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# Effect of *Ichthyophonus* on blood plasma chemistry of spawning Chinook salmon and their resulting offspring in a Yukon River tributary

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ABSTRACT: *Ichthyophonus* is a protozoan parasite of Alaska Chinook salmon *Oncorhynchus tshawytscha*. In this study, we determined whether spawning Chinook salmon in the Yukon River drainage exhibited a measurable stress response (i.e. elevated plasma cortisol concentrations) and detectable changes in selected blood plasma chemistry parameters when infected with *Ichthyophonus.* The resulting alevin were also analyzed for any differences in blood plasma chemistry caused by parental infection with *Ichthyophonus.* In 2010, 2011, and 2012, spawning adult Chinook salmon were collected from the Salcha River, Alaska, USA, and the prevalence of *Ichthyophonus* in these fish was 7.8, 6.3, and 8.3%, respectively. Fish with no clinical signs of *Ichthyophonus* and *Ichthyophonus*-positive parents were cross-fertilized to investigate potential second-generation effects as a result of *Ichthyophonus* infection. We found no significant difference in cortisol concentrations in blood plasma between *Ichthyophonus*-positive and -negative adults or between alevin from *Ichthyophonus*-positive and -negative parents. There were no significant differences in blood plasma parameters (e.g. alanine aminotransferase, creatine kinase, glucose) of *Ichthyophonus*-negative and -positive adults, with the exception of aspartate aminotransferase, which was significantly higher in plasma of *Ichthyophonus*-negative adults. All clinical chemistry parameters for alevin resulting from both *Ichthyophonus*-negative and -positive parents were not significantly different. Based on this study, which has a limited sample size and low prevalence of *Ichthyophonus,* offspring of Chinook salmon appear to suffer no disadvantage as a result of *Ichthyophonus* infection in their parents on the Salcha River.

KEY WORDS: *Oncorhynchus tshawytscha* · Protozoan · Parasite · Yukon River · Salcha River · Cortisol concentration · Clinical blood chemistry



## INTRODUCTION

Chinook salmon *Oncorhynchus tshawytscha* are a fundamental source of sustenance, economics, and culture in Alaska and the Pacific Northwest (USA), providing food for marine mammals, predatory fishes, seabirds, and terrestrial animals (including humans), and providing marine-derived nutrients to river banks after spawning (Cederholm et al. 1999). Chinook salmon spawning in the upper reaches of the Yukon River drainage undertake one of the longest migrations (between 2400 and 3200 km) known for any salmonid (Behnke 2002). Chinook salmon returns from 2007 to 2009 were lower than expected based on reported spawning escapements in the brood years (JTC 2008, 2009, 2011). In response, a Chinook salmon directed commercial fishery on the Yukon River mainstem did not occur in 2008 and 2009 for the first time in almost a decade (JTC 2011), and the subsistence fishery was reduced

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by up to 50% (JTC 2011, 2013, 2014). In 2014, all Chinook salmon harvest, including subsistence, was closed on the Yukon River. In 2015, the run was still below the historical average, but escapement into Canada was met and some subsistence fishing was allowed (ADF&G 2015). While the ultimate cause(s) for the continued run failures of Chinook salmon remain unknown, infectious diseases play a role in some other salmon population declines (Kocan et al. 2004, Kocan & Hershberger 2006). Disease may be a contributing factor, either due to pathogen-induced mortality, reduced fecundity, or the inability of Chinook salmon to successfully migrate and spawn.

*Ichthyophonus* is a marine-derived protozoan parasite infecting a variety of marine and anadromous fish species, including salmonids (McVicar 1999, Kocan et al. 2004, Tierney & Farrell 2004, Gavryuseva 2007). This parasite was identified in the mid-1980s after fishers reported white papules on the heart, liver, and musculature of Chinook salmon returning to spawning tributaries in Alaska (Kocan et al. 2004). By the early 2000s, *Ichthyophonus* prevalence had reached 25 to 30% in Chinook salmon in the mainstem of the Yukon River (Kocan et al. 2003, 2004), and appears to be cyclical over time (Zuray et al. 2012). As Chinook salmon abundance fluctuates, prevalence of the disease also changes (Kocan et al. 2004, Zuray et al. 2012), as is typical of a parasite cycle where increased numbers of individuals in an area can increase the incidence of disease and vice versa (Altizer et al. 2006). Evidence suggests that *Ichthyophonus* may cause pre-spawning mortality in Chinook salmon (Kocan et al. 2004, 2006, 2009). Since 2003, the prevalence of *Ichthyophonus* in Chinook salmon has declined continuously and appears to be correlated with a greater than 50% decline in the population abundance of Chinook salmon in the Yukon River (Zuray et al. 2012). Infection with *Ichthyophonus* reduces oxygen uptake and exercise capabilities (Tierney & Farrell 2004), and fish appear to compensate by increasing heart mass and cardiac output (Kocan & Hershberger 2006). This results in lower stamina and swim speed (Kocan et al. 2009). Due to these effects, *Ichthyophonus* prevalence can be higher in lower parts of the Yukon River compared with the spawning grounds as fish die before reaching their final destination (Kocan & Hershberger 2006).

Environmental stressors are well recognized for their impact on fish health (Marcogliese 2004), and disease caused by *Ichthyophonus* may be exacerbating morbidity of salmon in Alaska (Hamazaki et al. 2013). In-river conditions in the Yukon River have changed over the past 30 yr, with June water temperatures having increased by approximately 2.5°C (Kocan et al. 2004, Kahler et al. 2007). Temperature changes influence disease processes in poikilotherms (Finn & Nielson 1971), by causing higher parasite loads, faster die-offs, and reduced swimming performance with increased temperature (Kocan et al. 2009). Stress lowers the ability of fish to maintain homeostasis and carry out actions crucial for endurance, growth, and reproduction (Schreck 1982). The most common glucocorticoid hormone found in salmonids is cortisol (Donaldson 1981, Passino 1984), which is often utilized as a gauge of stress (Fevolden et al. 1993) and serves as a reliable bioindicator of stress in fishes. In general, increased cortisol levels are associated with increased disease susceptibility (Pickering & Pottinger 1989, Fevolden et al. 1993). Elevated levels of blood glucose (GLU) are another indicator of stress in fishes (Van Waarde et al. 1990, Wendelaar Bonga 1997).

Measurement of blood plasma parameters can be useful indicators of tissue damage due to disease or other stressors (Grizzle & Kiryu 1993, McPherson & Pincus 2011), and monitoring the health of free-ranging animal populations does not necessarily require lethal procedures when utilizing blood. Diseases, such as *Ichthyophonus*, cause heart and skeletal muscle inflammation, which has been positively correlated with increased levels of aspartate aminotransferase (AST) and creatine kinase (CK) (Marty et al. 1998, Yousaf & Powell 2012). The involvement of these enzymes in acute and chronic disease processes, in particular with regard to tissue damage, makes these blood plasma parameters good potential bioindicators for the necrotic and inflammatory action associated with *Ichthyophonus* infection.

