

**2013 Arctic-Yukon-Kuskokwim Sustainable Salmon Initiative
Project Final Product¹**

Effects of *Ichthyophonus* on Yukon River Chinook salmon

by:

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¹ Final products of AYK Sustainable Salmon Initiative-sponsored research are made available to the Initiatives Partners and the public in the interest of rapid dissemination of information that may be useful in salmon management, research, or administration. Sponsorship of the project by the AYK SSI does not necessarily imply that the findings or conclusions are endorsed by the AYK SSI.

ABSTRACT

The fish parasite *Ichthyophonus* sp. is one of the most ecologically and economically important diseases of wild marine fishes worldwide. *Ichthyophonus* was first reported in adult Chinook salmon (*Oncorhynchus tshawytscha*) during their spawning migration up the Yukon River in the mid-1980s. *Ichthyophonus* can severely impact salmon fillet quality and it has been hypothesized that *Ichthyophonus*-associated pre-spawning mortality may be one driver of Chinook salmon declines in the Yukon River. Salmon likely acquire infections by consuming infected prey in the marine environment before entering the river. A limited understanding of the interactions among the fish host, the pathogen, and the environment that determine the outcome of *Ichthyophonus* infections in Yukon River Chinook salmon has hindered development of predictive models that could be used in evaluation of potential management strategies.

This research investigated selected factors that are hypothesized to impact *Ichthyophonus* infections in Chinook salmon. For example, *Ichthyophonus* has not been a reported problem in genetically distinct Chinook salmon stocks outside the AYK region, despite high *Ichthyophonus* prevalence among prey fishes throughout the eastern North Pacific Ocean, including the Salish Sea. Here, we compared relative susceptibilities of Yukon River- and Salish Sea-origin Chinook salmon to *Ichthyophonus*. After exposure to *Ichthyophonus* via feeding of tissues from infected Pacific herring (*Clupea pallasii*), Chinook salmon from the Yukon River experienced higher mortality and more persistent and progressive infections compared to those from the Salish Sea. These results support the hypothesis of a longer co-evolutionary relationship between *Ichthyophonus* and the Salish Sea Chinook salmon resulting in increased resistance of this stock relative to the Yukon River population.

The influence of water temperatures and elevated levels of the stress hormone cortisol on disease progression of *Ichthyophonus*-infected Yukon River Chinook salmon were also investigated. Infected salmon were exposed to a range of temperatures (7°C–20°C) that would be typically encountered during movement from the marine to freshwater environment for spawning. No significant relationship between water temperature and *Ichthyophonus*-related mortality was observed in the juvenile salmon used, although increased degradation of parasites was observed in fish held at higher temperatures. However, these findings may not be predictive of results for adult salmon with compromised immune functions. A series of hormonal changes occur during the terminal phases of spawning migration, which include elevated levels of cortisol that may enhance freshwater homing but suppress the salmon's immune system. The USGS experiments showed that administration of cortisol to juvenile Yukon River Chinook salmon resulted in higher elevation of cortisol levels in the blood plasma in fish that had been previously exposed to *Ichthyophonus* relative to fish that were either given cortisol alone or exposed to *Ichthyophonus* alone. The cortisol-injected fish that had been exposed to *Ichthyophonus* also suffered significantly higher *Ichthyophonus*-associated mortality than fish in all other experimental groups, although fin and body fungus likely also contributed to the mortality. These results suggest that *Ichthyophonus* infections in Yukon River Chinook salmon could contribute to elevation of plasma cortisol levels, as well as increased mortality and susceptibility to secondary infections.

PRESS RELEASE

The economic benefits behind top-quality Yukon River Chinook salmon fillets are easy to understand. However, in recent years, this stock of high-value fish has suffered substantial reductions in abundance affecting both commercial and subsistence fisheries in Alaska. A new study by the U.S. Geological Survey identifies the fish parasite, *Ichthyophonus*, and its ability to suppress the salmon's immune system, as one potential culprit.

Initially identified as a significant cause of mortality among herring and other marine forage species, *Ichthyophonus* was first reported in adult Chinook salmon during their spawning migration up the Yukon River in the mid-1980s. Salmon likely became infected by consuming infected prey in the marine environment before entering the river. Nevertheless, a limited understanding of the factors that affect the severity of *Ichthyophonus* infections in Yukon River Chinook salmon has hindered development of the scientific predictive models that could be used to evaluate potential management strategies.

Recent research funded by the Arctic-Yukon-Kuskokwim Sustainable Salmon Initiative and conducted by scientists at the U.S. Geological Survey has been investigating selected factors that are thought to impact *Ichthyophonus* infections in Chinook salmon.

"*Ichthyophonus* has not been a reported problem in genetically distinct Chinook salmon stocks outside the AYK region, despite high *Ichthyophonus* prevalence among prey fishes throughout the eastern North Pacific Ocean, including the Salish Sea," said USGS Fishery Research Biologist Paul Hershberger. "We want to know why."

Scientists compared relative susceptibilities of Yukon River- and Salish Sea-origin Chinook salmon to *Ichthyophonus* and found that Chinook salmon from the Yukon River experienced higher mortality and more persistent and progressive infections compared to those from the Salish Sea. These results might indicate a longer co-evolutionary relationship between *Ichthyophonus* and the Salish Sea Chinook salmon resulting in increased resistance of this stock relative to the Yukon River population.

Scientists also investigated the influence of water temperatures and elevated levels of the stress hormone cortisol on disease progression of *Ichthyophonus*-infected Yukon River Chinook salmon. Infected salmon were exposed to a range of temperatures that would be typically encountered during migration from the marine to freshwater environment for spawning. While higher water temperatures were shown earlier to speed the progression of disease, no significant relationship between water temperature and total *Ichthyophonus*-related mortality was observed in the juvenile salmon used. However, these findings may not be predictive of results for adult salmon with compromised immune functions.

A series of hormonal changes occur during the terminal phases of spawning migration, which include elevated levels of cortisol that may suppress the salmon's immune system. "We took juvenile Yukon River Chinook salmon that had been exposed to the fish parasite and injected them with cortisol, mimicking the hormonal increase that would happen in nature," said Diane Elliott, Research Microbiologist with the USGS. The new USGS experiments showed that cortisol-injected fish that had been exposed to *Ichthyophonus* suffered significantly higher *Ichthyophonus*-associated mortality than fish in other experimental groups, although fin and body fungus likely also contributed to the mortality.

PROJECT EVALUATION

Experiments were completed to achieve all of the goals and objectives described in the proposal. For Objective 1 (effects of temperature on kinetics and outcome of *Ichthyophonus* infections in Yukon River Chinook salmon), experiments were originally planned only for freshwater. However, experiments for this objective were completed in both freshwater and seawater. For Objective 2 (effects of cortisol on *Ichthyophonus* infections on Yukon River Chinook salmon), experiments were originally planned for both freshwater and seawater. However, because elevated cortisol levels are principally relevant to adult salmon during their freshwater spawning migration, preliminary and final experiments were conducted in freshwater only. For Objective 3 (relative susceptibilities of Yukon River and Puget Sound [Salish Sea] Chinook salmon), experiments were completed in freshwater and seawater as planned, but two experiments, rather than a single experiment, were conducted in freshwater. The second freshwater experiment was done to achieve higher *Ichthyophonus* infection prevalence and levels in the two fish stocks.

This project began later than the scheduled start date (July 1, 2013) because the final contract paperwork was not approved and signed by all parties until August 8, 2013. Experimental work on Objective 1 was further delayed by WFRRC wetlab repairs and the U.S. government shutdown in October 2013. Therefore, data collection for the main experiment in this objective, which was scheduled to be completed in December 2013, could not be started until January 2014. The failure to initiate *Ichthyophonus* infection by gastric gavage treatment, and the unexpected confounding of results in the 20°C holding groups by an opportunistic parasite infection (*Ichthyophthirius multifiliis*) further delayed completion of this objective. However, *Ichthyophonus* infections were successfully initiated in later experiments by 6-day feeding exposures to minced tissues from infected herring, and all subsequent experiments were completed using this exposure method, which is likely the natural *Ichthyophonus* infection route for piscivorous fishes such as salmon. Experiments for Objective 2 were delayed until 2015 to allow fish to grow to sufficient size for cortisol implantation. The seawater challenge experiment for Objective 3 was delayed by initial problems with seawater adaptation of the fish, but the experiment was completed in late 2015. Delays caused by the problems described above resulted in a no-cost extension of the project until December 31, 2015. (The original scheduled end date was June 30, 2015.) The successful resolution of problems outlined above enabled completion of two draft manuscripts for submission to peer-reviewed journals.

DELIVERABLES

Deliverables for this project included this final report and several semiannual progress reports. These reports covered the following time periods:

July-December 2013
January-June 2014
July-December 2014
January-June 2015

Deliverables also included oral and poster presentations for scientific conferences:

Poster presentation: McKibben, C.L., P.K. Hershberger, M.K. Purcell, C.M. Conway, and D.G. Elliott. Influence of temperature and fish stock on progression of *Ichthyophonus* infections in Chinook salmon (*Oncorhynchus tshawytscha*). Seventh International Symposium on Aquatic Animal Health, Portland, Oregon, September 3, 2014.

Oral presentation: Elliott, D.G., C.L. McKibben, C.M. Conway, A.H. MacKenzie, L.M. Hart, M.K. Purcell, and P.K. Hershberger. Differential susceptibility of Yukon River and Salish Sea

Chinook salmon (*Oncorhynchus tshawytscha*) stocks to *Ichthyophonus*. American Fisheries Society-Fish Health Section Annual Meeting, Ithaca, New York, July 14, 2015.

Oral presentation: Elliott, D.G., C.L. McKibben, C.M. Conway, A.H. MacKenzie, L.M. Hart, M.K. Purcell, and P.K. Hershberger. Differential susceptibility of Yukon River and Salish Sea Chinook salmon (*Oncorhynchus tshawytscha*) stocks to *Ichthyophonus*. 17th International Conference on Diseases of Fish and Shellfish, European Association of Fish Pathologists, Las Palmas de Gran Canaria, Spain, September 7, 2015.

From the results of this research, two draft manuscripts have been prepared for submission to peer-reviewed journals (Appendix A and B). The manuscript titles are:

Differential susceptibility of Yukon River- and Salish Sea-origin Chinook salmon (*Oncorhynchus tshawytscha*) to ichthyophoniasis. Authors: D.G. Elliott, C.M. Conway, C.L. McKibben, A.H. MacKenzie, L.M. Hart, M.K. Purcell, J.L. Gregg, and P.K. Hershberger.

Effects of temperature and elevated plasma cortisol levels on progression of *Ichthyophonus* infections in Yukon River Chinook salmon *Oncorhynchus tshawytscha*. Authors: D.G. Elliott, C.L. McKibben, C.M. Conway, A.H. MacKenzie, L.M. Hart, M.K. Purcell, J.L. Gregg, and P.K. Hershberger.

PROJECT DATA SUMMARY

Data collected during this research were all generated during the performance of laboratory experiments. Hard copies of data including fish mortality records, *Ichthyophonus* culture results and histopathology observations are currently contained within laboratory notebooks at the Western Fisheries Research Center (WFRC), Seattle, WA, and the Marrowstone Marine Field Station (MMFS), Nordland, WA. Many of these data sets have been transferred to Microsoft Excel spreadsheets and are stored on government-owned computers at the WFRC or MMFS. Water temperature logger data and plasma cortisol measurements are also stored in Excel spreadsheets. The data sets are relatively small, and the most likely users would be fish health scientists interested in comparisons with similar data sets from other laboratory experiments.

APPENDICES (attached)

Appendix A. Draft manuscript: Differential susceptibility of Yukon River- and Salish Sea-origin Chinook salmon (*Oncorhynchus tshawytscha*) to ichthyophoniasis. Authors: D.G. Elliott, C.M. Conway, C.L. McKibben, A.H. MacKenzie, L.M. Hart, M.K. Purcell, J.L. Gregg, and P.K. Hershberger.

Appendix B. Draft manuscript: Effects of temperature and elevated plasma cortisol levels on progression of *Ichthyophonus* infections in Yukon River Chinook salmon *Oncorhynchus tshawytscha*. Authors: D.G. Elliott, C.L. McKibben, C.M. Conway, A.H. MacKenzie, L.M. Hart, M.K. Purcell, J.L. Gregg, and P.K. Hershberger.

Appendix A.

Draft Manuscript

Differential susceptibility of Yukon River- and Salish Sea-origin Chinook salmon (*Oncorhynchus tshawytscha*) to ichthyophthiriasis. Authors: D.G. Elliott, C.M. Conway, C.L. McKibben, A.H. MacKenzie, L.M. Hart, M.K. Purcell, J.L. Gregg, and P.K. Hershberger.



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March 10, 2016

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Subject: USGS Fundamental Science Practices: Courtesy Review and Non-Disclosure Policy
Prior to Publication

Dear Katie:

Attached is a draft manuscript entitled "Differential susceptibility of Yukon River- and Salish Sea-origin Chinook salmon (*Oncorhynchus tshawytscha*) to ichthyophthiriosis." This science product was generated as requested under AYK SSI Project AC-1335.

In being provided this courtesy copy of the information product prior to publication and release, parties are bound by the Bureau's non-disclosure policy as stated:

Survey Manual 502.1.D. *Non-disclosure prior to publication.* In agreeing to be a peer reviewer for the USGS information product, reviewers must agree to be bound by the strictest scientific ethics in ensuring confidentiality of the science that is being reviewed and to not disclose or divulge any results or conclusions, or to make any public statements regarding the science before it is published and released.

On behalf of the WFRC, I thank you in advance for your time and assistance in making the publication of this science product a success.

