7	2 ± 0.6	1 ± 0.8	2 ± 0.7	2.8	0.7
Aggression score	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.6 ± 0.1	7.2	0.2

at the Weaver Creek spawning channel, Hruska et al. (2010) related mortality to changes in physiological condition and activity levels, providing a baseline of variables that change as Pacific salmon (specifically sockeye salmon) senesce. To complement that work, the present study attempted to identify whether stressful conditions can cause pre-spawn mortality on spawning grounds. It seems plausible that since cortisol treatment in the present study increased cortisol values to those found in senescing fish and at the same time reduced longevity, then the premature mortality we observed was a function of this senescence-like physiological state, a state that was not reached via the imposition of acute stressors, even though exposure to acute stressors was expected to acutely elevate circulating cortisol levels.

Enclosure study

Unripe cortisol-treated fish spent less time on their territory than all other groups. In addition, cortisol-treated fish were significantly less aggressive than fish in the other treatment groups, and were frequently subjected to aggressive attacks from conspecifics. A decrease in aggressiveness is detrimental to reproductive success because a female benefits from guarding its territory from other females looking for suitable habitat, and aggressive behavior is often associated with reproductive success (Heard, 1991; Quinn and Foote, 1994). These results are supported by previous studies that found that cortisol treatment increased the probability of individual fish (rainbow trout in these cases) experiencing increased fin damage indicative of both aggressive attacks (Gregory and Wood, 1999) and becoming socially subordinate (DiBattista et al., 2005; Gilmour et al., 2005). No behavioral differences were detected among treatments for ripe fish. This finding suggests that even in the face of chronically elevated cortisol levels, reproductively mature fish maintain key reproductive behaviors, further supporting the idea that fish with limited reproductive opportunity will still engage in spawning in what would be regarded as extreme situations during other life-history phases.

Metyrapone treatment

Metyrapone inhibits the enzyme 11-β hydroxylase, thereby preventing synthesis of cortisol from 11-deoxycortisol (Mommsen et al., 1999). No significant changes in cortisol titers, reproductive behavior, reproductive success, or hormone levels occurred as a result of metyrapone treatment. Doyon et al. (2006) determined that metyrapone inhibits the cortisol response to a stressor but does not reduce baseline (non-stressed) cortisol levels. There is also a suggestion that plasma cortisol does not turn over rapidly for semelparous salmon on spawning grounds (Donaldson and Fagerlund, 1972). Therefore, it is possible that baseline (i.e. non-stressed) levels of cortisol were maintained, but increases in cortisol levels with stress were prevented (although this was not tested in the current study). For future studies, responsiveness could be observed following injection to determine whether metyrapone-treated fish respond to acute stressors. This approach would provide a useful means of distinguishing between the effects of baseline cortisol and stress-induced cortisol on reproductive success.

There was evidence that metyrapone treatment caused some egg retention and delayed senescence, as observed in the enclosure study (i.e., significantly lower cortisol values compared with other treatment groups in ripe fish). If cortisol spikes immediately prior to ovulation (Milla et al., 2009), this process could have been inhibited through the action of metyrapone in blocking cortisol synthesis. Additionally, cortisol rises again during senescence (Hruska et al., 2010), and this process also could have been inhibited by the action of metyrapone. To examine these possibilities, more detailed time course of plasma hormone levels is needed. Ideally, metyrapone-treated fish should be monitored just prior to ovulation, immediately following egg release and before morbidity.

Elevated cortisol levels on spawning grounds

One of the most notable findings of this study was that exposure of pink salmon to acute stressors on spawning grounds did not alter spawning ground longevity, reproductive success, or behavior, in accordance with theory that semelparous animals in general should resist stress (i.e. attenuate stress responses and/or exhibit resistance to the effects of elevated stress hormone levels) in favor of allocating energy to their current, and only, reproductive opportunity (Wingfield and Sapolsky, 2003). Behavioral and physiological profiles of spawning Pacific salmon are well documented, but the function of (baseline) cortisol elevation in semelparous fish in their natural spawning habitat is not well understood. From a mechanistic standpoint, it has yet to be determined how semelparous salmon successfully breed despite circulating cortisol being elevated to a level that would inhibit reproduction in other species. However, our data indicate that there is a limit to this capacity because cortisol treatment did impair reproduction.