The purpose of this study was to determine whether Chinook salmon exhibit a measurable stress and physiological response to infection with *Ichthyophonus*. We hypothesized that spawning Chinook salmon infected with *Ichthyophonus* would exhibit a stress response resulting in higher concentrations of cortisol and GLU compared with *Ichthyophonus*-negative salmon, as well as measureable increases in tissue damage indicators (e.g. alanine aminotransferase [ALT], AST, and CK). We further hypothesized that cortisol concentrations and some blood plasma parameters, such as CK and AST, propagate to the second generation, such that offspring produced by *Ichthyophonus*-positive parents would differ from those of *Ichthyophonus*-negative parents.

## MATERIALS AND METHODS

### Sample collection

In 2010 and 2011, Chinook salmon were sampled via electrofishing from the Salcha River, Alaska (approximately river km 39 at the following coordinates: 64.472°N, 146.971°W). In summer 2010, we collected 51 Chinook salmon (27 females, including 3 freshly dead, and 24 males) from 29 July to 1 August. On 19 July 2011, we sampled 32 Chinook salmon (15 females and 17 males). On 26 July 2012, we sampled 12 Chinook salmon (6 females and 6 males) at the Salcha River boat ramp (64.464°N, 146.967°W); these fish were captured using rod and reel sport fishing gear. The lower sample size and different capture gear used in 2012 was due to permit restrictions as a result of low Chinook salmon returns to the Yukon and Salcha Rivers.

After capture, adult Chinook salmon were held in net pens (1.2 × 1.2 × 2.4 m, with a 3.8 cm mesh) for 1 to 2 d, and were maintained in the river current for continuous water flow. Fish were held at a maximum density of 12 fish pen$^{-1}$, with males and females kept separated. The ripeness of females was characterized by loss of skein structure, and was determined by gentle palpation of the belly and deposition of eggs through the ovipositor. Milt was readily available from males upon capture; therefore, female ripeness determined when fertilization could commence. Fish were euthanized by cranial concussion, followed by exsanguination via the caudal vein by cutting the peduncle. Whole blood was collected in sterile BD Vacutainers® coated with sodium heparin and centrifuged at 1900 × *g* for 10 min (VWR® Clinical 50 Centrifuge). Next, plasma was pipetted into cryovials and immediately flash frozen in liquid nitrogen. Once all samples were collected and the site was thoroughly cleaned for the day, samples were transported to the University of Alaska Fairbanks and stored at −80°C until analysis.

To avoid cross-contamination with *Ichthyophonus* across fish and samples, tissues were collected using extreme care with sterile, disposable sampling supplies. The ventral surface of each fish was cleaned of mucus and blood and cut with a sterile blade to expose the heart. All fish were examined internally for typical clinical signs of *Ichthyophonus* infection, which can be observed as white papules on the heart, liver, or kidney (Fig. 1A). A piece of cardiac muscle (~1 g) was removed with a second sterile blade and cultured in MEM-5 supplemented with 5% fetal bovine serum, penicillin, streptomycin, and gentamycin (Kocan & Hershberger 2006). The tissue was then incubated at 14°C and examined daily for *Ichthyophonus* schizonts (Fig. 1B) to confirm clinical infection with *Ichthyophonus* at the State University of New York, Syracuse, NY (Whipps et al. 2006). A second subsample of cardiac muscle was placed in 95% ethanol and shipped to Purdue University, West Lafayette, IN, for molecular confirmation of infection. The presence of *Ichthyophonus* 18S rDNA was evaluated using polymerase chain reaction (PCR) following the procedure described by Whipps et al. (2006). While molecular confirmation positively identifies *Ichthyophonus,* the culture method determines whether the parasite is viable in the host.

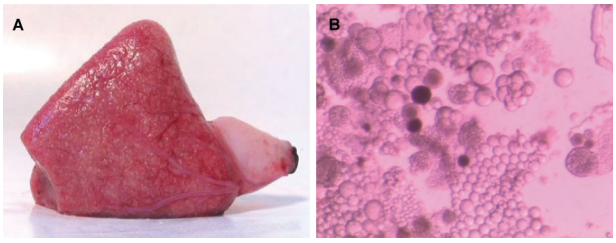


Fig. 1. (A) Clinical signs of *Ichthyophonus* infection in the heart of a spawning Chinook salmon *Oncorhynchus tshawytscha*; white diffuse lesions are visible. (B) *Ichthyophonus* spores at 50× magnification. Photos by L. Horstmann-Dehn (A) and C. Whipps (B)

### Analysis of plasma chemistry and alevin homogenates

Spawning Chinook salmon blood plasma was analyzed with an Abaxis VetScan using the comprehensive diagnostic profile reagent rotor and the avian/reptilian profile plus rotor. The following diagnostic parameters were analyzed (usually within 8 h of collection) in Chinook salmon plasma samples: albumin (ALB), alkaline phosphatase (ALP), ALT, amylase (AMY), AST, bile acids (BA), blood urea nitrogen (BUN), calcium (Ca), CK, cortisol, creatinine (CRE), globulin (GLOB), GLU, potassium (K), sodium (Na), phosphorus (P), uric acid (UA), total bilirubin (TB), and total protein (TP). For alevin, the diagnostic parameters listed above were determined on total body homogenates after Franson (1982). In 2010, alevin were obtained from this study's fertilization trial (Floyd-Rump 2015). In 2011 and 2012, the Alaska Department of Fish and Game (ADF&G) Division of Sport Fish in Fairbanks collected spawning Chinook salmon from the Salcha River for rearing

offspring in the state hatchery, and the resulting alevin were acquired for analysis in this study; parentage (i.e. *Ichthyophonus*-positive or -negative was known for all alevin). Approximately 1 g of tissue (or ~5 alevin) was placed in a glass homogenization tube, and then 9 ml of cold phosphate buffer (0.1 M, pH 7.4) were added to the tube for homogenization (Franson 1982). Using a glass rod, the sample was homogenized for 2 to 3 min, or until all large particulates were dissolved. Next, the sample was centrifuged for 20 min at 1000 × *g*, and supernatants were removed using a serological pipette. Samples were refrigerated at 4°C immediately after preparation and were analyzed on an Abaxis VetScan Classic, using a comprehensive diagnostic profile reagent rotor and an avian/reptilian profile plus rotor, within 4 h of homogenization.

### Cortisol radioimmunoassay

Cortisol concentrations were determined using a solid phase single antibody radioimmunoassay (RIA) (Siemens Coat-A-Count® Kit). Plain 12 × 75 mm polypropylene tubes were used in duplicate for all samples and standards. Cortisol calibrators containing 0.0, 5, 10, 50, 100, 200, and 460 ng of cortisol per ml in processed human serum were used to create a standard curve. Before Chinook salmon plasma samples were tested, the assay was validated with tests for parallelism (Fig. 2A) and accuracy (Fig. 2B). For each sample, 25 µl of each standard was added to each tube in duplicate, with the zero calibrator added to the nonspecific binding tube. Then 1 ml of $^{125}$I cortisol was added to every tube, and all tubes were incubated in a water bath for 45 min at 37°C. Sample reactivity was determined with a gamma counter. Any samples that had a 10% or greater difference in counts between duplicates or counts outside the range of the standard curve were diluted and reanalyzed.