Sincerely,

Jill B. Rolland
Center Director

1 **Differential susceptibility of Yukon River- and Salish Sea-origin Chinook salmon**
2 **(*Oncorhynchus tshawytscha*) to ichthyophoniasis**

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13
14 Running page head: *Ichthyophonus* susceptibility of two Chinook salmon stocks

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Draft: Do Not Distribute

Abstract

The fish parasite *Ichthyophonus* sp. has been hypothesized as a driver of long-term population declines in Chinook salmon (*Oncorhynchus tshawytscha*) from the Yukon River by contributing to pre-spawning mortality. Interestingly, ichthyophoniasis has not been a reported problem in Chinook salmon outside the Arctic-Yukon-Kuskokwim region, despite occurring in high prevalence among forage fishes throughout the eastern North Pacific Ocean, including the Salish Sea (northwestern Washington State, USA and southwestern British Columbia, Canada). This study was intended to address this apparent paradox by comparing relative susceptibilities of Yukon River- and Salish Sea-origin Chinook salmon to *Ichthyophonus*. Juvenile Chinook salmon from Yukon River and from Kendall Creek (Salish Sea) stocks were exposed to *Ichthyophonus* in freshwater during two 6-day trials by feeding with minced tissues from infected Pacific herring (*Clupea pallasii*). Control groups were fed with tissues from specific pathogen-free herring. Periodic subsampling for *Ichthyophonus* detection was conducted up to 64 days after initial exposure. Infection prevalence was determined by microscopic examination of heart and liver explant cultures for characteristic schizonts, and disease progression was evaluated by histopathology. A third feeding trial with the two fish stocks was conducted in seawater. *Ichthyophonus*-related mortality was $\leq 24\%$ in Yukon River-origin salmon and rarely observed in Kendall Creek-origin salmon. Overall *Ichthyophonus* infection rates were higher in Yukon River fish than in Kendall Creek fish ($P \leq 0.02$) during all three trials. Initial infection prevalence up to 100% and 73% occurred in the Yukon River and Kendall Creek treatments, respectively. Infection prevalence remained near initial levels in Yukon River fish throughout the post-challenge holding period, but prevalence

39 decreased to 0 – 8% in Kendall Creek fish by the end of each experiment. Culture and
40 histopathology results indicated that *Ichthyophonus* caused persistent and progressive
41 infections in Chinook salmon from the Yukon River but more transient infections in those
42 from Kendall Creek. The demonstration of higher *Ichthyophonus* resistance of Kendall
43 Creek Chinook salmon relative to Yukon River Chinook salmon suggested a longer
44 sympatric relation between the Salish Sea stock and the parasite resulting in genetic
45 adaptation of the host.

48

49 INTRODUCTION

50 The first reports of ichthyophoniasis in Chinook salmon (*Oncorhynchus tshawytscha*)
51 from the Yukon River occurred in the mid-1980s, when characteristic lesions of the
52 disease were observed in the skeletal muscle of affected individuals, and fillets were
53 reported to have an off-odor and failed to dry properly using traditional techniques
54 (Kocan et al. 2004). The etiological agent associated with the condition was eventually
55 diagnosed as *Ichthyophonus* sp., and a series of studies was initiated to better understand
56 the kinetics of the disease and identify any host and population-level effects. Although
57 causation between the presence of *Ichthyophonus* and the decline in the Yukon River
58 Chinook salmon population was not conclusively determined, the projects did provide
59 several lines of supporting evidence indicating that *Ichthyophonus* may impact the health
60 and survival of infected cohorts (Kocan et al., 2006, 2009; Zuray et al., 2012).

61 Preliminary studies suggested that Chinook salmon from the Yukon River
62 demonstrated higher susceptibility to *Ichthyophonus* than other genetically distinct strains
63 of Chinook salmon (Jones and Dawe 2002). Higher susceptibility of Yukon River
64 Chinook salmon might explain the high occurrence of ichthyophoniasis in these fish in
65 comparison to other Chinook salmon stocks, such as those originating from the Salish
66 Sea or British Columbia. Ichthyophoniasis has not been reported as a problem in Chinook
67 salmon in British Columbia or the Salish Sea despite a high prevalence of the parasite (up
68 to 50-70%) in Pacific herring *Clupea pallasii* (Kocan et al., 1999; Jones and Dawe,
69 2002), a major prey item for Chinook salmon (Kocan et al. 2004). Stated issues in the
70 Jones and Dawe (2002) research involved confounding co-infections and sub-optimal

diagnostic techniques that caused difficulties in interpreting the results of laboratory exposure studies.

We repeated Chinook salmon susceptibility studies using laboratory-reared, specific-pathogen-free host animals. Two experiments were conducted in freshwater and one in seawater to compare the susceptibility of Yukon River Chinook salmon and a stock of Salish Sea Chinook salmon to *Ichthyophonus* sp. infection. Parameters measured included mortality, infection prevalence, parasite load in tissues, and host tissue response to infection.

METHODS

Experimental fish. Eyed eggs of Yukon River Chinook salmon stock were transported from the Creative Salmon Sea Spring Hatchery, Chemainus, B.C., Canada, to the Western Fisheries Research Center (WFRC), Seattle, Washington, on September 26, 2013. Eyed eggs of a Salish Sea, Washington Chinook salmon stock (North Fork Nooksack River) were obtained from the Kendall Creek Hatchery, Deming, Washington (Washington Department of Fish And Wildlife) on September 27, 2013. Eggs were hatched and fry were reared in sand-filtered, UV-treated water at the WFRC and fed a commercial pellet diet (Bio-Vita; BioOregon) until they reached sufficient size for experimentation. Freshwater experiments were conducted at the WFRC, and the seawater experiment was conducted at the Marrowstone Marine Stateion (MMFS), Nordland, Washington, in tanks supplied with sand-filtered seawater.

Preparation of challenge material. Pacific herring were used as a source of *Ichthyophonus*-infected donor tissues for subsequent feeding trials with Chinook salmon.

Briefly, specific pathogen-free (SPF) Pacific herring (Beaulaurier et al. 2012) were exposed to *Ichthyophonus* by interperitoneal (IP) injections with parasite life stages that were suspended in phosphate buffered saline (PBS). *Ichthyophonus* life stages were obtained from naturally infected wild Pacific herring; parasite isolates were generated by tissue explant culture in Tris-buffered Eagle's Minimum Essential Medium, supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 100 µg/mL gentamicin (MEM-5). External signs of infection (Hart et al. *in press*) were evident in some exposed herring after 3-6 weeks, at which time donor herring were euthanized with an overdose of buffered tricaine methanesulfonate (MS-222), diced into small pieces, and respective experiments were initiated by feeding the diced tissues to Chinook salmon. Control SPF herring were euthanized and frozen at -20°C, then thawed and minced as needed for Chinook salmon feeding trials.

Ichthyophonus exposure of Chinook salmon. Fish were weaned from commercial pellet feed to minced SPF herring over a 1-week to 2-week period prior to each experiment. Then, fish were fed fresh minced SPF herring (control) or *Ichthyophonus*-infected herring (treatment) daily for a period of 6 days. *Ichthyophonus*-infected herring were euthanized every 2 days and kept refrigerated to provide the fresh tissues for a 6-day feeding period. Following the exposure period, fish were returned to the commercial diet and fed *ad libitum*. Freshwater experiments were conducted at 15°C, and the seawater experiment was conducted at 8.5°C.

Culture of Ichthyophonus from fish. Prevalence of *Ichthyophonus* in each experimental group was determined by culture in MEM-5 medium of heart and liver tissues from euthanized fish. Heart and liver tissues of fish that died during each

experiment were also cultured for detection of *Ichthyophonus*. The tissues were placed into MEM-5 broth at a ratio of ≤ 1 part tissue: 5 parts medium (w/v), incubated at 15°C, and screened by microscopy at weekly intervals over a 21-day period for *Ichthyophonus* growth as described by Hershberger (2012).

Histopathology. For examination of parasite density and host response, fish were euthanized and tissues were fixed for histopathology in 10% neutral buffered formalin and subsequently embedded in paraffin wax according to standard protocols. Tissues were sectioned at 5 μ m, and slides were stained with Gill's hematoxylin and eosin (H&E) for assessment of host response or periodic acid-Schiff (PAS) (Carson, 1997) for evaluation of parasite load. The slides were examined with a Zeiss Axiophot light microscope (Carl Zeiss, Inc.). The criteria of Kocan (2013) were used to identify morphological stages of *Ichthyophonus*. The abundance of *Ichthyophonus* in tissues was determined by the procedure of Hart et al. (*in press*) and was reported as the total number of parasites (or number of parasites of a given morphological stage) per 10 microscope fields at 200x magnification. The distribution and degree of the host response to the parasite in a given tissue was quantified at 200x magnification by the method of Hart et al. (*in press*). Scores of lesion distribution (0-3) and degree (0-3) of inflammation, fibrosis, and necrosis were added together to obtain an overall host response severity measure for each tissue type (maximum score = 12).

Freshwater experiment 1. The average weight of fish from the Yukon River and Kendall Creek stocks at the beginning of the experiment was 2.0 g. Feeding of minced infected or SPF herring tissues at a rate of 2% of body weight per day occurred while fish were held in 278-L tanks. Eight days after the beginning of the feeding exposure period,

subgroups of fish from each stock were moved to 119-L tanks for mortality monitoring (three tanks per treatment group and one tank per control group; 40 fish per tank) and sampling (one tank per treatment or control group; 100 fish per tank). All fish that died in a treatment or control group were cultured for detection of *Ichthyophonus* as described above. In addition, 10 fish were sampled and euthanized from each treatment or control group for culture at 7, 20, and 34 days after the beginning of the feeding challenge, and 15 fish per group were sampled at the termination of the experiment 62 days after the beginning of the challenge. On the same dates, five fish were sampled from each treatment or control group for histopathology.

Freshwater experiment 2. Fish from each stock (4.5 g average weight) were held in 278-L tanks (140 fish per stock per treatment or control tank) and fed minced infected herring or SPF herring at 6% body weight per day. Fish were euthanized from the tanks for culture and histopathology during and after the 6-day feeding period; samples were taken on days 3, 7, 10, 14, 24 and 64 after the beginning of the feeding challenge, and the remaining fish were sampled on day 64. The target sample number per fish stock on each sample date included 15 fish for culture and 8 fish for histopathology.

Seawater experiment. At smoltification, groups of fish from each stock were transported to the MMFS, held in 593-L tanks, and acclimated to flow-through, sand-filtered seawater (29‰) over a 10-day period. At the beginning of the experiment the average weights of the Yukon River fish and the Kendall Creek fish were 7.0 g and 15.6 g, respectively, and the feeding rates during the 6-day challenge with minced herring were 7.1% and 6.4% of body weight per day, respectively. Fish were euthanized for culture from the treatment and control tanks for each stock on days 8, 31 and 45 after the

beginning of the feeding trial. Target sample numbers were 15 fish per tank at each time point; no samples were taken for histopathology.

Statistical analysis. Statistical analyses were performed using InStat 3 (GraphPad Software, Inc.) The Fisher exact test (two groups) and chi-square test (three or more groups) were used to compare *Ichthyophonus* prevalence between or among groups in the experiments. Mean parasite loads in histological sections from *Ichthyophonus*-infected fish in various treatment groups were compared by Student's *t* test (two groups) or ANOVA (three or more groups) following log transformation of the data. Comparisons of host response severity score data between fish stocks were analyzed by the nonparametric Mann-Whitney test.

RESULTS

Ichthyophonus-associated Mortality

In the mortality monitoring tanks for freshwater experiment 1, mortality only occurred in groups exposed to *Ichthyophonus*. Total fish mortality in the Yukon River stock treatment tanks (24%; 30 of 124 fish) was significantly higher ($P < 0.0001$) than mortality in the Kendall Creek stock treatment tanks (1%; 1 of 120 fish). All of the Yukon River mortalities tested positive for *Ichthyophonus* by culture, whereas the single Kendall Creek mortality tested negative. There was no significant difference in mortality among the three Yukon River treatment tanks ($P = 0.9$). In the sampling tanks for freshwater experiment 1, cultures from all 16 *Ichthyophonus*-exposed Yukon River stock mortalities were *Ichthyophonus*-positive, whereas the single mortality cultured from the

Ichthyophonus-exposed Kendall Creek tank in this experiment was *Ichthyophonus*-negative. No mortality occurred in the control sampling tanks.

Separate mortality monitoring tanks were not included in freshwater experiment 2 or the seawater experiment, but heart and liver cultures were made from all mortalities that occurred in the treatment and control sampling tanks. In freshwater experiment 2, each of the seven Yukon River treatment mortalities tested *Ichthyophonus*-positive, but none of the three mortalities each in the Kendall Creek treatment and control tanks tested positive. Tail rot of unknown etiology was the presumed cause of death in the Kendall Creek treatment fish but not the control fish. In the seawater experiment, both of the mortalities from the Yukon River treatment tank and one of the three mortalities from the Kendall Creek treatment tank tested *Ichthyophonus*-positive, but none of the four mortalities from the Yukon River control tank or the single mortality from the Kendall Creek control tank tested positive.

***Ichthyophonus* Infection Prevalence (Live Fish Samples)**

Ichthyophonus was detected in heart and liver tissues cultured from live fish sampled from both the Yukon River and the Kendall Creek Chinook stocks shortly after feeding exposures in both freshwater and seawater (Figure 1). The initial *Ichthyophonus* prevalence in both stocks was higher in the experiments with higher feeding rates of infected tissue (freshwater experiment 2 and the seawater experiment). Whereas the detected *Ichthyophonus* prevalence showed relatively minor fluctuations in the Yukon River fish throughout a given experiment, the infection prevalence in the Kendall Creek fish declined at later sample time points during each experiment, and was not detected

from Kendall Creek fish ≥ 20 d post-exposure in freshwater experiment 1, or from fish sampled on day 64 in freshwater experiment 2 (Figure 1). Although *Ichthyophonus* was cultured from a higher proportion of Yukon River Chinook salmon than from Kendall Creek Chinook salmon in all experiments at all sample time points, in freshwater experiment 2 prevalence first showed significant differences on day 24 ($P = 0.03$), and in the seawater experiment prevalence showed significant differences on day 31 ($P = 0.02$) and day 45 ($P < 0.0001$). The overall *Ichthyophonus* prevalence determined by culture for all sample time points combined was significantly higher in the Yukon River fish than that in the Kendall Creek fish in freshwater experiment 1 (27% and 7%, respectively; $P = 0.02$), freshwater experiment 2 (97% and 56%, respectively; $P < 0.0001$), and the seawater experiment (85% and 24%, respectively; $P < 0.0001$). No cultures from control fish sampled from either stock were positive for *Ichthyophonus*.

For the seawater experiment, separate cultures of heart and liver tissue were made from each sampled fish. Among the culture-positive Yukon River fish, *Ichthyophonus* was detected in heart tissue only for 11 fish, liver tissue only for 3 fish, and in both tissue types for 31 fish. Overall, there was no significant difference ($P = 0.13$) in *Ichthyophonus* detection rates between heart and liver cultures. Among the Kendall Creek fish that tested *Ichthyophonus*-positive by culture, the parasite was detected in heart tissue only for four fish, liver tissue only for three fish, and from both tissues for six fish, and there was no significant difference ($P = 1.00$) in rates of *Ichthyophonus* detection between the tissue types.