The scope of the present study does not enable us to speculate about the mechanism of cortisol elevation on spawning grounds. Moreover, we did not measure cortisol receptor occupancy or sensitivity, factors that will affect the ability of (high) cortisol levels to mediate target tissue responses, and an issue that ideally would be addressed in future studies. We can conclude, however, that acute elevation of cortisol levels does not hinder reproductive behaviors and outcome. In addition, it seems that the second spike in cortisol is an indicator of impending senescence, as noted in previous studies (e.g., Hruska et al., 2010). If high cortisol levels are evident before spawning is complete, key reproductive behaviors and outcome can be negatively affected, as evidenced in this study by the use of semi-chronic cortisol implants. It would have been useful to collect blood immediately following exposure of fish to the acute stressors to assess the extent of the stress response elicited. In a similar study on stress responsiveness, Cook et al. (2011) observed an increase in cortisol levels from 333 ± 17 to 497 ± 22 ng ml^{-1} following 2 min of air exposure using Weaver Creek sockeye salmon. Other Pacific salmonids (including sockeye, chum [*O. keta*], coho [*O. kisutch*] and Chinook [*O. tshawtscha*]), as well as pink salmon, all have been found to experience an acute stress

response when exposed to short-term stressors, with cortisol levels recovering within 2–4 h (Mike Donaldson, UBC, personal communication). Therefore, the pink salmon in this study likely experienced an acute stress response with chasing and air exposure, but were not negatively impacted by these acute stressors in terms of reproductive physiology, behavior, or outcome.

Study limitations

Sham-treated fish were negatively affected by the administration of a cocoa-butter implant alone. Although longevity was not altered, sham-treated fish released only ~70% of their eggs on average in the channel experiment, somewhat less (but not significantly so) than control fish, and released only ~15% in the unripe enclosure trials, a value significantly lower than that of control fish. When fish were dissected afterwards, some eggs were observed within the body cavity intermingled with the cocoa butter, creating a mass that might not be easily expelled through the vent during spawning. This unexpected outcome might be prevented in future studies by using a vehicle with a lower melting point or by using less volume than used in the present study. Indeed, a recent study on brown trout (*Salmo trutta*) revealed that cocoa butter implants reduced egg and hatchling size (Hoogenboom et al., 2011) relative to controls, further emphasizing the need for additional research on improving the mechanisms for experimental delivery of lipophilic hormones, a technique that is becoming increasingly common in fish physiology research (reviewed in Gamperl et al. 1994). In our study, because cortisol-treated fish exhibited high cortisol levels with reduced longevity together with a decrease in the number of eggs released, we believe that our results support a real and significant effect of cortisol itself.

Conclusion

Because the migratory and spawning processes of Pacific salmon are regarded as remarkable challenges, we strive to understand the links among physiology, behavior and fitness in these animals. Salmon migrations historically have shown a large degree of consistency, but any environmental changes or anthropogenic perturbations are considered a potential threat to reproduction, and thus survival, of a given population. Our results suggest that acute stressors do not influence behavior or reproductive outcome when experienced upon arrival at spawning grounds. However, there is a limit to the ability of these fish to tolerate elevated cortisol levels because experimental cortisol elevation for several days negatively affected reproductive success and longevity. Collectively, our results address a void in current research, explaining how varying degrees of cortisol elevation can influence reproductive behavior and spawning success of Pacific salmon. Finally, our study is among the first field studies conducted to investigate the ecological consequences of stress during reproduction for a semelparous species.

Acknowledgments

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