### Cortisol extraction and assays of alevin homogenates

Cortisol concentrations were assessed in total body-homogenized alevin using the solid phase single antibody RIA as described above. One individual alevin, weighing approximately 0.2 g, was placed in a 2 ml cryovial with 1.7 mm ceramic homogenizing beads, and then 1 ml of 200 proof ethanol was added to this cryovial. All samples were homogenized for 45 s using a Disruptor Genie® at a speed of 6.5 m s$^{-1}$. The samples were then iced, and these steps were repeated a total of 8 times to ensure proper homogenization. Samples were centrifuged for 10 min at 3000 × *g* (Beckman GS-6R), and then 0.5 ml of the supernatant was aspirated into 13 × 100 mm disposable glass culture tubes and evaporated under compressed nitrogen gas, while incubating in a 25°C water bath. The supernatant residue was purified in a series of extractions using ethanol:acetone (4:1 v/v), diethyl ether, acetonitrile, and hexane (Kellar et al. 2006). After the final extraction, hexane was added, and the bottom layer was aspirated and then evaporated under nitrogen. The final sample was stored at –80°C.

Before analysis, the sample homogenates were reconstituted in 500 µl of phosphate-buffered saline

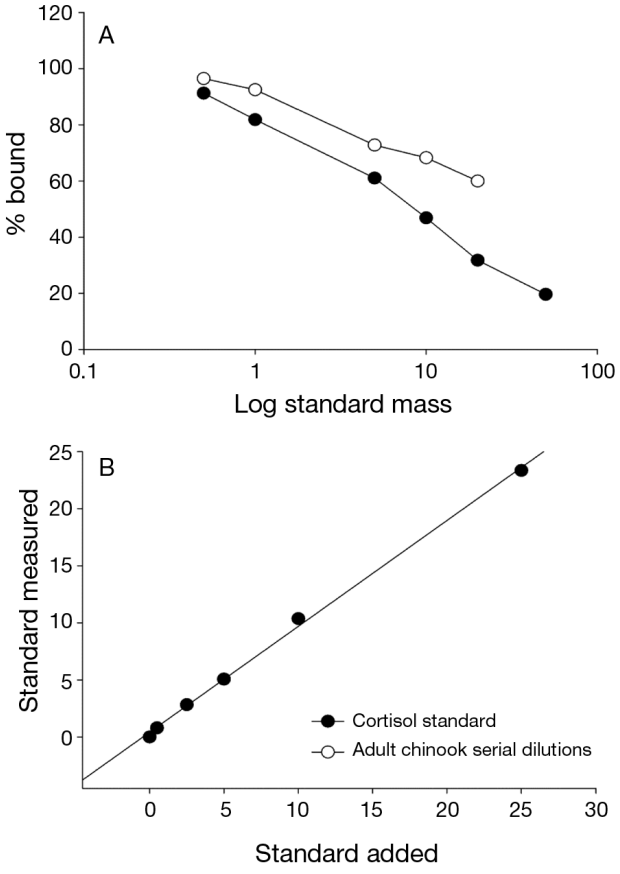


Fig. 2. (A) Serial dilutions of cortisol (% binding) from undiluted to 1:16 pooled spawning Chinook salmon *Oncorhynchus tshawytscha* plasma (open circles), plotted with a cortisol kit standard (closed circles) versus log-standard mass (ng ml$^{-1}$) indicating displacement by the pool parallel to that of the standard. (B) Chinook salmon plasma cortisol (ng ml$^{-1}$) added 1:1 to each of the assay standards (ng ml$^{-1}$), with a slope of 0.93 ($R^2$ = 0.99) indicating 93% accuracy of the assay in the measurement of plasma cortisol

(pH 7.5) with 1% bovine serum albumin. Samples were sonicated using a Tissue Tearor (BSP 780CL-07) for 3 min at the highest setting. Cortisol concentrations were then measured as described above. Because some samples contained low cortisol concentrations, samples were rerun at a higher sample volume. Cortisol assays on alevin were validated (i.e. parallelism and accuracy) as described for plasma samples. The parallelism test passed, and the accuracy of the assay in the measurement of Chinook salmon alevin cortisol of total body homogenates was 85%.

### Statistical analyses

Standard descriptive statistics were compiled, i.e. mean, median, standard deviation (±1 SD), and data range. The statistical program package R was used for all statistical tests, with an alpha of 0.05 considered significant (R Development Core Team 2008). Within R, a Shapiro-Wilk's normality test and Bartlett's test for equal variances were performed to assess if data met assumptions for ANOVA. Data were not normally distributed with unequal variances; therefore, a non-parametric Kruskal-Wallis test was used to assess differences between *Ichthyophonus*-positive and -negative spawning adults, male and female spawning adults (*Ichthyophonus*-negative only), and their resulting offspring. Principal components analysis (PCA) was performed on all clinical chemistry parameters for adults and alevin using PRIMER-E (Version 6.1.16) to assess the combined effects of all variables when comparing *Ichthyophonus*-negative and -positive fish and their offspring. PCA done for alevin was normalized. In addition, multidimensional scaling on a Bray-Curtis similarity matrix without data transformation was performed for adult Chinook salmon plasma and alevin clinical chemistry parameters using PRIMER-E (stress of 0.10 is fair and >0.2 is poor; Kruskal 1964).

## RESULTS

In 2010, we collected 51 Chinook salmon (27 females [3 dead], 24 males), of which 4 were *Ichthyophonus*-positive (3 females, 1 male; 7.8% prevalence). Note that 1 male and 3 female plasma samples were hemolyzed and therefore were not usable for blood chemistry analysis; these fish are not included in the sample sizes, and none of these fish tested positive for *Ichthyophonus*. In 2011, 15 females and 17 males were sampled, of which 2 males were infected with *Ichthyophonus* (6.3%). In 2012, we sampled 12 salmon (6 males, 6 females), and only 1 male was positive for *Ichthyophonus* (8.3%). There was complete concordance between the 2 diagnostic methods, i.e. culture and PCR, in all years. However, 1 individual sampled in 2010 displayed clinical signs of *Ichthyophonus*, but was not positive by either culture or PCR; this fish was considered *Ichthyophonus*-negative. Plasma chemistry parameters and cortisol concentrations for spawning Chinook salmon are given in Table 1. Variables were not significantly ($p > 0.05$) different between *Ichthyophonus*-positive and -negative spawning Chinook salmon (Table 1), with the exception of AST, which was significantly lower in *Ichthyophonus*-positive adults ($p = 0.03$). CK ranged from 0.0 ± 0.0 U $l^{-1}$ for *Ichthyophonus*-positive adults to 1041.1 ± 2678.2 U $l^{-1}$ for *Ichthyophonus*-negative adults, but differences were not significantly different ($p = 0.27$) due to large SDs. PCA supports the overall lack of difference between *Ichthyophonus*-positive and -negative adult Chinook salmon (Fig. 3). The first principal component (PC) using blood parameters of spawning Chinook salmon explained 88.0% of the variability, and the second PC explained 10.2% (Fig. 3A). However, PCA showed 2 groups of salmon that separated due to high concentrations of CK, but no clear pattern emerged (Fig. 3A) after this analysis. When CK was removed from the PCA (Fig. 3B), 2 groups were still noticeable, and separation appeared to be mostly due to sex, although not in a uniform manner (Fig. 3B). The separation was driven by a positive loading of AST and PHOS in PC1, and a negative loading of cortisol in PC1 (Fig. 3B; PC1 explained 84.5% of the variation, and PC2 explained 13.0%).