Histopathology

The most common *Ichthyophonus* life stages observed in histological sections of fish sampled in freshwater experiments 1 and 2 included multinucleate plasmodia and schizonts, as well as degenerated schizonts (Figure 2). Germinating multinucleate bodies were only rarely observed in Yukon River fish (freshwater experiment 2, 14 days post-exposure only); these were characterized by the protrusion of short hyphal structures through the capsule of schizonts (photo not shown).

Freshwater experiment 1. By histopathology, *Ichthyophonus* infections were first observed in tissues of fish from both the Yukon River and Kendall Creek stocks 34 days after the initial feeding exposure (Table 1). Whereas multiple *Ichthyophonus* life stages at densities up to 60 parasites per 10 microscope fields at 200x magnification were observed in several tissues of infected Yukon River fish sampled on day 34, the only parasite stage observed in the single *Ichthyophonus*-positive Kendall Creek fish sampled on this day was a degenerated schizont in red skeletal muscle. By day 62, the few parasites observed in tissues of the single infected fish from each stock were degenerated schizonts (Table 1).

The most predominant host response to *Ichthyophonus* infection both fish stocks on days 34 and 62 was granulomatous inflammation. Fibrosis was also observed in lesions in some tissues of Yukon River fish sampled on day 34, and mild necrosis was observed in lesions in the single infected fish in each stock on day 62. The occurrence of host response usually corresponded with the presence of *Ichthyophonus* (Table 1), although focal areas of granulomatous host response were observed in the absence of visible parasites in one fish each from both stocks on day 62. The highest severity of host response (score ≥ 6) was observed in the Yukon River fish on day 34 (Table 1).

Freshwater experiment 2. *Ichthyophonus* parasites were first observed by histopathology in both fish stocks on the first sample day, 3 days after the beginning of the feeding exposure, and continued to be detected in both stocks through the final sampling on day 62, although the parasite prevalence in a particular tissue was usually higher in the Yukon River stock than in the Kendall Creek stock on a given sample day (Table 2). *Ichthyophonus* infections were observed in histological sections of heart, liver, gastrointestinal tract, pancreas, kidney hematopoietic tissue, spleen, and red and white skeletal muscle of Yukon River fish, and in all of these tissues except liver and spleen in Kendall Creek fish (Table 2). The tissues most consistently infected in both stocks were heart, red and white skeletal muscle, and kidney hematopoietic tissue.

Mean *Ichthyophonus* loads (mean number of parasites in 10 microscope fields at 200x magnification) in the four most consistently infected tissues were calculated for both stocks at each sample time point; the highest mean densities in both stocks were observed in heart tissue (Figure 3). For the Yukon River stock, the prevalence and mean parasite loads in each tissue peaked 14 days after the beginning of the feeding exposure, followed by declines through the last sample time point 62 days after the beginning of exposure. For the Kendall Creek stock, the *Ichthyophonus* prevalence reached its highest point on days 10 to 14, but mean parasite loads remained low throughout the experiment. On day 14, mean parasite loads in red skeletal muscle of the Yukon River fish were significantly higher than those in the Kendall Creek fish ($P = 0.03$), but there were no significant differences between the two stocks in mean parasite densities in heart or white skeletal muscle ($P \geq 0.07$).

277 Considerable variation in parasite densities in a given tissue was observed among
278 individual fish in each stock. For example, despite overall declines in mean parasite
279 densities (values for all positive fish combined) in tissues of Yukon River Chinook
280 salmon sampled after day 14, the total numbers of parasites observed in a few Yukon
281 River fish in the day 24 sample were higher for kidney (2 fish) and red and white skeletal
282 muscle (1 fish each) than the highest numbers of parasites observed in any individual fish
283 from this stock on day 14. This resulted in higher numbers of total parasites observed in
284 these tissues (values for all fish combined) on day 24 than on day 14 (Figure 4). For the
285 Kendall Creek stock, the highest total parasite numbers were observed on day 14 for all
286 tissues except kidney, where parasite numbers remained low throughout the experiment
287 (Figure 4).

288 Among the *Ichthyophonus* life stages observed by histopathology, the proportion of
289 degenerated schizonts in the four most highly infected tissues increased in both stocks by
290 the last sample time point 64 days after the beginning of the feeding exposure (Figure 4).
291 For the Yukon River stock, 13% to 27% of the parasites observed in heart, kidney, red
292 skeletal muscle and white skeletal muscle in the day 64 sample were degenerated
293 schizonts. In the Kendall Creek fish, few visible parasites remained in each of these
294 tissues on day 64, and 67% to 100% of the parasites observed were degenerated
295 schizonts.

296 The principal host response to *Ichthyophonus* infections in tissues of fish from both
297 stocks was granulomatous inflammation (Figure 2), often accompanied by fibrosis and
298 (usually mild) necrosis. Although *Ichthyophonus* was first observed in a single fish in
299 each stock on day 3, no host response to the parasite was observed in host tissues until

day 7 (Figure 5). Whereas the highest mean parasite densities in heart, kidney, and red and white skeletal muscle of Yukon River fish were observed on day 14, the mean severity of the host response to the parasite in these tissues continued to increase after this time point and remained high through day 64 (Figure 5). For the Kendall Creek stock, the mean severity of the host response varied by tissue type (Figure 5). The host response was mild or absent on all sample days in kidney tissue, where the parasite prevalence detected by histopathology was low (0–25%) throughout the experiment. In the other three tissues, in which the *Ichthyophonus* prevalence in the Kendall Creek fish was $\geq 63\%$ by histopathology on day 14, differing patterns of host response were observed. The mean severity of host response in heart tissue followed a pattern very similar to that of the Yukon River fish, with no evidence of decline through day 64. In red and white skeletal muscle, the highest mean severity of host response was observed on days 14 and 24, respectively, followed by apparent declines in mean severity at later time points. At all time points in which host response to *Ichthyophonus* was observed in a given tissue in five or more fish from each stock, there was no significant difference in host response between the two stocks ($P \geq 0.22$)

DISCUSSION

The results of our research corroborated and expanded the findings of Jones and Dawe (2002), which showed higher prevalence and intensity of *Ichthyophonus* infection by histopathology in juvenile Yukon River Chinook salmon relative to a Vancouver

Island, British Columbia, Canada stock (Big Qualicum River), in a freshwater trial, following IP injection of fresh tissue homogenate from naturally infected Pacific herring. However, no *Ichthyophonus* infections were observed in seawater-adapted juvenile Chinook salmon from another Vancouver Island stock (Little Qualicum River) that were injected IP at the same time with the same herring tissue homogenate used in the freshwater trial. Jones and Dawe (2002) also achieved infections in Yukon River Chinook salmon in a freshwater trial by a single feeding of fresh tissue homogenates from *Ichthyophonus*-infected herring, but were unsuccessful in an attempt to infect Big Qualicum Chinook salmon by the same method. The authors cautioned that the results for the Big Qualicum fish, particularly in the feeding trial, may have been compromised by the presence of *Flavobacterium* sp. in the fish. In contrast to the results of Jones and Dawe (2002) with Vancouver Island Chinook salmon, our experiments demonstrated that both Yukon River and Salish Sea (Kendall Creek) Chinook salmon juveniles could be infected with *Ichthyophonus* by a 6-day feeding exposure with fresh tissue homogenates from infected Pacific herring, in trials conducted in both freshwater and seawater. Similar to the findings of their IP injection trial with fresh tissue homogenate, our feeding exposure experiments revealed higher *Ichthyophonus* prevalence and infection intensity in Yukon River relative to the Kendall Creek Chinook salmon. The results of our mortality monitoring in freshwater experiment 1 also showed significantly higher *Ichthyophonus*-associated mortality in Yukon River fish than in Kendall Creek fish; no separate mortality monitoring tanks were included in the experiments of Jones and Dawe (2002).

As demonstrated by these experiments and others (e.g., Kocan et al. 2009, 2013, 2014; Gregg et al., 2012), the principal natural route of *Ichthyophonus* infection for salmonids and other piscivorous fishes is probably the gastrointestinal tract via ingestion of infected prey. Although differences in prevalence between the stocks as determined by culture were not significant in early samples taken 3-8 days after the beginning of each *Ichthyophonus* feeding exposure, the consistently lower prevalence observed in the Kendall Creek stock at these time points suggested that these fish may be better able than the Yukon River fish at preventing entry and dissemination of the parasite to tissues. The observation of degenerated schizonts, but no intact parasites, in the intestinal contents of two Kendall Creek fish sampled on day 3 during *Ichthyophonus* feeding exposure, and observation of intact parasites in intestinal tissues of one Yukon River fish sampled one day after the end of the feeding exposure provide limited support for this hypothesis. However, the possibility exists that infectious stages had already exited the empty schizonts observed in the intestinal contents of the Kendall Creek fish, and entered host tissues (Kocan et al., 2013).

The components of the innate immune system important for defense against adhesion and entry of *Ichthyophonus* through the gastrointestinal mucosa are unknown, but primary immunological barriers in fish that are resistant to histozoic parasites that infect fish via gastrointestinal entry are believed to involve mucus protein composition, epithelial cells, and goblet cells in the intestine (Gómez et al., 2014). Conversely, it is possible that a parasite can inhibit the recognition mechanism in a susceptible fish host as a strategy for successful entry into gastrointestinal tissue (Gómez et al., 2014). In our freshwater experiment 2, the observation of several *Ichthyophonus* plasmodia and a

schizont directly beneath the epithelium in the lamina (tunica) propria of the intestine of a Yukon River fish on day 7 (one day after the end of feeding exposure), without associated host response such as necrosis or inflammation, provides limited support for the hypothesis that the parasite may inhibit host recognition mechanisms to gain entry into gastrointestinal tissues. A mild inflammatory host response with or without accompanying fibrosis was observed in all other infected tissues of this fish except pancreas. Similarly, Kocan et al. (2013) observed *Ichthyophonus* amoeboid plasmodia and cells resembling small schizonts in the tunica propria of the stomach wall in rainbow trout (*Oncorhynchus mykiss*) and Pacific staghorn sculpin (*Leptocottus armatus*) 48 hours after ingestion of infected herring tissues. Clear “halos” surrounded the schizont-like cells in both the stomach contents and the tunica propria of the stomach, and a thin translucent zone surrounded the plasmodia observed in the tunica propria, but otherwise no alterations were seen in host tissue.

After entry through the gastrointestinal mucosa, the parasites likely spread throughout the body via the blood and become lodged in capillary beds, particularly in blood-rich organs (McVicar, 1999; Kocan et al., 2013). Kocan et al. (2013) hypothesized that the earlier detection of *Ichthyophonus* in organ cultures relative to histological samples results from the presence of an infectious stage of the parasite in blood circulating through the organs. These authors further hypothesized that the timing of the first detection of *Ichthyophonus* in tissues by histopathology is dependent on parasite dose. The results of our research supported both of these hypotheses. While *Ichthyophonus* was detected by organ culture in fish from both stocks at the first sample time point (3 or 7 days after the beginning of feeding exposure) in each of the experiments in which

histological samples were also taken, the parasite was first detected by histopathology in tissues of fish from both stocks on day 3 when fish were fed *Ichthyophonus*-infected herring tissues for 6 days at a rate of 6% of body weight per day, but not until day 34 when fish were fed infected tissues for the same time period at a rate of 2% of body weight per day. Although we did not sample blood from fish for *Ichthyophonus* detection, *Ichthyophonus* DNA has been detected in blood samples from adult Chinook salmon sampled from the Yukon River (Whipps et al., 2006).

From primary infection sites, further parasite spread is achieved by proliferation and invasion of adjacent tissue, and by release of further infective stages into the circulatory system (McVicar, 1999; Kocan et al., 2013). Our research showed that *Ichthyophonus* attained higher mean infection intensities in a greater number of tissues in the Yukon River fish than in the Kendall Creek fish, similar to the observations of Jones and Dawe (2002). Our finding that mean parasite densities in tissues of Yukon River peaked at day 14 in freshwater experiment 2 and then showed declines for the remainder of the experiments indicated that juvenile Yukon River Chinook salmon can mount a defense against *Ichthyophonus*, but not as effectively as the Kendall Creek fish, which had low tissue parasite densities throughout the experiment. Further evidence of the effectiveness of the host response was the increase in proportions of degenerated parasites observed in tissues of fish from both stocks, but especially in the Kendall Creek fish, toward the end of freshwater experiment 2. Though viable parasites remained in all of the Yukon River fish cultured at the end of the experiment, negative cultures from all of the Kendall Creek fish further suggested that they had cleared most of the viable parasites. It is unknown whether reductions in parasite loads similar to what we observed in juvenile fish would

occur in adult Yukon River Chinook salmon, which like other Pacific salmon exhibit elevated levels of the immunosuppressive hormone cortisol during their spawning migration (Carruth et al., 2002; Horstmann-Dehn et al., 2012).

Although a marked humoral immune response is elicited by *Ichthyophonus* in some fish species (MacVicar, 1999), the prominent cellular response is likely more important in defense against spread of the parasite in tissues (Sitjà-Bobadilla, 2008). This author hypothesized that if the fish host can successfully encapsulate *Ichthyophonus* “spherical resting bodies” (schizonts) within a thick wall of host tissue, the parasite will be unable to reproduce and will eventually die. If the cellular host reaction is weak, however, the parasite will continue to proliferate. A vigorous and persistent cellular host response, comprised largely of granulomatous inflammation and fibrosis and often resulting in encapsulation of parasites at later time points, was observed in *Ichthyophonus*-infected fish of both stocks in our experiments. The host response was first detected 4 days after the first observation of parasites in fish tissues in freshwater experiment 2, and persisted in tissues of infected fish 64 days after the beginning of exposure. The lack of significant differences between stocks in the magnitude of host response in freshwater experiment 2 at sample time points with sufficient numbers of infected fish in both stocks for statistical comparisons, despite the lower mean densities of *Ichthyophonus* in tissues of the Kendall Creek fish at these time points, supports the hypothesis of Sitjà-Bobadilla (2008) that a robust host response is an important factor in resistance to *Ichthyophonus* infections. Our results suggest that greater resistance (ability to limit parasite burden) is more important than tolerance (ability to limit tissue damage caused by a given parasite burden) in the

higher survival of Kendall Creek Chinook salmon relative to Yukon River Chinook salmon following *Ichthyophonus* exposure (Råberg et al., 2007; Metzger et al., 2010).

Intraspecific differences in resistance to pathogenic organisms can be indicative of genetic adaptation of the host. Natural evolution of such adaptation is evident in the relative resistance of fish populations sympatric with certain parasites (Bower et al. 1995; Bartholomew, 1998; Quigley and McArdle, 1998). The findings of our study suggested the existence of a longer sympatric relationship between the Kendall Creek Chinook salmon stock and *Ichthyophonus* than between the Yukon River Chinook salmon stock and the parasite. *Ichthyophonus* is endemic in populations of Pacific herring, an important prey species for Chinook salmon (Davis et al., 2009), in various regions of the northeast Pacific Ocean (Hershberger et al., 2015) including British Columbia (Jones and Dawe, 2002) and the Salish Sea (Hershberger et al., 2002), but has not been detected in herring from the Bering Sea (Kocan et al., 2004). However, *Ichthyophonus* has been present in walleye pollock (*Theragra chalcogramma*) in the Bering Sea for at least 20 years (White et al., 2014). These authors have hypothesized that walleye pollock likely is a key reservoir of *Ichthyophonus* for infection of susceptible species including Yukon River Chinook salmon, based on several lines of evidence. Walleye pollock forms a significant portion of the diets of ocean-phase Chinook salmon (Davis et al., 2009), and the geographic ocean distribution of *Ichthyophonus*-infected pollock overlaps the ocean range of Chinook salmon (White et al., 2014). Potential for exposure of Chinook salmon to *Ichthyophonus*-infected pollock may occur through two trophic pathways—ingestion of whole fish as prey and scavenging on discharged offal from at-sea fish processing ships—though measures implemented during the past decade have reduced waste from

processing ships (White et al., 2014). Based on a multiyear epidemiological study that revealed simultaneous increases and decreases in *Ichthyophonus* prevalence and population abundance of Yukon River Chinook salmon, Zuray et al. (2012) suggested that a prey item in the food web critical to Chinook salmon survival may be the source of infection, and White et al. (2014) proposed that walleye pollock is that infection source. Ultimately the ecology of ichthyophoniasis in Bering Sea fishes including Chinook salmon is likely affected by complex interactions among factors such as climate variables, oceanographic conditions, food web dynamics, and anthropogenic pressures (Kocan et al., 2009; Zuray et al., 2012; Burge et al., 2013; White et al., 2014).

In summary, our research demonstrated increased *Ichthyophonus* resistance of a Salish Sea Chinook salmon stock relative to Yukon River Chinook salmon. The study results indicated that disease resistance, as evidenced by a robust cellular host response and apparent clearance of parasites, rather than tolerance of high parasite loads, was a more important determinant of higher survival in the Salish Sea Chinook salmon. However, the mechanisms of host resistance to ichthyophoniasis are poorly understood, and further research is necessary to identify biomarkers that measure host immune response at molecular, cellular or tissue levels, and to evaluate prognostic capabilities of such biomarkers to predict disease outcome. We hypothesize that a longer co-evolutionary relationship between Salish Sea Chinook salmon and *Ichthyophonus* has resulted in greater genetic adaptation to ichthyophoniasis in those fish than in Yukon River Chinook salmon. Identification and mapping of genetic markers associated with susceptibility to *Ichthyophonus* infections could provide information useful for

incorporation into predictive models of impacts of ichthyophoniasis on Chinook salmon populations.

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632 Table 1. Prevalence and mean parasite densities of *Ichthyophonus*, and occurrence and severity of host response to *Ichthyophonus*
633 infections, observed in histological sections of tissues from Yukon River Chinook salmon and Kendall Creek Chinook salmon in
634 freshwater experiment 1. Sample day was the number of days after the date of the first feeding exposure. Slides examined for parasite
635 detection were stained with periodic acid-Schiff (PAS), and slides examined for host response were stained with hematoxylin and
636 eosin (H&E). Scores of lesion distribution (0-3) and degree (0-3) of inflammation, fibrosis, and necrosis were added together to obtain
637 an overall host response severity measure for each tissue type. No *Ichthyophonus* or host response was detected by histopathology in
638 fish in the day 7 or day 20 samples, or in any control fish (data not shown). YR = Yukon River, KC = Kendall Creek.
639

| Sample day | Fish Stock | Number fish positive for <i>Ichthyophonus</i> /total examined (%) | | | | | | | |
|------------|------------|--|---------------|-----------------------|-----------------------|---------------------|--------|------------------------|------------------------|
| | | Mean number of parasites in 10 microscope fields at 200x magnification (range) | | | | | | | |
| | | Number of fish positive for host response/total examined (%) | | | | | | | |
| | | Mean host response severity score \pm SD | | | | | | | |
| | | Heart | Liver | GI tract ¹ | Pancreas ² | Kidney ³ | Spleen | Red skeletal muscle | White skeletal muscle |
| 34 | YR | 2/5 (40%) | 2/5 (40%) | 0/5 | 1/5 (20%) | 1/5 (20%) | 0/4 | 2/5 (40%) | 3/5 (60%) ⁴ |
| | | 35 (20–50) | (4–53) | --- | 60 | 2 | --- | 33 (20–47) | 6 (1–16) |
| | | 2/5 (40%) | 2/5 (40%) | 0/5 | 1/5 (20%) | 1/5 (20%) | 0/4 | 2/5 (40%) | 3/5 (50%) |
| | KC | 4.5 \pm 0.7 | 3.0 \pm 1.5 | --- | 6.0 | 7.0 | --- | 6.0 \pm 1.4 | 3.3 \pm 1.5 |
| | | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 (20%) ⁴ | 0/5 |
| | | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 (20%) | 0/5 |
| 62 | YR | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 (20%) ⁴ |
| | | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 (20%) | 1/5 (20%) |
| | | --- | --- | --- | --- | --- | --- | 2.0 | 4.0 |
| | KC | 1/4 (25%) ⁴ | 0/4 | 0/4 | 0/4 | 0/4 | 0/3 | 0/4 | 0/4 |
| | | 1 | | | | | | | |
| | | 1/3 (33%) | 0/3 | 0/4 | 0/4 | 0/4 | 0/3 | 0/4 | 1/4 (25%) |
| | | 4.0 | | | | | | | 4.0 |

640 Table 1.

641

642 ¹GI tract = gastrointestinal tract

643 ²Includes inter-pancreatic connective tissue

644 ³Parasites in hematopoietic tissue

645 ⁴Degenerated schizont(s) only parasite(s) observed in infected tissues of one fish

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648 Table 2. Prevalence of *Ichthyophonus* in Yukon River (YR) and Kendall Creek (KC) Chinook salmon as determined by histological
649 examination of fish from freshwater experiment 2. The sample day was the number of days after the date of the first feeding exposure.
650 Slides were stained with periodic acid-Schiff (PAS) to facilitate detection of parasites. No parasites were detected in any control fish
651 (data not shown).
652

| Sample day | Fish stock | Number of positive fish/total examined (%) | | | | | | | |
|------------|------------|--|-----------|------------------------|-----------------------|-----------------------------|-----------|---------------------|-----------------------|
| | | Heart | Liver | Gastrointestinal tract | Pancreas ¹ | Kidney hematopoietic tissue | Spleen | Red skeletal muscle | White skeletal muscle |
| 3 | YR | 1/7 (14%) | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 2/8 (25%) | 2/8 (25%) |
| | KC | 1/7 (14%) | 0/8 | 0/8 ² | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |
| 7 | YR | 3/8 (38%) | 2/8 (25%) | 1/8 (13%) | 2/8 (25%) | 2/8 (25%) | 0/7 | 5/8 (63%) | 3/8 (38%) |
| | KC | 2/8 (25%) | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 4/8 (50%) | 4/8 (50%) |
| 10 | YR | 4/7 (57%) | 0/7 | 0/7 | 2/7 (29%) | 3/7 (43%) | 1/7 (14%) | 5/7 (71%) | 5/7 (71%) |
| | KC | 5/8 (63%) | 0/8 | 0/8 | 1/8 (13%) | 1/8 (13%) | 0/8 | 6/8 (75%) | 3/8 (38%) |
| 14 | YR | 7/8 (88%) | 6/8 (75%) | 1/8 (13%) | 4/8 (50%) | 7/8 (88%) | 2/8 (25%) | 8/8 (100%) | 8/8 (100%) |
| | KC | 5/8 (63%) | 0/8 | 0/8 | 0/8 | 1/8 (13%) | 0/8 | 6/8 (75%) | 6/8 (75%) |
| 24 | YR | 5/8 (63%) | 3/8 (38%) | 1/8 (13%) | 5/8 (63%) | 6/8 (75%) | 1/8 (13%) | 8/8 (100%) | 8/8 (100%) |
| | KC | 3/8 (38%) | 0/8 | 1/8 (13%) | 0/8 | 1/8 (13%) | 0/8 | 3/8 (38%) | 4/8 (50%) |
| 64 | YR | 6/8 (75%) | 4/8 (50%) | 0/8 | 4/8 (50%) | 4/8 (50%) | 1/8 (13%) | 5/8 (63%) | 5/8 (63%) |
| | KC | 1/4 (25%) | 0/4 | 0/4 | 0/4 | 1/4 (25%) | 0/4 | 3/4 (75%) | 3/4 (75%) |

653 ¹Includes inter-pancreatic connective tissue

654 ²Degenerated schizonts observed only in intestinal contents of two fish

List of Figures

Figure 1. Prevalence of *Ichthyophonus* detected by culture of heart and liver tissues in juvenile Chinook salmon from Yukon River and Kendall Creek stocks sampled at various times in two freshwater experiments and one seawater experiment (after 6-day exposures by feeding of minced tissue from *Ichthyophonus* infected Pacific herring. The numbers of fish sampled from each stock at each time point were: freshwater experiment 1, 10 fish; freshwater experiment 2, 15 fish; seawater experiment, 15 fish; exceptions are noted above bars. No *Ichthyophonus* was cultured from control fish at any time point (data not shown).

Figure 2. *Ichthyophonus* life stages in histological sections from Yukon River Chinook sampled at (a) 14 days and (b and c) 64 days post exposure from freshwater experiment 2. (a). Plasmodium in white skeletal muscle. Note the smaller size and lack of a multilaminar capsule. (b). Schizont in kidney tissue, showing multilaminar capsule (arrow). (c). Degenerated schizont in kidney tissue, showing remaining capsule (arrow), fibrosis (arrowhead) and inflammation (asterisk). Sections were stained with periodic acid-Schiff (PAS).

Figure 3. *Ichthyophonus* density (mean number of parasites per 10 microscope fields at 200x magnification) in histological sections from *Ichthyophonus*-positive Yukon River and Kendall Creek Chinook salmon sampled at various times after the first exposure day during freshwater experiment 2. The graphs show data from the four tissues (heart, kidney hematopoietic tissue, red skeletal muscle and white skeletal muscle) that had the

highest *Ichthyophonus* prevalence and infection intensity in both fish stocks. Sections were stained with periodic acid-Schiff (PAS) to facilitate parasite detection. The mean of the positive fish for each stock is shown on the y-axis, and parasite prevalence at each time point is shown above each data point (the number of positive fish divided by the number of fish examined). Note the differences in scale for the different tissues examined. No *Ichthyophonus* was detected in any control fish (data not shown).

Figure 4. Percentage of degenerated schizonts among total *Ichthyophonus* parasites observed in histological sections of tissues of Yukon River and Kendall Creek stock Chinook salmon sampled at various times after the first exposure day during freshwater experiment 2; data are from the four tissues (heart, kidney hematopoietic tissue, red skeletal muscle and white skeletal muscle) that had the highest *Ichthyophonus* prevalence and infection intensity in both fish stocks. The numeral above each bar is the total number of parasites observed in a given fish stock at a given sample time point. No *Ichthyophonus* was detected in any control fish (data not shown).

Figure 5. Occurrence and mean severity of host response to *Ichthyophonus* infections, observed in histological sections of tissues from Yukon River and Kendall Creek Chinook salmon sampled at various times after the first exposure day during freshwater experiment 2; data are from the four tissues (heart, kidney hematopoietic tissue, red skeletal muscle and white skeletal muscle) that had the highest *Ichthyophonus* prevalence and infection intensity in both fish stocks. Slides were stained with hematoxylin and eosin (H&E), and scores of lesion distribution (0-3) and degree (0-3) of inflammation,

fibrosis, and necrosis were added together to obtain an overall host response severity
measure for each tissue type.

DRAFT

Figure 1.