All plasma chemistry parameters for *Ichthyophonus*-negative spawning Chinook salmon were analyzed between males and females (Table 2). Variables were not significantly different ($p > 0.05$) between male and female salmon, with the exception of ALB ($p = 0.0004$), ALP ($p = 0.0003$), and cortisol ($p = 0.01$), all of which were significantly higher in females compared with males.

All clinical chemistry parameters as well as cortisol for Chinook salmon alevin produced by *Ichthyophonus*-positive and -negative adults were not significantly different ($p > 0.05$); concentrations of all parameters are summarized in Table 3. The PCA of clinical chemistry parameters of alevin showed no separation of the 2 groups. PC1 explained 22.4% of variability and PC2 explained 13.7% for a cumula-

Table 1. Plasma chemistry parameters of spawning Chinook salmon *Oncorhynchus tshawytscha* (*Ichthyophonus*-positive: n = 3 females, 4 males; and *Ichthyophonus*-negative: n = 39 females, 42 males) sampled from the Salcha River, 2010 to 2012. A Kruskal-Wallis test was used, and $p \leq 0.05$ was considered significant (given in **bold**); $H_0$: mean ranks of the 2 populations are equal. For plasma parameters that did not have any variation in value, NA replaces the range for that parameter. Literature values for plasma or serum of teleost fishes are provided for comparison

| | *Ichthyophonus*-positive | *Ichthyophonus*-negative | p | Literature values for teleosts |
|---|---|---|---|---|
| Albumin (g ml$^{-1}$) | | | | |
| Mean | 0.04 ± 0.01 | 0.03 ± 0.01 | 0.17 | 0.018–0.024 |
| Median | 0.04 | 0.03 | | Folmar (1993) |
| Range | 0.03–0.05 | 0.01–0.07 | | Atlantic salmon |
| Alkaline phosphatase (U l$^{-1}$) | | | | |
| Mean | 100.7 ± 14.5 | 95.0 ± 83.5 | 0.14 | 100.0–300.0 |
| Median | 102.5 | 77.0 | | Folmar (1993) |
| Range | 76.0–114.0 | 12.0–702.0 | | Rainbow trout |
| Alanine aminotransferase (U l$^{-1}$) | | | | |
| Mean | 324.2 ± 190.5 | 278.6 ± 350.9 | 0.23 | 74.7–118.8 |
| Median | 302.5 | 186.0 | | Chen et al. (2003) |
| Range | 109.0–587.0 | 2.5–2000.0 | | Nile tilapia |
| Amylase (U l$^{-1}$) | | | | |
| Mean | 9.8 ± 5.5 | 14.8 ± 9.7 | 0.20 | 819.0 |
| Median | 9.0 | 13.0 | | Gu et al. (2013) |
| Range | 2.5–18.0 | 0.0–55.0 | | Atlantic salmon |
| Aspartate aminotransferase (U l$^{-1}$) | | | | |
| Mean | 227.7 ± 557.7 | 1043.3 ± 865.5 | **0.03** | 202–351 |
| Median | 0.0 | 1364.0 | | Folmar (1993) |
| Range | 0.0–1366.3 | 0.0–2192.0 | | Atlantic salmon |
| Bile acids (µmol l$^{-1}$) | | | | |
| Mean | 17.5 ± 0.0 | 17.5 ± 0.0 | 1.00 | 21.0 |
| Median | 17.5 | 17.5 | | Gu et al. (2013) |
| Range | NA | NA | | Atlantic salmon |
| Blood urea nitrogen (mg ml$^{-1}$) | | | | |
| Mean | 0.03 ± 0.03 | 0.03 ± 0.02 | 0.93 | 0.11 |
| Median | 0.03 | 0.03 | | Davidson et al. (2014) |
| Range | 0.01–0.08 | 0.00–0.16 | | Rainbow trout |
| Total calcium (mg ml$^{-1}$) | | | | |
| Mean | 0.13 ± 0.02 | 0.12 ± 0.02 | 0.21 | 0.53 |
| Median | 0.13 | 0.12 | | Hasler et al. (2011) |
| Range | 0.10–0.16 | 0.08–0.16 | | Chinook salmon |
| Creatine kinase (U l$^{-1}$) | | | | |
| Mean | 0.0 ± 0.0 | 1041.1 ± 2678.2 | 0.27 | 900–4100 |
| Median | 0.0 | 0.0 | | Folmar (1993) |
| Range | NA | 0.0–9826.0 | | Striped mullet |
| Cortisol (ng ml$^{-1}$) | | | | |
| Mean | 390.0 ± 430.0 | 560.0 ± 440.0 | 0.29 | 297.0 |
| Median | 310.0 | 440.0 | | McConnachie et al. (2012) |
| Range | 3.0–104.0 | 50.0–174.0 | | Pink salmon |
| Creatinine (mg ml$^{-1}$) | | | | |
| Mean | 0.001 ± 0.0 | 0.001 ± 0.0008 | 0.93 | 0.005 |
| Median | 0.001 | 0.001 | | Sandnes et al. (1988) |
| Range | NA | 0.000–0.006 | | Atlantic salmon |
| Globulin (g ml$^{-1}$) | | | | |
| Mean | 0.010 ± 0.007 | 0.010 ± 0.008 | 0.34 | 0.034 |
| Median | 0.020 | 0.010 | | Lepic et al. (2014) |
| Range | 0.002–0.020 | 0.000–0.030 | | Vimba bream |
| Glucose (mg ml$^{-1}$) | | | | |
| Mean | 2.62 ± 0.95 | 2.59 ± 1.14 | 0.18 | 0.3–0.8 |
| Median | 2.80 | 2.47 | | Folmar (1993) |
| Range | 1.10–3.50 | 0.32–5.87 | | Rainbow trout |

(Table continued on next page)

Table 1 (continued)

| | *Ichthyophonus*-positive | *Ichthyophonus*-negative | p | Literature values for teleosts |
|---|---|---|---|---|
| Potassium (mmol $l^{-1}$) | | | | |
| Mean | 5.6 ± 2.3 | 4.7 ± 2.8 | 0.11 | 3.65 |
| Median | 4.8 | 4.6 | | Hasler et al. (2011) |
| Range | 3.4–8.5 | 0.0–8.5 | | Chinook Salmon |
| Sodium (mmol $l^{-1}$) | | | | |
| Mean | 133.5 ± 25.0 | 114.9 ± 35.9 | 0.07 | 165.3 |
| Median | 130.0 | 122.5 | | Hasler et al. (2011) |
| Range | 112.0–180.0 | 1.0–180.0 | | Chinook salmon |
| Phosphorus (mg $ml^{-1}$) | | | | |
| Mean | 0.15 ± 0.05 | 0.12 ± 0.04 | 0.79 | 0.61 |
| Median | 0.13 | 0.11 | | Hasler et al. (2011) |
| Range | 0.11–0.25 | 0.06–0.26 | | Chinook salmon |
| Uric acid (mg $ml^{-1}$) | | | | |
| Mean | 0.01 ± 0.003 | 0.02 ± 0.002 | 0.71 | 0.006 |
| Median | 0.02 | 0.02 | | Haman et al. (2012) |
| Range | 0.01–0.02 | 0.01–0.02 | | Atlantic sharpnose |
| Total bilirubin (mg $ml^{-1}$) | | | | |
| Mean | 0.006 ± 0.002 | 0.007 ± 0.009 | 0.82 | 0.003–0.080 |
| Median | 0.050 | 0.006 | | Folmar (1993) |
| Range | 0.004–0.008 | 0.002–0.070 | | Rainbow trout |
| Total protein (g $ml^{-1}$) | | | | |
| Mean | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.38 | 0.05 |
| Median | 0.04 | 0.05 | | Hasler et al. (2011) |
| Range | 0.03–0.05 | 0.03–0.08 | | Chinook salmon |