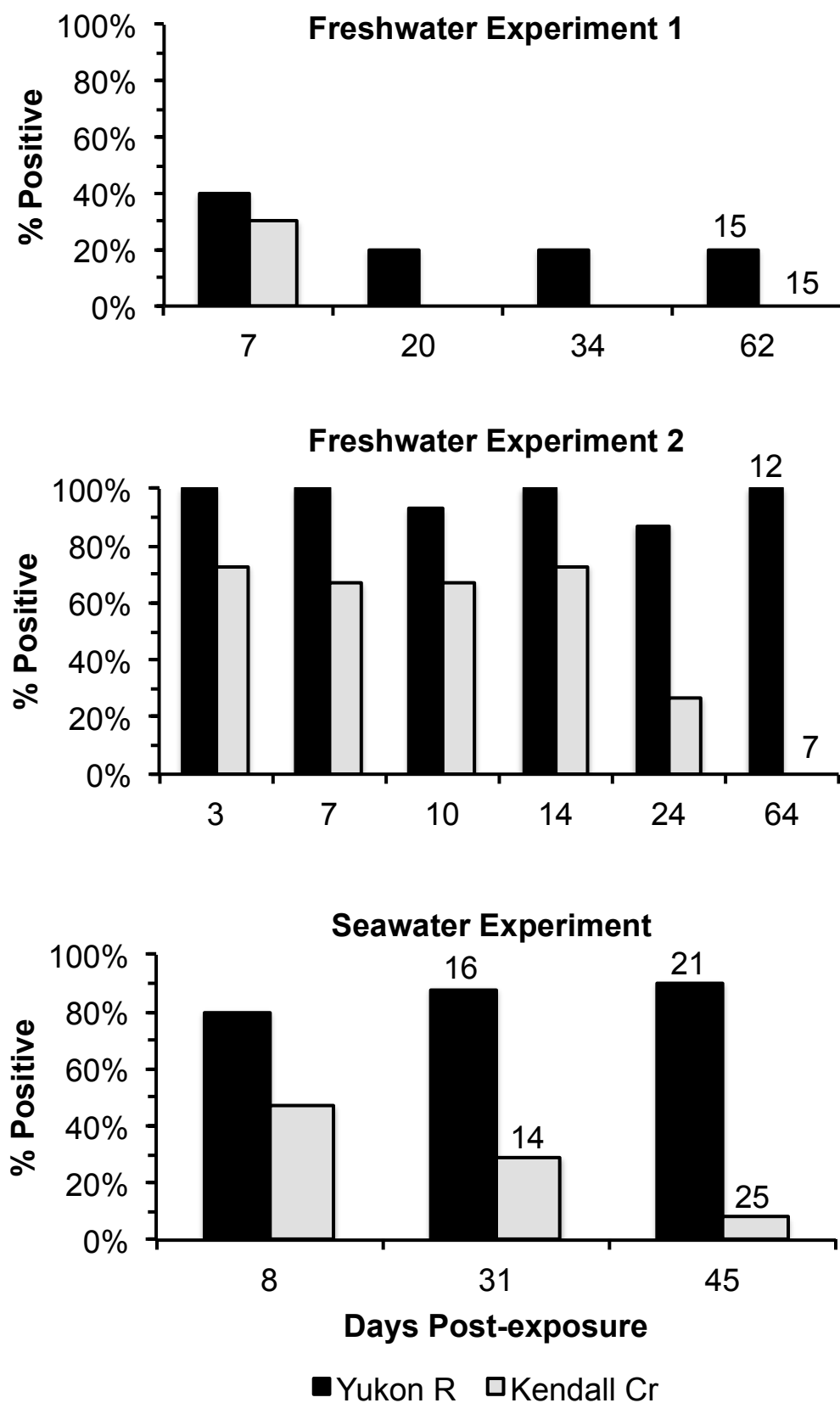


Figure 2.

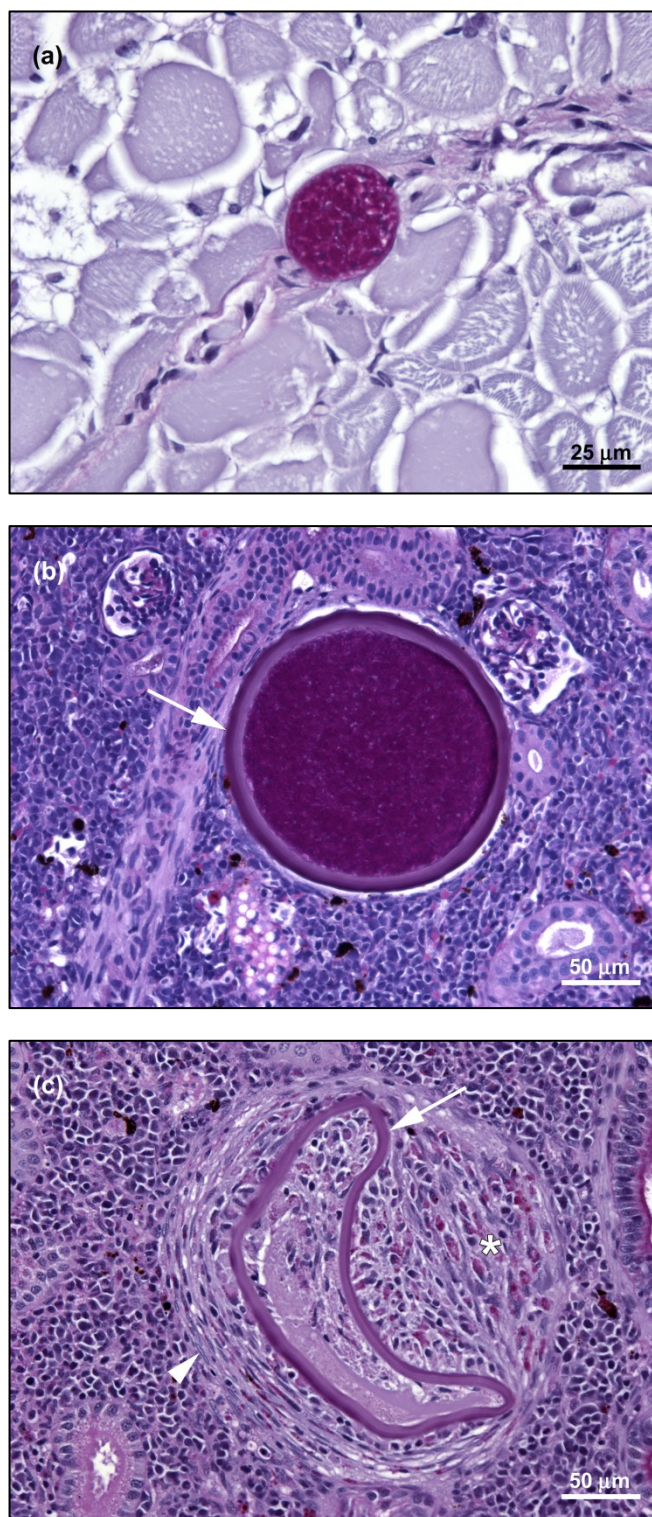


Figure 3.

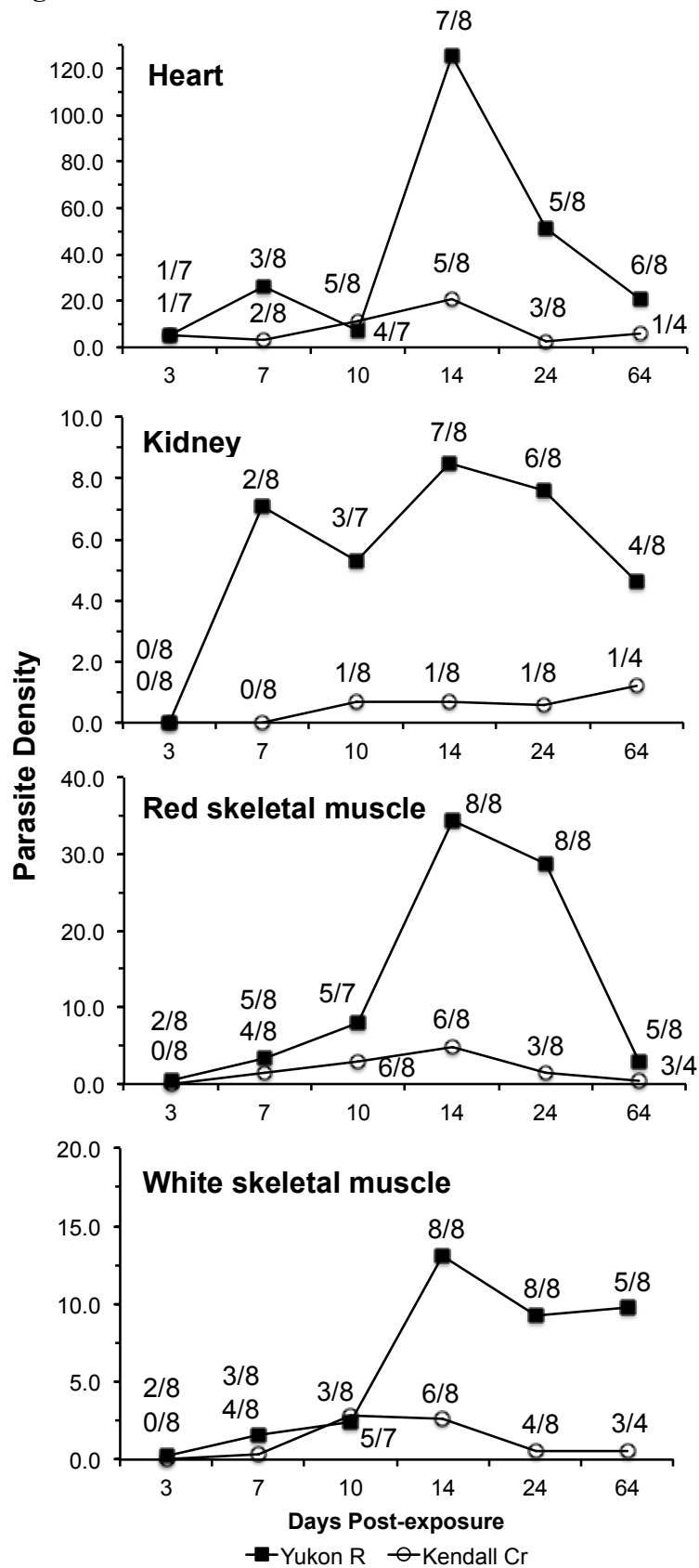


Figure 4.

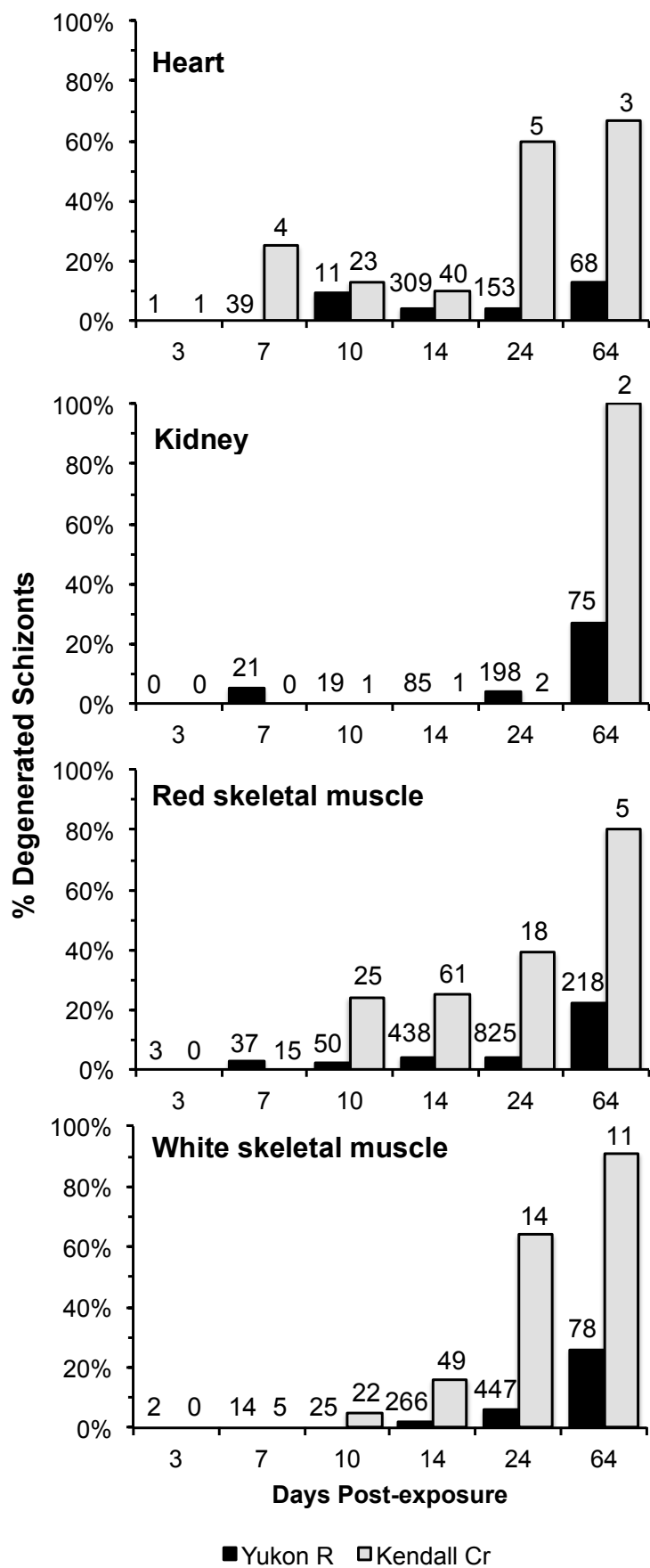
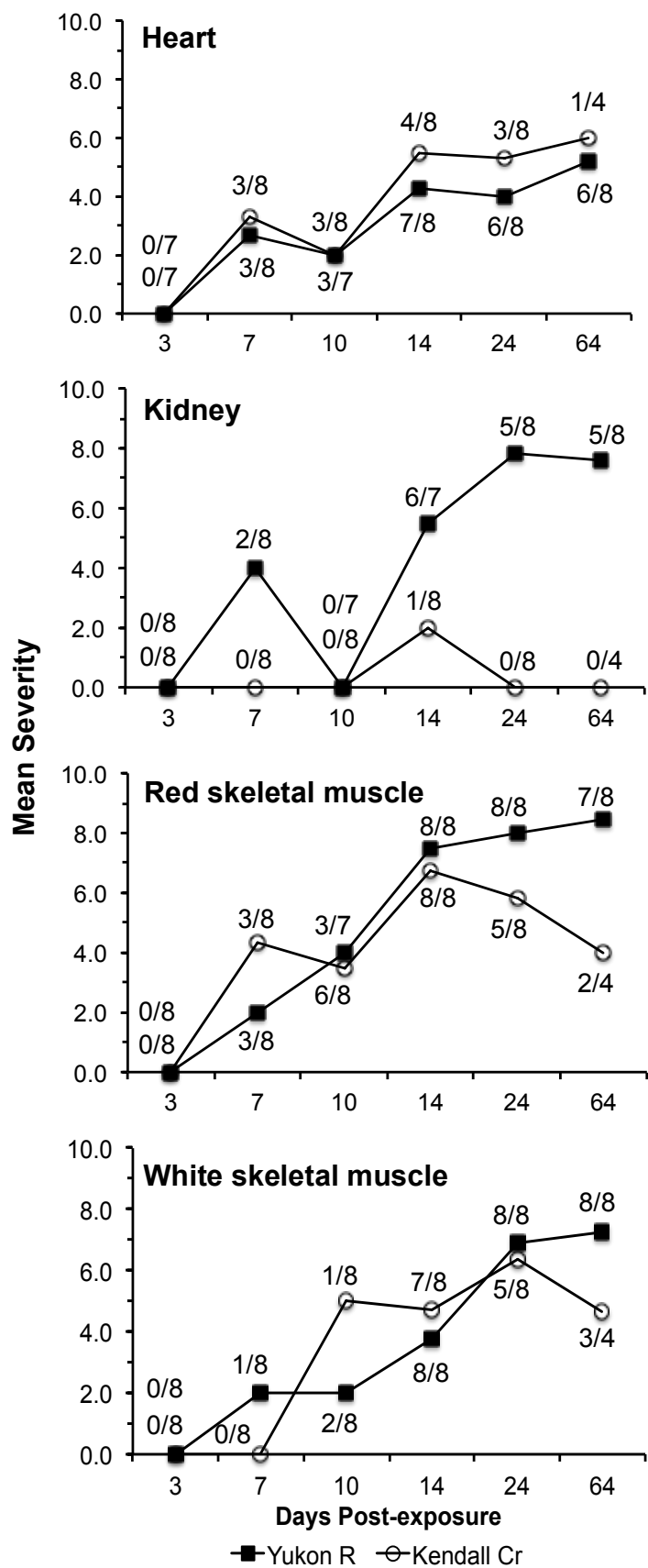


Figure 5.



Appendix B.

Draft Manuscript

Effects of temperature and elevated plasma cortisol levels on progression of *Ichthyophonus* infections in Yukon River Chinook salmon *Oncorhynchus tshawytscha*. Authors: D.G. Elliott, C.L. McKibben, C.M. Conway, A.H. MacKenzie, L.M. Hart, M.K. Purcell, J.L. Gregg, and P.K. Hershberger.



United States Department of the Interior

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Biological Resources Discipline

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March 10, 2016

Katie Williams

Project Manager

Arctic-Yukon-Kuskokwim Sustainable Salmon Initiative (AYK SSI)

Bering Sea Fish Fishermen's Association

431 West 7th Avenue, Suite 204

Anchorage, AK 99501

Subject: USGS Fundamental Science Practices: Courtesy Review and Non-Disclosure Policy
Prior to Publication

Dear Katie:

Attached is a draft manuscript entitled "Effects of temperature and elevated plasma cortisol levels on progression of *Ichthyophonus* infections in Yukon River Chinook salmon *Oncorhynchus tshawytscha*." This science product was generated as requested under AYK SSI Project AC-1335.

In being provided this courtesy copy of the information product prior to publication and release, parties are bound by the Bureau's non-disclosure policy as stated:

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On behalf of the WFRC, I thank you in advance for your time and assistance in making the publication of this science product a success.

Sincerely,

Jill B. Rolland
Center Director

1 **Effects of temperature and elevated plasma cortisol levels on progression of**
2 ***Ichthyophonus* infections in Yukon River Chinook salmon *Oncorhynchus***
3 ***tshawytscha***

4
5 Diane G. Elliott^{1*}, Constance L. McKibben¹, Carla M. Conway¹, Ashley H. MacKenzie²,
6 Lucas M. Hart², Maureen K. Purcell¹, Jacob L. Gregg², and Paul K. Hershberger²

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14
15 Running page head: Temperature and cortisol effects on ichthyophoniasis

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16 ***Abstract***

17 *Ichthyophonus* sp. was first reported in adult Chinook salmon (*Oncorhynchus*
18 *tshawytscha*) during their spawning migration up the Yukon River in the mid-1980s.
19 *Ichthyophonus* can severely impact salmon fillet quality and it has been hypothesized that
20 *Ichthyophonus*-associated pre-spawning mortality may be one driver of Chinook salmon
21 declines in the Yukon River. Salmon likely acquire infections by consuming infected
22 prey in the marine environment before entering the river. A limited understanding of the
23 interactions among the fish host, the pathogen, and the environment that determine the
24 outcome of *Ichthyophonus* infections in Yukon River Chinook salmon has hindered
25 development of predictive models that could be used in evaluation of potential
26 management strategies. In this research, the influence of water temperatures and elevated
27 levels of the stress hormone cortisol on disease progression of *Ichthyophonus*-infected
28 Yukon River Chinook salmon were investigated. Infected salmon were exposed to a
29 range of temperatures (7°C–20°C) that would be typically encountered during movement
30 from the marine to freshwater environment for spawning. No significant relationship
31 between water temperature and *Ichthyophonus*-related mortality was observed in the
32 juvenile salmon used, although increased degradation of parasites was observed in fish
33 held at higher temperatures. However, these findings may not be predictive of results for
34 adult salmon with compromised immune functions. A series of hormonal changes occur
35 during the terminal phases of spawning migration, which include elevated levels of
36 cortisol that may enhance freshwater homing but suppress the salmon's immune system.
37 Our experiments showed that administration of cortisol to juvenile Yukon River Chinook
38 salmon resulted in a greater increase in cortisol levels in the blood plasma in fish that had

39 been previously exposed to *Ichthyophonus* relative to fish that were given cortisol alone
40 or exposed to *Ichthyophonus* alone. The cortisol-injected fish that had been exposed to
41 *Ichthyophonus* also suffered significantly higher *Ichthyophonus*-associated mortality than
42 fish in all other experimental groups, although fin and body fungus likely also contributed
43 to the mortality. These results suggest that *Ichthyophonus* infections in Yukon River
44 Chinook salmon can contribute to elevation of plasma cortisol levels, as well as increased
45 mortality and susceptibility to secondary infections.

46

47 INTRODUCTION

48 The fish parasite *Ichthyophonus* has been hypothesized as a driver of long-term
49 declines in Yukon River Chinook salmon by reducing spawning effectiveness and
50 contributing to pre-spawning mortality (Kocan et al., 2004). However, most evidence
51 supporting this hypothesis is circumstantial, based either on field survey data that show
52 only correlations, or laboratory studies conducted with surrogate host species that may
53 exhibit different host responses to *Ichthyophonus*. Cause-and-effect relationships can
54 only be demonstrated by a combination of laboratory and field research specifically
55 targeting the Yukon River Chinook salmon strain, placing particular emphasis on
56 understanding the host, parasite, and environmental factors influencing *Ichthyophonus*
57 disease progression and host mortality.

58 The scientific literature indicates that water temperature can have a significant
59 influence on the progression of *Ichthyophonus* infections in fishes; however, reported
60 findings are often contradictory. For example, Okamoto et al. (1987) reported that
61 cumulative mortality of rainbow trout (*Oncorhynchus mykiss*) increased with increasing
62 temperature after a feeding exposure to *Ichthyophonus*. An analogous trend in mortality
63 was not detected in rainbow trout by Kocan et al. (2009); however, higher temperatures
64 did result in more rapid onset of the disease, higher parasite load, and increased disease
65 severity. Further confounding the information on effects of temperature is the report by
66 Gregg et al (2011) that the infectivity of *Ichthyophonus* to Pacific herring (*Clupea*
67 *pallasii*) decreased as temperature increased. Available evidence indicates that the
68 effects of temperature on *Ichthyophonus* may differ with respect to host species or stock

and strain of pathogen, such that use of a host / pathogen model specific to the Yukon River system is required for investigations of the effects of temperature on *Ichthyophonus* infections in Yukon River Chinook salmon. A better understanding of effects of temperature on initiation and progression of *Ichthyophonus* infections in Yukon River Chinook salmon would assist in development of models to predict prevalence and outcome of *Ichthyophonus* infections in Yukon River Chinook salmon under currently observed temperature ranges and those that might be expected with continued climate change.

In fishes and other animals, elevated levels of circulating corticosteroids, a class of naturally occurring hormones with immunosuppressive as well as adaptive functions, occur in response to chemical, physical, and / or biological stressors (both natural and anthropogenic). As adult salmon enter freshwater on their spawning migration, energy is allocated from some basal functions (including the immune system) to more immediate needs including up-river swimming and spawning. During the terminal phases of this migration, a series of hormonal changes occur, including elevated levels of the hormone cortisol. Although the high levels of cortisol observed in Pacific salmon during the spawning migration are hypothesized to have evolutionary significance in enhancement of the olfactory system for freshwater homing (Carruth et al., 2002), the immunosuppressive effects of elevated cortisol levels can result in increased pathogen proliferation (Bullock and Stuckey, 1975; Houghton and Matthews, 1986; Antonio and Hedrick, 1994; Gadan et al., 2012). Elevated cortisol levels may be a significant factor in progression and outcome of *Ichthyophonus* infections in Yukon River Chinook salmon during the spawning migration. Evidence exists that administration of high levels of

synthetic cortisol to starry flounder (*Platichthys stellatus*), concomitant with exposure to *Ichthyophonus*, results in an increased prevalence of infection and severity of resulting disease (Perry et al., 2004). However, repeated efforts to administer corticosteroids to Pacific herring carrying chronic infections with *Ichthyophonus* have failed to exacerbate the infections to overt disease (Hershberger, unpublished). Therefore, the effects of cortisol on the progression or severity of *Ichthyophonus* infections in fish appear to be host-specific; as such, these relationships require further investigation in Yukon River Chinook salmon.

Laboratory experiments were conducted in freshwater and in seawater to investigate effects of temperature on the progression of *Ichthyophonus* infections on Yukon River Chinook salmon. An additional freshwater experiment investigated effects of elevated levels of cortisol on the progression of *Ichthyophonus* infections in this stock of salmon.

METHODS

Experimental fish. Yukon River Chinook salmon were obtained as eyed eggs from the Creative Salmon Sea Spring Hatchery, Chemainus, B.C., Canada, and transported to the Western Fisheries Research Center (WFRC), Seattle, Washington, on September 26, 2013. The eggs and fry were maintained at the WFRC in sand-filtered, UV-treated water. Fry were fed a commercial pellet diet (BioOregon) until they reached the appropriate size for experiments. Freshwater experiments were conducted at the WFRC, and the seawater experiment was conducted at the Marrowstone Marine Station (MMFS), Nordland, Washington, in tanks supplied with sand-filtered seawater.

114 *Preparation of challenge material.* Pacific herring were used as a source of
115 *Ichthyophonus*-infected donor tissues for subsequent feeding trials with Chinook salmon.
116 Briefly, specific pathogen-free (SPF) Pacific herring (Beaulaurier et al. 2012) were
117 exposed to *Ichthyophonus* by interperitoneal (IP) injections with parasite life stages that
118 were suspended in phosphate buffered saline (PBS). *Ichthyophonus* life stages were
119 obtained from naturally infected wild Pacific herring; parasite isolates were generated by
120 tissue explant culture in Tris-buffered Eagle's Minimum Essential Medium,
121 supplemented with 5% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL
122 streptomycin, and 100 µg/mL gentamicin (MEM-5). External signs of infection (Hart et
123 al. *in press*) were evident in some exposed herring after 3-6 weeks, at which time donor
124 herring were euthanized with an overdose of buffered tricaine methanesulfonate (MS-
125 222), diced into small pieces, and respective experiments were initiated by feeding the
126 diced tissues to Chinook salmon. Control SPF herring were euthanized and frozen at -
127 20°C, then thawed and minced as needed for Chinook salmon feeding trials.

128 *Ichthyophonus exposure of Chinook salmon.* Fish were weaned from commercial
129 pellet feed to minced SPF herring over a 1-week to 2-week period prior to each
130 experiment. Then, fish were fed fresh minced SPF herring (control) or *Ichthyophonus*-
131 infected herring (treatment) daily for a period of 6 days (exception noted below).

132 *Ichthyophonus*-infected herring were euthanized every 2 days and kept refrigerated to
133 provide the fresh tissues for a 6-day feeding period. Following the exposure period, fish
134 were returned to the commercial diet and fed *ad libitum*.

135 *Culture of Ichthyophonus from fish.* Prevalence of *Ichthyophonus* in an experimental
136 group was determined by culture of heart and liver tissues from euthanized fish. Heart

and liver tissues of fish that died during an experiment were also cultured for detection of *Ichthyophonus*. The tissues were placed into MEM-5 broth at a ratio of ≤ 1 part tissue to 5 parts medium (w/v), incubated at 15°C, and screened by microscopy at weekly intervals over a 21-day period for *Ichthyophonus* growth as described by Hershberger (2012).

Histopathology. For examination of parasite density and host response, fish were euthanized and tissues were fixed for histopathology in 10% neutral buffered formalin and embedded in paraffin wax according to standard protocols. Tissues were sectioned at 5 μ m, and slides were stained with Gill's hematoxylin and eosin (H&E) for assessment of host response or periodic acid-Schiff (PAS) (Carson, 1997) for evaluation of parasite load. The slides were examined with a Zeiss Axiophot light microscope (Carl Zeiss, Inc.). The criteria of Kocan (2013) were used to identify morphological stages of *Ichthyophonus*. The abundance of *Ichthyophonus* in tissues was determined by the procedure of Hart et al. (*in press*) and was reported as the total number of parasites (or number of parasites of a given morphological stage) per 10 microscope fields at 200x magnification. The distribution and degree of the host response to the parasite in a given tissue was quantified at 200x magnification by the method of Hart et al. (*in press*). Scores of lesion distribution (0-3) and degree (0-3) of inflammation, fibrosis, and necrosis were added together to obtain an overall host response severity measure for each tissue type (maximum score = 12).

Freshwater temperature experiment. Yukon River Chinook salmon (2.0 g average weight) were fed infected or SPF herring tissues at a rate of 2% of body weight per day during holding in 278-L tanks at 15°C. Eight days after the beginning of the feeding exposure period, subgroups of fish were moved to three sets of 119-L tanks for holding at

three different temperatures. Four tanks in each set were used for mortality monitoring at a given temperature (three tanks for the treatment group and one tank for the control group; 40 fish per tank), and two tanks per set were used for sampling (one tank each for the treatment and control group; 100 fish per tank). The temperature for one set of control and treatment tanks was increased to 20°C over a 3-day period, the temperature for another set was reduced to 10°C over 3 days, and the temperature for the third set of tanks remained at 15°C.

Ten fish were sampled from each treatment or control group for culture at 7 days after the beginning of the feeding challenge (while all fish were still maintained at 15°C), and also at days 20 and 32, after the temperature changes. Fifteen fish per group were sampled at the termination of the experiment 62 days after the beginning of the challenge. On the same dates, five fish were sampled from each treatment or control group and preserved for histopathology. Tissues examined for histopathology assessments included heart, liver, kidney, spleen, gastrointestinal tract, exocrine pancreas and inter-pancreatic connective tissue, skin and skeletal muscle.

Seawater temperature experiment. Seawater-adapted Yukon River Chinook salmon (60.4 g average weight) were held at 7°C in 593-L tanks of 29‰ seawater (120 fish per tank; one treatment tank and one control tank), and fed minced *Ichthyophonus*-infected or SPF herring four times over a 6-day period (average feeding rate 4% body weight per day for treatment fish 3% body weight per day for control fish on the feeding days).