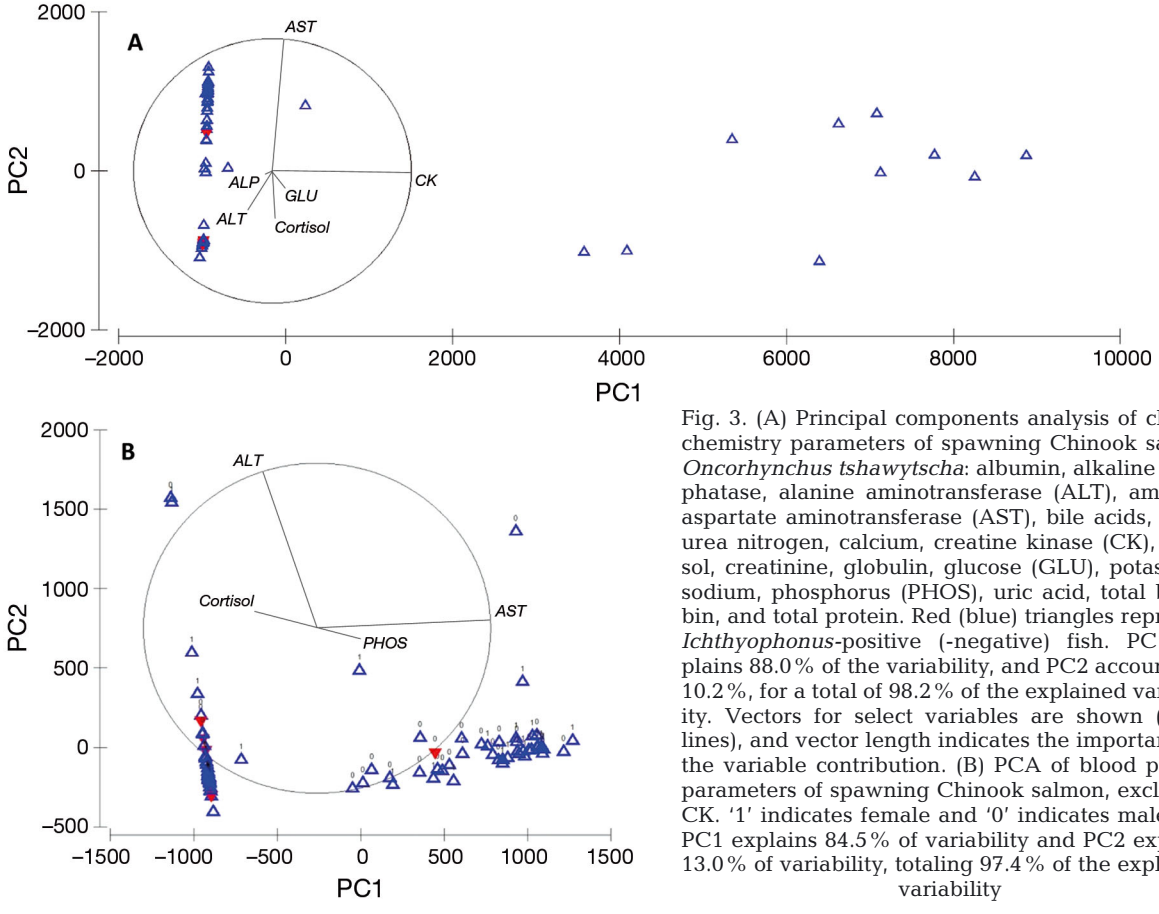


Fig. 3. (A) Principal components analysis of clinical chemistry parameters of spawning Chinook salmon *Oncorhynchus tshawytscha*: albumin, alkaline phosphatase, alanine aminotransferase (ALT), amylase, aspartate aminotransferase (AST), bile acids, blood urea nitrogen, calcium, creatine kinase (CK), cortisol, creatinine, globulin, glucose (GLU), potassium, sodium, phosphorus (PHOS), uric acid, total bilirubin, and total protein. Red (blue) triangles represent *Ichthyophonus*-positive (-negative) fish. PC1 explains 88.0% of the variability, and PC2 accounts for 10.2%, for a total of 98.2% of the explained variability. Vectors for select variables are shown (black lines), and vector length indicates the importance of the variable contribution. (B) PCA of blood plasma parameters of spawning Chinook salmon, excluding CK. '1' indicates female and '0' indicates male fish. PC1 explains 84.5% of variability and PC2 explains 13.0% of variability, totaling 97.4% of the explained variability

Table 2. Plasma chemistry parameters of *Ichthyophonus*-negative male (n = 42) and female (n = 39) spawning Chinook salmon *Oncorhynchus tshawytscha* from the Salcha River, 2010 to 2012. A Kruskal-Wallis test was used, and p ≤ 0.05 considered significant (given in **bold**); $H_0$: mean ranks of the 2 populations are equal. For plasma parameters that did not have any variation in value, NA replaces the range for that parameter

| | Male | Female | p |
|---|---|---|---|
| Albumin (g ml$^{-1}$) | | | |
| Mean | 0.003 ± 0.001 | 0.004 ± 0.001 | **0.0004** |
| Median | 0.003 | 0.004 | |
| Range | 0.001–0.005 | 0.002–0.007 | |
| Alkaline phosphatase (U l$^{-1}$) | | | |
| Mean | 66.1 ± 36.2 | 126.0 ± 101.0 | **0.0003** |
| Median | 53.0 | 101.0 | |
| Range | 12.0–184.0 | 56.0–702.0 | |
| Alanine aminotransferase (U l$^{-1}$) | | | |
| Mean | 293.5 ± 396.9 | 269.4 ± 277.0 | 0.74 |
| Median | 192.0 | 203.0 | |
| Range | 34.0–2000.0 | 2.5–1526.0 | |
| Amylase (U l$^{-1}$) | | | |
| Mean | 14.4 ± 7.1 | 14.5 ± 11.6 | 0.33 |
| Median | 15.0 | 12.0 | |
| Range | 2.5–29.0 | 0.0–55.0 | |
| Aspartate aminotransferase (U l$^{-1}$) | | | |
| Mean | 1182.8 ± 790.9 | 786.7 ± 911.5 | 0.12 |
| Median | 1481.5 | 0.0 | |
| Range | 0.0–2131.0 | 0.0–2192.0 | |
| Bile acids (µmol l$^{-1}$) | | | |
| Mean | 17.5 ± 0.0 | 17.5 ± 0.0 | 1.00 |
| Median | 17.5 | 17.5 | |
| Range | NA | NA | |
| Blood urea nitrogen (mg ml$^{-1}$) | | | |
| Mean | 0.03 ± 0.02 | 0.04 ± 0.03 | 0.24 |
| Median | 0.03 | 0.03 | |
| Range | 0.01–0.07 | 0.0–0.2 | |
| Total calcium (mg ml$^{-1}$) | | | |
| Mean | 0.12 ± 0.02 | 0.12 ± 0.02 | 0.96 |
| Median | 0.12 | 0.11 | |
| Range | 0.09–0.16 | 0.08–0.16 | |
| Cortisol (ng ml$^{-1}$) | | | |
| Mean | 39.5 ± 34.9 | 68.0 ± 46.7 | **0.01** |
| Median | 31.8 | 55.2 | |
| Range | 4.9–141.0 | 0.3–174.2 | |
| Creatine kinase (U l$^{-1}$) | | | |
| Mean | 1169.7 ± 2941.4 | 761.3 ± 2196.9 | 0.28 |
| Median | 0.0 | 0.0 | |
| Range | 0.0–9826.0 | 0.0–8725.0 | |
| Creatinine (mg ml$^{-1}$) | | | |
| Mean | 0.001 ± 0.005 | 0.001 ± 0.009 | 0.20 |
| Median | 0.001 | 0.001 | |
| Range | 0.0–0.003 | 0.0–0.006 | |
| Globulin (g ml) $^{-1}$ | | | |
| Mean | 0.01 ± 0.08 | 0.05 ± 0.2 | 0.27 |
| Median | 0.01 | 0.09 | |
| Range | 0.0–0.03 | 0.0–1.5 | |
| Glucose (mg ml$^{-1}$) | | | |
| Mean | 2.6 ± 1.1 | 2.6 ± 1.2 | 0.78 |
| Median | 2.4 | 2.5 | |
| Range | 0.3–5.0 | 0.3–5.9 | |
| Potassium (mmol l$^{-1}$) | | | |
| Mean | 5.2 ± 2.8 | 4.3 ± 2.7 | 0.11 |
| Median | 5.8 | 4.3 | |
| Range | 0.0–8.5 | 0.0–8.5 | |
| Sodium (mmol l$^{-1}$) | | | |
| Mean | 121.7 ± 26.0 | 110.3 ± 42.7 | 0.44 |
| Median | 124.0 | 121.0 | |
| Range | 50.0–180.0 | 1.0–180.0 | |
| Phosphorus (mg ml$^{-1}$) | | | |
| Mean | 0.13 ± 0.046 | 0.12 ± 0.033 | 0.65 |
| Median | 0.12 | 0.12 | |
| Range | 0.055–0.26 | 0.063–0.19 | |
| Uric acid (mg ml$^{-1}$) | | | |
| Mean | 0.01 ± 0.05 | 0.01 ± 0.03 | 0.93 |
| Median | 0.01 | 0.01 | |
| Range | 0.01–0.02 | 0.01–0.02 | |
| Total bilirubin (mg ml$^{-1}$) | | | |
| Mean | 0.006 ± 0.003 | 0.009 ± 0.011 | 0.13 |
| Median | 0.005 | 0.007 | |
| Range | 0.002–0.001 | 0.002–0.007 | |
| Total protein (g ml$^{-1}$) | | | |
| Mean | 0.05 ± 0.01 | 0.04 ± 0.01 | 0.33 |
| Median | 0.05 | 0.04 | |
| Range | 0.03–0.06 | 0.03–0.06 | |

tive 36.1% (Fig. 4). There was no apparent influence of parental sex for *Ichthyophonus*-positive adults on the resulting alevin, but sample sizes were too small for a meaningful comparison. Multidimensional scaling on a Bray-Curtis similarity matrix without data transformation on clinical chemistry parameters for adults and alevin also showed no dissimilarities between *Ichthyophonus*-positive or negative individuals or offspring (stress = 0.09 and 0.01 for adults and alevin, respectively).

## DISCUSSION

Analyzing blood plasma parameters, such as cortisol, GLU, and AST, are simple techniques that can provide important information about the physiological status of an animal (Chen et al. 2004). In this study, we investigated the effect of infection with *Ichthyophonus* on spawning Chinook salmon plasma chemistry and potential second-generation effects. We did not find any differences in basic clinical

Table 3. Total body homogenate clinical chemistry parameters for alevin produced by *Ichthyophonus* (*Ich*)-positive (n = 5) and *Ich*-negative (n = 30) Chinook salmon *Oncorhynchus tshawytscha* sampled in 2010 to 2012. A Kruskal-Wallis test was used, and $p \leq 0.05$ was considered significant; $H_0$: ranks of the 2 populations are equal. For clinical chemistry parameters that did not have any variation in value, NA replaces the range for that parameter