To examine effects of a rapid increase in water temperature on progression of early *Ichthyophonus* infections, 20 fish each were moved from the 7°C (cold) treatment and control tanks into additional 593-L tanks 7 days after the beginning of the feeding

challenge, and the temperature was raised over a 1-week period to 17°C (warm early transfer) tanks. On the same date that the fish were moved into the warm early transfer tanks, 21 and 20 additional fish were euthanized from the cold treatment and cold control tanks, respectively, for culture and histopathology. Half of the heart and a portion of the liver from each fish were cultured, and the remaining heart and liver tissue from each fish were placed in fixative in histology cassettes identified by fish number. Additional tissues preserved in numbered cassettes from each fish for histopathology included kidney, spleen, red and white skeletal muscle, and skin. Remaining fish in the warm early transfer treatment and control tanks were euthanized on day 51, and 17 and 18 fish from the treatment and control tanks, respectively, were sampled for culture and histopathology. On the same date, 17 fish each from the cold treatment and control tanks were euthanized and sampled for culture and histopathology.

To investigate effects of a rapid water temperature increase on progression of later stage, established *Ichthyophonus* infections, 20 fish each were moved from the cold treatment and control tanks to additional 593-L tanks 28 days after the beginning of the feeding challenge, and the temperature was increased over a 1-week period to 17°C (warm late transfer) tanks. On the same date that fish were moved into the warm late transfer tanks, 20 fish each were euthanized from the cold treatment and cold control tanks and processed for culture and histopathology as previously described. Remaining fish in the warm late transfer tanks were euthanized on day 71 (control) and day 72 (treatment), and 16 fish from each tank were sampled for culture and histopathology. Remaining fish in the respective cold control and treatment tanks were euthanized on the

same dates, and 19 fish from the cold treatment tank and 11 fish from the cold control tank were sampled for culture and histopathology.

Cortisol experiment (freshwater). To examine effects of elevated plasma cortisol levels on progression of *Ichthyophonus* infections in Yukon River Chinook salmon, 550 Yukon River Chinook salmon (22.0 g average weight) were distributed equally into each of two 593-L tanks, acclimated to 15°C water, and fed minced *Ichthyophonus*-infected or SPF herring at 6% body weight per day over a 6-day period. Seven days after the beginning of the feeding exposure, 10 fish per tank were euthanized quickly with MS-222 for plasma cortisol analysis. The caudal peduncle was severed and blood was collected in a heparinized Natelson tube within 5 min of euthanasia, then transferred to a micro-centrifuge tube and held on ice until centrifugation for 6 min at 6,000 x g at 4°C to separate the plasma. Plasma was stored at -80°C until cortisol testing. After blood samples were taken, heart and liver tissue was removed from these 10 fish and 5 additional fish euthanized from each tank for culture. Tissues (heart, liver, kidney, spleen, gastrointestinal tract, exocrine pancreas and inter-pancreatic connective tissue, skin and skeletal muscle) from six more fish euthanized from each tank were preserved for histopathology.

Thirteen days after the beginning of the feeding exposure, fish from the *Ichthyophonus* treatment and feeding control groups were distributed into six 278-L tanks per group (20 fish in one mortality monitoring tank and 65 fish in one sampling tank for each group). Fish in one mortality monitoring tank and one sampling tank each for the *Ichthyophonus* and feeding control groups were injected IP with cortisol (hydrocortisone; Sigma H4001) emulsified in a vegetable shortening and vegetable oil mixture according

to the method of Specker et al. (1994). This mixture is fluid at 24°C (and is still injectable at 15°C) but solidifies in the peritoneal cavity, where it forms a slow-releasing cortisol implant that can maintain cortisol at elevated levels for up to 1 month (Specker et al., 1994). Briefly, the shortening:oil vehicle was prepared by heating equal parts (v/v) of vegetable shortening and vegetable oil to liquification (45°C). Cortisol (10 mg per mL of vehicle) was dissolved in the vehicle by stirring at 45°C. Syringes (1 mL) were filled when the emulsification was at 25°–30°C, bubbles were removed, and the syringes were fitted with 20-gauge needles for injection of fish. Each fish was weighed before injection and injected with the cortisol emulsion at a rate of about 50 µL/10 g body weight, which provided a cortisol dose of 50 µg/g body weight. In preliminary experiments, this dose was found to result in elevated plasma cortisol levels up to 500 ng/mL after 7 days, within the range seen in Pacific salmon during their spawning migrations (Carruth et al., 2002). Injection control tanks (one mortality monitoring tank and one sampling tank each for the *Ichthyophonus* and feeding control groups) consisted of fish that were weighed and injected with the vehicle only (vehicle injection control), and fish that received no injection (no injection control). Heart and liver tissue was cultured for *Ichthyophonus* detection from all fish that died in any tank during the experiment.

Twenty-three days after the beginning of the feeding exposure (10 days after cortisol or vehicle injection), fish were euthanized from each of the sample tanks for plasma cortisol analysis (10 fish), culture (15 fish), and histopathology (6 fish) as previously described. On day 49 after the beginning of the feeding exposure (36 days after cortisol or vehicle implantation), the remaining fish in the sample tanks were euthanized and sampled for cortisol analysis, culture, and histopathology. Tissues examined for

histopathology assessments included heart, liver, kidney, spleen, gastrointestinal tract, exocrine pancreas and inter-pancreatic connective tissue, skin and skeletal muscle.

Plasma cortisol assay. An enzyme-linked immunosorbent assay (ELISA) kit (ADI-900-071) from Enzo Life Sciences was used for plasma cortisol measurement. The assay employs a monoclonal antibody to cortisol to competitively bind to plasma cortisol, and is reported by the manufacturer to have a sensitivity of 56.72 pg/mL. The assay reagents, standards and controls were prepared according to the manufacturer's instructions. A standard curve of cortisol concentrations was generated via serial dilution of a standard provided by the manufacturer to achieve seven concentrations between 156 and 10,000 pg/mL cortisol.

The blood plasma samples from experimental fish were thawed and 60 µL of each sample was diluted to 1:50 and 1:100 (v/v) in sample buffer, and assayed in duplicate in a 96-well microplate. The absorbance or optical density (OD) values of samples and standards were read on an ELx808 Ultra microplate reader connected to Gen5TM microplate data collection and analysis Ssoftware (Bio-Tek Instruments). Plasma cortisol levels were reported as ng/mL cortisol.

Statistical analysis. Statistical analyses were performed using PASW V.18 (IBM Inc.) and InStat 3 (GraphPad Software, Inc.). Differences in arcsine transformed cumulative percent mortality (CPM) among temperature groups in the freshwater experiment were assessed by analysis of variance (ANOVA). Only a single mortality tank was included in the experimental design for the cortisol experiment; differences in final mortality in this experiment were assessed by Fisher's exact test. A general linear model (GLM) was

employed to evaluate differences in plasma cortisol levels using *Ichthyophonus* exposure (exposed vs. non-exposed) and cortisol treatment (uninjected, vehicle only or cortisol) as explanatory fixed factors. The Fisher exact test (two groups) and chi-square test (three or more groups) were used to compare *Ichthyophonus* prevalence between or among groups in the experiments. Mean parasite densities in histological sections from *Ichthyophonus*-infected fish in various treatment groups were compared by Student's *t* test (two groups) or ANOVA (three or more groups) following log transformation of the data. Host response severity score data were analyzed by the nonparametric Mann-Whitney (two groups) or Kruskal-Wallis (three or more groups) tests.

RESULTS

Does temperature affect progression of *Ichthyophonus* infections?

Freshwater experiment. In the freshwater experiment, no mortality was recorded before transfer of fish to the final holding temperatures, but *Ichthyophonus*-associated mortality, as indicated by positive cultures from heart and liver tissues, occurred in challenged fish held at 10°C, 15°C, and 20°C (Table 1). In two of three replicate tanks held at 20°C, fish suffered high mortality associated with skin and gill infections with the protozoan parasite *Ichthyophthirius multifiliis*. There was no significant difference in cumulative percent *Ichthyophonus*-associated mortality in fish challenged with *Ichthyophonus* and held at the three different temperatures ($p=0.89$). *Ichthyophonus* was not recovered from the single mortality that occurred in the 10°C control tank.

Ichthyophonus prevalence, determined by culture of heart and liver tissue culture, was low (20-40%) in all temperature groups in the freshwater experiment at all sample time points (Table 2). There were no significant differences in prevalence among the temperature groups at any sample time ($P \geq 0.36$). *Ichthyophonus* was not detected in cultures from any of the control groups.

Ichthyophonus was observed by histopathology in challenged fish held at each of the three temperatures in freshwater. Common life stages observed included the schizont, a large, PAS-positive, multinucleate cell surrounded by a non-cellular, multi-laminate capsule; the plasmodium, a small, PAS-positive multinucleate cell that lacked a capsule; and the degenerated schizont, which lacked an intact multinucleate, PAS-positive center but retained a capsule or recognizable pieces thereof. The center of the degenerated schizont often appeared amorphous, or was replaced by infiltration of inflammatory cells. Rarely, germinating multinucleate bodies were observed; these were characterized by the protrusion of short hyphal structures through the capsule of schizonts.

The *Ichthyophonus* prevalence detected by histopathology in tissues examined from fish in the freshwater temperature experiment was highly variable and generally low (Table 3); *Ichthyophonus* was not detected in any control fish (data not shown).

Ichthyophonus was not detected in histological samples until day 20, when it was observed in the red skeletal muscle of a single fish in the 10°C group. In the day 34 sample, none of the fish in the 10°C group were *Ichthyophonus*-positive by histopathology, but parasites were observed in at least one examined tissue in three of five fish in the 15°C group and two of five fish in the 20°C group. In the day 62 sample, *Ichthyophonus* was observed in at least one examined tissue in one of five fish sampled

from both the 10°C and 15°C groups, and in three of five fish sampled from the 20°C group.

The parasite prevalence in individual tissues examined by histopathology was also variable and generally low (Table 3). The highest parasite prevalence (60%) was observed in red skeletal muscle of fish in the 15°C group from the day 34 sample, and in the red and white skeletal muscle of fish in the 20°C group from the day 62 sample. The *Ichthyophonus* infection intensity was also highly variable (Table 3). The highest parasite densities (≥ 50 parasites per 10 microscope fields at 200x magnification) were recorded from heart and liver tissue of a fish in the 15°C group on day 34, from heart and kidney tissue of one fish and liver tissue of another fish in the 20°C group on day 34, and from heart and kidney tissue of a fish in the 10°C group on day 62.

The most predominant host response to *Ichthyophonus* infection, first observed in histological sections at 20 days after exposure (Table 3), consisted of granulomatous inflammation, often accompanied by fibrosis. Tissue necrosis was less frequently observed in areas of infection. The occurrence of host response usually corresponded with the presence of *Ichthyophonus* (Table 3), although tissues with low parasite densities occasionally showed no host reaction to the parasites, and focal areas of granulomatous host response were rarely observed in the absence of visible parasites. The highest severity of host response (score ≥ 8.0) was observed in heart tissue of the single infected fish in the 10°C group on day 62, and in kidney, red skeletal muscle and white skeletal muscle of the *Ichthyophonus*-positive fish in the 20°C group on day 62 (Table 3). No host response typical of *Ichthyophonus* infection was observed in any control fish.

Histopathological examination of fish tissues revealed that the proportions of degenerated *Ichthyophonus* parasites in the tissues increased in all temperature groups by day 62 (Figure 1A), although there was not a clear association with temperature. While only 14% of parasites detected in the single *Ichthyophonus*-positive fish in the 10°C group on day 62 were degenerated in comparison to 32% of parasites detected in three positive fish the 20°C group at this time point, the only two parasites detected in the single positive fish in the 15°C at this time point were degenerated.

Seawater experiment. Separate mortality monitoring tanks were not included in the seawater temperature experiment, but all of the heart and liver cultures of mortalities from the control tanks (five from the 7°C tank, two from the 17°C early transfer tank and three from the 17°C late transfer tank) tested negative for *Ichthyophonus*. None of the cultures from the five mortalities in the 7°C *Ichthyophonus*-exposed tank tested positive for *Ichthyophonus*, but cultures from one of two mortalities from the 17°C early transfer tank and one of four mortalities from the 17°C late transfer tank tested *Ichthyophonus*-positive.

In the seawater experiment, *Ichthyophonus* prevalence as determined by culture from live fish ranged from 6% to 47% (Table 4). There was no significant difference ($P = 0.7$) in prevalence between the 7°C group and the early transfer 17°C group 44 days after transfer (day 51), but the *Ichthyophonus* prevalence was significantly higher ($P = 0.02$) in the 7°C group compared with the late transfer 17°C group 44 days after transfer (day 72). No *Ichthyophonus* was cultured from any of the control fish (data not shown).

Comparative histopathological examination of tissues from the 17°C early transfer treatment group and the 7°C treatment group on day 51 revealed the presence of

Ichthyophonus parasites in at least one tissue from 75% (six of eight) fish from the 17°C group and 63% (five of eight) fish from the 7°C group. Examination of tissues from the 17°C late transfer treatment group and the 7°C treatment group on day 72 showed the presence of *Ichthyophonus* parasites in at least one tissue from 50% (four of eight) fish in the 17°C group and 88% (seven of eight) fish from the 7°C group. Tissues with the highest infection prevalence were the heart, kidney and red and white skeletal muscle (Table 5). No *Ichthyophonus* was detected by histopathology from any control fish.

Mean parasite densities in tissues of *Ichthyophonus*-exposed fish examined by histopathology ranged from less than 1 to 46 per 10 microscope fields at 200x magnification (Table 5). There were no significant differences in mean parasite densities between any of the treatment groups ($P \geq 0.15$). The proportion of observed parasites in all tissues that were degenerated in each treatment group was also calculated (Figure 1B). The proportion of degenerated parasites was higher in the 17°C early transfer group than in the 7°C group on day 51 ($P = 0.0006$), and the proportion of degenerated parasites was higher in the 17°C late transfer group than in the 7°C group on day 72 ($P < 0.0001$). The proportion of degenerated parasites in the 17°C late transfer group on day 72 was also higher than the proportion of degenerated parasites in the 17°C early transfer group on day 51 ($P = 0.0017$). Notably, for the heart and liver, portions of which were cultured from each fish examined by histopathology, 82% (9 of 11) of parasites detected in stained heart tissue sections and 75% (3 of 4) of parasites detected in liver tissue sections from the 17°C late transfer group fish on day 72 were degenerated, in comparison to 10% (2 of 21) degenerated parasites detected in heart tissue sections and no (0 of 1) degenerated parasite detected in liver tissue sections from the 17°C early transfer group on day 52. In

contrast to the results for the 17°C groups, the proportions of degenerated parasites in the 7°C group did not significantly differ between the samples taken on days 51 and 72 ($P = 0.08$).