| | *Ich*-positive | *Ich*-negative | p |
|---|---|---|---|
| Albumin (g ml$^{-1}$) | | | |
| Mean | 0.005 ± 0.001 | 0.005 ± 0.001 | 0.93 |
| Median | 0.005 | 0.005 | |
| Range | 0.002–0.005 | 0.002–0.005 | |
| Alkaline phosphatase (U l$^{-1}$) | | | |
| Mean | 2.5 ± 0.0 | 2.2 ± 0.8 | 0.14 |
| Median | 2.5 | 2.5 | |
| Range | NA | 0.0–2.5 | |
| Alanine aminotransferase (U l$^{-1}$) | | | |
| Mean | 30.9 ± 14.7 | 30.5 ± 12.7 | 0.65 |
| Median | 28.0 | 26.3 | |
| Range | 17.3–58.0 | 14.7–57.3 | |
| Amylase (U l$^{-1}$) | | | |
| Mean | 2.1 ± 0.9 | 2.3 ± 0.7 | 0.68 |
| Median | 2.5 | 2.5 | |
| Range | 0.0–2.5 | 0.0–2.5 | |
| Aspartate aminotransferase (U l$^{-1}$) | | | |
| Mean | 62.5 ± 38.8 | 51.4 ± 21.6 | 0.37 |
| Median | 42.7 | 44.0 | |
| Range | 38.5–144.0 | 32.1–117.7 | |
| Bile acids (µmol l$^{-1}$) | | | |
| Mean | 17.5 ± 0.0 | 17.5 ± 0.0 | 1.00 |
| Median | 17.5 | 17.5 | |
| Range | NA | NA | |
| Blood urea nitrogen (mg ml$^{-1}$) | | | |
| Mean | 0.01 ± 0.0 | 0.01 ± 0.0 | 1.00 |
| Median | 0.01 | 0.01 | |
| Range | NA | NA | |
| Total calcium (mg ml$^{-1}$) | | | |
| Mean | 0.02 ± 0.00 | 0.02 ± 0.00 | 1.00 |
| Median | 0.02 | 0.02 | |
| Range | NA | NA | |
| Cortisol (ng ml$^{-1}$) | | | |
| Mean | 0.9 ± 0.4 | 0.9 ± 0.7 | 0.66 |
| Median | 0.9 | 0.8 | |
| Range | 0.3–1.0 | 0.3–4.0 | |
| Creatine kinase (U l$^{-1}$) | | | |
| Mean | 3342.0 ± 1556.8 | 5900.1 ± 4162.4 | 0.48 |
| Median | 3146.8 | 4273.3 | |
| Range | 2119.8–11097.3 | 2681.3–14462.0 | |
| Creatinine (mg ml$^{-1}$) | | | |
| Mean | 0.001 ± 0.000 | 0.001 ± 0.000 | 1.00 |
| Median | 0.001 | 0.001 | |
| Range | NA | NA | |
| Globulin (g ml$^{-1}$) | | | |
| Mean | 0.0 ± 0.0 | 0.0 ± 0.0 | 1.00 |
| Median | 0.0 | 0.0 | |
| Range | NA | NA | |
| Glucose (mg ml$^{-1}$) | | | |
| Mean | 0.40 ± 0.09 | 0.46 ± 0.16 | 0.68 |
| Median | 0.39 | 0.44 | |
| Range | 0.27–0.59 | 0.28–0.80 | |
| Potassium (mmol l$^{-1}$) | | | |
| Mean | 6.9 ± 2.0 | 5.6 ± 1.8 | 0.13 |
| Median | 6.9 | 4.9 | |
| Range | 4.7–10.5 | 3.0–8.9 | |
| Sodium (mmol l$^{-1}$) | | | |
| Mean | 180.0 ± 0.0 | 180.0 ± 0.0 | 1.00 |
| Median | 180.0 | 180.0 | |
| Range | NA | NA | |
| Phosphorus (mg ml$^{-1}$) | | | |
| Mean | 0.48 ± 0.16 | 0.58 ± 16.40 | 0.17 |
| Median | 0.45 | 0.50 | |
| Range | 0.30–0.83 | 0.29–0.89 | |
| Uric acid (mg ml$^{-1}$) | | | |
| Mean | 0.015 ± 0.006 | 0.014 ± 0.002 | 0.82 |
| Median | 0.014 | 0.014 | |
| Range | 0.011–0.018 | 0.012–0.018 | |
| Total bilirubin (mg ml$^{-1}$) | | | |
| Mean | 0.002 ± 0.004 | 0.002 ± 0.002 | 0.52 |
| Median | 0.002 | 0.002 | |
| Range | 0.002–0.003 | 0.002–0.003 | |
| Total protein (g ml$^{-1}$) | | | |
| Mean | 0.010 ± 0.001 | 0.010 ± 0.001 | 0.94 |
| Median | 0.010 | 0.010 | |
| Range | 0.007–0.01 | 0.005–0.01 | |

chemistry parameters of spawning adults on their terminal spawning grounds as a function of infection with *Ichthyophonus*, with the exception of AST, which was substantially higher in *Ichthyophonus*-negative salmon. Similarly, no differences were observed in clinical chemistry parameters of the resulting offspring.

Cortisol has been identified as one of the principal steroid hormones in spawning salmonids (Idler et al. 1964), and corticosteroids play a vital role in the catabolism of protein for delivery of metabolic energy in salmonids (Freeman & Idler 1973). Female salmonids generally have higher levels of cortisol than males (Idler et al. 1959, Folmar 1993, Berg et al. 2004), and this was observed in the current study as well (Table 2). Higher cortisol levels in female Chinook salmon could be due to increased estrogen experienced during spawning, which can trigger the salmon hypothalamic-pituitary-interrenal axis, whereas in males, exogenous androgens do not have

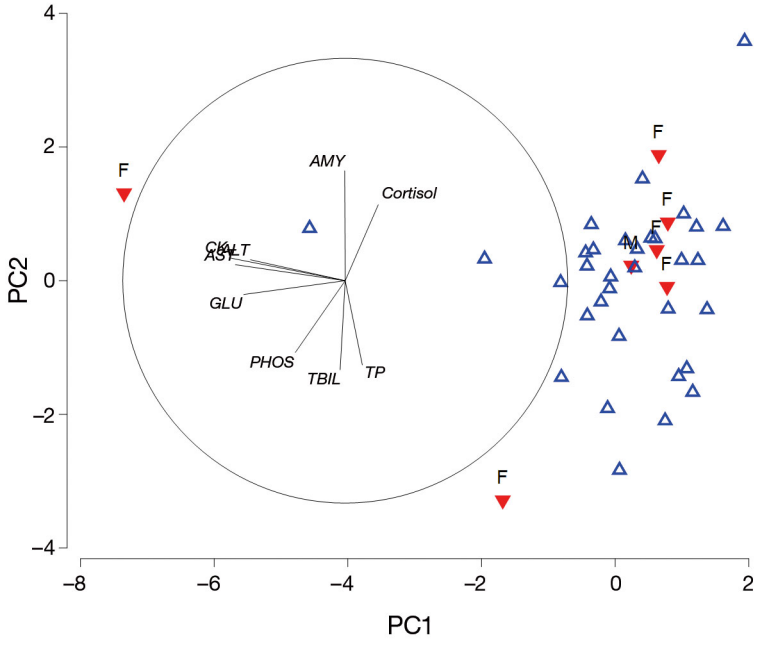


Fig. 4. Principal components analysis of clinical diagnostic parameters of Chinook salmon *Oncorhynchus tshawytscha* alevin total body homogenates (as listed in Fig. 3). Red (blue) triangles represent alevin from *Ichthyophonus*-positive (-negative) parents (F: female; M: male). PC1 explained 22.4% of variability, PC2 explained 13.7% of variability, for a total of 36.1% variability explained. Vectors for select variables are shown (black lines), and vector length indicates the importance of the variable contribution

this effect (Carruth et al. 2000). Cortisol has also been identified as an important bioindicator for disease (Pickering et al. 1982, Pickering & Pottinger 1989). Chronic increased levels of cortisol increase susceptibility to disease, decrease immune response, and increase wound healing time (Pickering & Pottinger 1989, Godbout & Glaser 2006). Cortisol reduces inflammation in the body, and will increase in blood with infection or disease (Barnes & Adcock 2009). Chinook salmon infected with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease, had significantly higher blood plasma cortisol levels than healthy fish (Mesa et al. 1999). We did not find a significant difference in blood plasma cortisol concentrations between *Ichthyophonus*-negative and -positive spawning Chinook salmon. This could be due to adrenal exhaustion; Chinook salmon cortisol is high at the beginning of migration, and prolonged chronic high cortisol levels could lead to adrenal exhaustion, resulting in low cortisol levels at the end of their spawning migration (McConnachie et al. 2012). Adrenal failure may occur due to the initial increase in number of adrenal cells due to gonadal development followed by marked hyperplasia in the adrenal gland, followed by rapid degeneration (Robertson & Wexler 1959). It is also feasible that the limited sample size of *Ichthyophonus*-infected salmon in this study reduced the ability to detect a difference.

Chinook salmon that spawn in Yukon River tributaries travel over 1600 km to their spawning areas (Healey 1991). Blood enzyme levels fluctuate throughout the duration of their spawning migration (Wagner & Congleton 2004), with AST levels reaching their maximum levels during spawning (Hlavová 1989). However, as Chinook salmon reach their terminal spawning grounds, they are undergoing a down-regulation of protein biosynthesis and enzyme activity and continued down-regulation of muscle proteins and most glycolytic enzymes (Hane et al. 1966, Miller et al. 2009). AST is a vitamin B6 dependent enzyme that is important for amino acid metabolism (Ford et al. 1980) and is found in liver, heart, and skeletal muscle (Ford et al. 1980). Although female salmonids generally have higher levels of AST (Shahsavani et al. 2010), we did not detect a significant difference between males and females in the current study. We showed that *Ichthyophonus*-negative fish had significantly higher AST levels compared with positive fish. Concentrations of AST in farmed Atlantic salmon were 202 to 351 U $l^{-1}$ (Sandnes et al. 1988), similar to the *Ichthyophonus*-positive fish in this study, while AST in negative fish was more than 4 times higher (Table 1). Similarly, Nile tilapia *Oreochromis niloticus* affected with nephrocalcinosis also had lower AST concentrations than healthy fish, which was attributed with disease progression (Chen et al. 2003). In contrast, Marty et al. (1998) found significantly higher AST levels in *Ichthyophonus*-positive Pacific herring *Clupea pallasii* due to severe inflammation. The same effect may not have been seen in our study due to decreased enzyme activity at the end of a long spawning migration. It is also possible that deficiency in zinc or magnesium, as well as vitamin B12, as a direct result of inflammation (John & Mahajan 1979), can down-regulate AST activity (Yousaf & Powell 2012), thus explaining lower levels of inflammation indicators in *Ichthyophonus*-positive fish, particularly at the end of spawning migration.

CK has been used as a bioindicator of damage to CK-rich tissues, such as the heart, skeletal muscle, brain, and spermatozoa, and it is also an indicator of

cardiac infarction (Baird et al. 2012). In our study, CK presented with large SDs and was either present or not measurable. As a result, we found no difference in CK between disease presence or between sexes. However, it is interesting that all *Ichthyophonus*-positive fish had no detectable levels of CK, while negative fish had a wide range of concentrations. These results could indicate that diseased fish experience a range of enzyme depletion, which could be caused by many things, such as blood pH or temperature shift (Okamoto et al. 1987, Wagner & Congleton 2004). Reduced CK activity has been observed in patients with liver disease, connective tissue disease, and rheumatoid arthritis, and reflects the overall decline in muscle mass and reduced physical activity (Stucki et al. 1996, Rosalki 1998). A decline in muscle mass after the long spawning migration could be a reasonable explanation for low CK levels in some individuals, especially *Ichthyophonus*-positive salmon. However, there is no significant difference in muscle protein of *Ichthyophonus*-positive and -negative salmon (Floyd-Rump 2015). Reduced physical activity (and associated low CK) in salmon after reaching the spawning ground is a possibility as well, and the non-detectable enzyme activities found in *Ichthyophonus*-positive fish could indicate that they are moribund. Similar to our study, Chen et al. (2003) found that diseased tilapia had significantly lower CK enzyme activity compared with healthy controls. However, CK levels in healthy lake trout *Salvelinus namaycush* were on average 2315.8 ± 1401.9 U $l^{-1}$ (Edsall 1999), which is higher than the values we detected (Table 1). These results also show that there can be high variability in CK activity. Fish exposed to low environmental oxygen concentrations had increased levels of CK to make ATP available to anoxic muscles (Myers et al. 1985, Van Waarde et al. 1990). It is therefore possible that salmon in the current study with elevated levels of CK experienced anoxic conditions in their musculature due to exercise associated with the long migration. At the end of their spawning migration, fish may not be able to maintain high blood oxygen levels, thereby driving other enzymes, such as CK, to higher levels. CK levels can also be high during spawning (Hlavová 1989), and as mentioned previously, spawning activities depend on accumulated glycogen stores (French et al. 1983, Miller et al. 2009). The reliance on glycogen in turn indicates anaerobic metabolism (Pagnotta & Milligan 1991). During burst-type exercise in fish, muscles rely almost entirely on anaerobic metabolism of glycogen (Pagnotta & Milligan 1991). Overall, physiological changes associated with spawning and the spawning migration may confound any differences due to disease in these fish. Alternatively, severely diseased fish may be experiencing pre-spawning mortality and do not reach their final spawning grounds.

Maternal body condition at the time of spawning can affect the quality of salmon offspring (Bromage et al. 1992). Steroids, such as cortisol, are taken up by developing oocytes and may affect the developing eggs (Fevolden et al. 1991, de Jesus et al. 1993, Hwang & Wu 1993, Eriksen et al. 2006). Stressed fish produce eggs with higher cortisol concentrations, and this can hinder growth and development of the oocyte (Pankhurst & Van Der Kraak 1997). However, as described previously, females in general have higher cortisol concentrations (Idler et al. 1959, Folmar 1993), and there could be a built-in protective mechanism against the transfer of maternal steroids (Schreck et al. 2001), particularly in salmonids where chronic stress is part of their life history. Mechanisms, such as regulation of substance transfer, from the mother to the egg and controlling timing of reproduction may have been developed to buffer eggs from deleterious effects of stressors, such as migration (Schreck et al. 2001). In the current study, we did not detect any differences in whole-body clinical chemistry parameters of alevin from *Ichthyophonus*-positive and -negative parental Chinook salmon. This result is not completely unexpected, as we did not find any differences in blood plasma chemistry in spawning adult Chinook salmon between *Ichthyophonus*-positive and -negative fish, with the exception of AST. Maternal effects of spawning are likely outweighing any disease-related effects. Cortisol concentrations in healthy, unstressed juvenile Chinook salmon were near zero (Strange et al. 1978), which is similar to the results of this study (Table 3).

In the current study, several caveats must be taken into consideration. Most importantly, only a limited sample size was available for analysis. Samples obtained during this study relied on adequate escapement to the Salcha River spawning tributary and associated permission to sample. Yukon River Chinook salmon returns have been below expectations for several years, so in 2011 and 2012, we were unable to obtain independent samples separate from ADF&G. In addition, *Ichthyophonus* prevalence during the study years was low, and combined with an overall low availability of salmon led to sample size limitation in the *Ichthyophonus*-positive group. A larger sample size may have enabled us to describe variability associated with spawning as well as disease-related changes in blood chemistry parameters to greater effect. It is likely that values for uninfected

fish are accurate because of the sample size evaluated, but given the small sample size for infected fish, it is unlikely to capture that information. Further, sampling *Ichthyophonus*-infected fish on the spawning grounds may inadvertently select for the successfully surviving or less affected part of the salmon population, while other fish infected with the parasite may die during their migration.

We have shown that infection with *Ichthyophonus* does not appear to influence blood plasma chemistry parameters of Chinook salmon or their resulting alevin on the Salcha River. However, physiological changes and stress associated with spawning may confound any effects as a result of *Ichthyophonus* infection. Future studies should compare terminal spawning ground blood chemistry values to values at the beginning and mid-way through their freshwater migration. It is likely that Chinook salmon females may have a protective mechanism that shields the eggs (and resulting offspring) from any deleterious changes in plasma chemistry connected with spawning. The stress imposed on Chinook salmon during their freshwater spawning migration may be greater than the effects of disease, therefore masking any effects caused by *Ichthyophonus.*

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