Do elevated plasma cortisol levels affect progression of *Ichthyophonus* infections?

Plasma cortisol levels. Statistical analysis revealed temporal changes in plasma cortisol levels associated with cortisol injection and *Ichthyophonus* exposure (Figure 2). Plasma cortisol levels were not significantly different ($P \geq 0.31$) between fish exposed to *Ichthyophonus* relative to control fish on day 7, one day after the termination of the feeding exposure but prior to hydrocortisone or vehicle injection. On day 23, 10 days after hydrocortisone or vehicle injection of some groups, there was a significant effect of both *Ichthyophonus* exposure ($P = 0.015$) and cortisol treatment ($P = 0.007$) on plasma cortisol levels. Post-hoc testing revealed that plasma cortisol levels were significantly higher in fish given cortisol relative to fish that were not injected or were injected with only vehicle. By the final sample time point on day 49 (36 days after hydrocortisone or vehicle injection), there was no significant variation in cortisol levels among the treatment groups.

Ichthyophonus-associated mortality. In the mortality monitoring tanks, mortality only occurred in the cortisol-injected group exposed to *Ichthyophonus* and in the non-injected group exposed to *Ichthyophonus* (Table 6). *Ichthyophonus* was detected in heart and liver cultures from all but one mortality in the cortisol-injected group. However, external body and tail fungus was also observed on all dead fish from the cortisol-injected, *Ichthyophonus*-exposed tank, but not on the single dead fish from the non-injected,

Ichthyophonus-exposed group (Table 6). Mortality in the group injected with cortisol and exposed to *Ichthyophonus* was significantly greater than mortality in all other treatment or control groups ($P < 0.001$ for all comparisons).

In the sampling tanks, mortality also only occurred in groups exposed to *Ichthyophonus*. The highest mortality (19 fish) among *Ichthyophonus*-exposed groups was recorded in the cortisol-injected group. Ten of these fish (53%) tested positive for *Ichthyophonus* by heart and liver culture, but external fungus was observed on 18 of the fish (95%). *Ichthyophonus* and external fungus were detected from two of three mortalities in the vehicle-injected group. *Ichthyophonus* was detected in two of three mortalities in the uninjected group, and external fungus was observed on all three mortalities from this group.

Ichthyophonus infection prevalence (live fish samples). *Ichthyophonus* prevalence in groups that were exposed to the parasite by feeding minced infected herring tissues ranged from 50% to 87% (Table 7); no *Ichthyophonus* was detected in heart and liver cultures from fish that were fed minced tissues from SPF herring (data not shown). There were no significant differences in *Ichthyophonus* prevalence among the *Ichthyophonus*-exposed treatment groups in fish sampled at 23 and 49 days after the beginning of exposure ($P \geq 0.29$). Because of the high mortality in the sampling tank of fish that were exposed to *Ichthyophonus* and injected with cortisol, additional fish (nine per tank) were sampled from the mortality monitoring tanks for culture of heart and liver tissues 51 days after the beginning of parasite exposure and 38 days after cortisol and vehicle injection (Table 7), but there were no significant differences in *Ichthyophonus* prevalence among the groups at this time point ($P = 0.86$).

Histopathology. Histopathological examination of 6 fish per group sampled 23 days after the beginning of the *Ichthyophonus* exposure and 10 days after cortisol or vehicle injection revealed the presence of *Ichthyophonus* parasites in at least one tissue in all 6 fish injected with hydrocortisone and in 5 of 6 fish that were injected with the vehicle only or were not injected. No *Ichthyophonus* was observed in any non-*Ichthyophonus*-exposed control fish (data not shown). Among the tissues examined from *Ichthyophonus*-exposed fish, the highest parasite prevalence and infection intensity were usually observed in heart and in red and white skeletal muscle (Table 8). There was no significant difference in overall mean parasite densities in tissue sections among the treatment groups ($P = 0.69$). The proportion of total parasites that were degenerated was also calculated for each treatment group, and there were no significant differences ($P = 0.32$) in the proportions of degenerated parasites among the cortisol-injected group (8.7%, 54 of 618 parasites), the vehicle-injected group (9.4%, 52 of 554 parasites), and the non-injected group (8.4%, 49 of 580 parasites) in the sample taken on day 23.

Host response, consisting of granulomatous inflammation, fibrosis, and necrosis, was observed in fish in all *Ichthyophonus*-exposed groups on day 23 (Table 8) but was not observed in any fish that had not been exposed to the parasite (data not shown). The occurrence of host response generally corresponded with the presence of parasites in a given tissue (Table 8). The highest severity of host response (≥ 6) was observed in liver tissue of cortisol-injected fish, in red skeletal muscle of vehicle-injected fish, and in kidney and red and white skeletal muscle tissue of non-injected fish. There was no significant difference in overall median host response scores among treatment groups ($P = 0.29$) at this sample time point.

DISCUSSION

Water temperature can impact the course of disease in fish via effects on both the replication of pathogens (Noe and Dickerson, 1995; Spanggaard and Huss 1996) and the physiology of the fish host (Brett, 1956), including innate and adaptive immunity (Le Morvan et al., 1998; Alcorn et al. 2002). Some of our findings suggested decreased progression of *Ichthyophonus* infections in Yukon River Chinook salmon held at higher temperatures ($\geq 15^{\circ}\text{C}$). This was evidenced by higher proportions of degenerated parasites detected by histopathology at later sample time points in tissues of fish held at higher temperatures in comparison to those held at lower temperatures in both the freshwater and seawater temperature experiments. Additionally, in the seawater experiment, a significant reduction in *Ichthyophonus* infection prevalence detected by culture occurred in the 17°C late transfer group in comparison to the 7°C group 44 days after transfer, although a similar reduction in prevalence was not observed in the 17°C early transfer group 44 days after transfer, or at later sample time points in the group held at 20°C in the freshwater experiment. The *in vitro* experiments conducted by Spanggaard and Huss (1996) showed the ability of *Ichthyophonus* to grow at temperatures from 0°C to 25°C , with higher growth rates at 15°C and 25°C than at 0°C or 5°C , suggesting that increased effectiveness of the host immune response, rather than decreased ability of the parasite to survive and replicate, was a likely factor in the greater proportion of degenerated parasites observed in fish held at the higher temperatures in our experiments.

Although it is generally considered that a direct relation exists between immunocompetence and water temperature within the homeostatic range of the fish host

(Watts et al., 2001; Magnadóttir, 2006; Bowden et al., 2007), there is some evidence that at low temperatures, certain innate immune functions may be enhanced to compensate for suppressed adaptive immunity (Le Morvan et al., 1998). For histozoic parasite conditions such as ichthyophoniasis, a cellular rather than humoral immune response is likely the most effective defense against proliferation of the parasites in tissues (Alvarez-Pellitero et al., 2008; Sitjà-Bobadilla, 2008). In *Ichthyophonus* infections, cellular responses consisting of granulomatous inflammation and fibrosis often result in encapsulation of the parasites, which may eventually result in their death if the parasites cannot escape encystment and proliferate (Sitjà-Bobadilla, 2008). In our study, granulomatous inflammation and fibrosis were observed in *Ichthyophonus*-infected fish held at all temperatures, but the host response appeared more successful in causing degradation of the parasites at higher temperatures, although effector mechanisms were not identified. Gregg et al. (2011) hypothesized that a temperature-enhanced innate response may have resulted in reduced *Ichthyophonus* prevalence in Pacific herring held at high temperatures (15.3°C) relative to those held at lower temperatures (12.0°C or 9.3°C) following a 56-day fast and IP injection with *Ichthyophonus* one day before resumption of feeding. Culture results indicated that a higher proportion of herring in the high temperature group was able to clear the infection in comparison to the other groups when the infectious dose was relatively low (17 schizonts/g body weight of fish).

The results of histopathological analyses in our study differed from those of Kocan et al. (2009), who observed a direct relation between temperature, parasite densities in host tissues, and the progression and intensity of the host inflammatory response in *Ichthyophonus*-infected rainbow trout held at 10°C, 15°C, and 20°C following feeding

exposure to *Ichthyophonus*. The experimental results in the two studies may have been influenced by differences in infectious dose (not determined for either study), host fish species, and the *Ichthyophonus* isolates utilized for the research. Sequencing of *Ichthyophonus* internal transcribed spacer loci by Rasmussen et al. (2010) demonstrated that marine *Ichthyophonus* isolates such as the Pacific herring isolate used in our research are phylogenetically distinct from the freshwater rainbow trout isolate used by Kocan et al. (2009), and the marine and freshwater isolates also exhibit phenotypic differences in stability in freshwater and seawater (Hershberger et al., 2008).

In contrast to the studies of Okamoto et al. (1987), our research did not demonstrate a significant influence of temperature on *Ichthyophonus*-related mortality in previously infected Yukon River Chinook salmon held at temperatures between 10°C and 20°C. In groups of rainbow trout fed about 100 *Ichthyophonus* spherical bodies (schizonts) per fish from culture, Okamoto et al. (1987) observed 100% cumulative mortality in fish held at 15°C and 20°C compared to 10% mortality in fish held at 10°C and no mortality in fish held at 4°C for 35 days. When the *Ichthyophonus* dosage was increased to about 3,000 schizonts per fish fed as minced infected tissues, however, cumulative mortality was greater than 90% in all temperature groups except 4°C, where no mortality occurred, suggesting that temperature effects on mortality could be masked by high parasite loads. We did not quantify the dose of schizonts fed to fish in minced tissues in our freshwater experiment, but the relatively low *Ichthyophonus*-associated mortality ($\leq 26\%$) in the mortality monitoring tanks, the relatively low *Ichthyophonus* prevalence ($\leq 40\%$) by culture in live fish tested from the sampling tanks, and the inconsistent presence of the parasite in tissues examined by histopathology from all temperature groups, all were

indicative that the fish had received a low infectious dose of parasites, which may have masked any effects of temperature on *Ichthyophonus*-related mortality. In addition, the infections with the protozoan *Ichthyophthirius multifiliis* that occurred in *Ichthyophonus*-exposed fish in two of the three 20°C mortality monitoring tanks may have influenced the course of ichthyophoniasis in the fish.

Experiments with Pacific herring also showed that temperature impacts on disease outcome varied depending on the stage of *Ichthyophonus* infections in fish (Gregg et al., 2008). In groups of herring with established disease as evidenced by the presence of clinical signs, temperature manipulation had little effect on *Ichthyophonus*-related mortality. In newly infected fish, however, *Ichthyophonus*-related mortality was suppressed at both elevated temperature (12.4°C) and low temperature (5.6°C), suggesting the importance of relationships between temperature optima for parasite growth and host immune function in the establishment and outcome of *Ichthyophonus* infections. Culture results and external examination of fish in our research indicated that a portion of the fish were infected before temperature manipulations were applied, but they were not clinically diseased.

Although the juvenile Yukon River Chinook salmon used in our study demonstrated a robust host response to *Ichthyophonus* infections resulting in significant parasite degradation in tissues of fish held at higher temperatures for prolonged periods, it is unknown whether adult fish with diminished immune function can mount an effective defense against the parasite. Our experiment with cortisol injection of juvenile fish was an attempt to mimic the elevation of the stress hormone that occurs in adult fish during their spawning migration (Carruth et al., 2002), and to evaluate effects of elevated

cortisol on the progression of *Ichthyophonus* infections. Hydrocortisone injection resulted in significant elevation of plasma cortisol 10 days after injection, but exposure to *Ichthyophonus* also had a significant contribution to the elevation of plasma cortisol levels. Horstmann-Dehn et al. (2012) also showed significantly higher plasma cortisol levels ($P = 0.03$) in *Ichthyophonus*-infected adult Yukon River Chinook salmon relative to levels in uninfected salmon in fish collected at the river mouth (Emmonak, Alaska), but there was no significant difference in plasma cortisol levels ($P = 0.24$) between *Ichthyophonus*-infected and uninfected fish collected at river kilometer 1,200 (Eagle, Alaska). The mean and range of plasma cortisol levels detected by Horstmann-Dehn et al. (2012) in *Ichthyophonus*-infected (430 ng/mL; 24 to 837 ng/mL) and uninfected (260 ng/mL; 1 to 787 ng/mL) salmon at Emmonak were generally higher than the values achieved in our study for cortisol-injected fish that were *Ichthyophonus*-infected (120 ng/mL; 2 to 366 ng/mL) or uninfected (23 ng/mL; 0 to 122 ng/mL). In comparison, the mean and range of plasma cortisol levels in adult Chinook salmon sampled by Horstmann-Dehn et al. (2012) at Eagle were 98 ng/mL (8 to 447 ng/mL) for *Ichthyophonus*-infected fish and 54 ng/mL (54 to 330 ng/mL) for uninfected fish.

Our results also indicated that *Ichthyophonus* exposure combined with cortisol injection contributed significantly to fish mortality, although external fungus also was likely a factor in mortality. Skin lesions such as ulceration associated with clinical *Ichthyophonus* infections were observed in some fish in the current research and have been previously reported in salmonids (Hershberger, 2012). These lesions may provide a portal of entry to secondary pathogens such as fungus; fungal infections were only observed in *Ichthyophonus*-exposed groups in our cortisol experiment. Nevertheless, it is

unknown whether *Ichthyophonus* infections contribute to increased susceptibility of immunocompromised adult salmon to opportunistic pathogens during their spawning migration.

In contrast to the findings of Perry et al. (2004), who injected starry flounders IP with the synthetic corticosteroid dexamethasone sodium phosphate in saline solution 1–2 days prior or 1 day after IP injection with *Ichthyophonus*, we did not observe a significant increase in *Ichthyophonus* infection prevalence and intensity, relative to *Ichthyophonus*-challenged control fish, in Chinook salmon that had been injected with hydrocortisone 13 days after the beginning of a 6-day feeding exposure to *Ichthyophonus*. However, the dexamethasone was injected into starry flounder as a pharmacological rather than a physiological dose as was done with hydrocortisone implants in our study, and dexamethasone levels achieved in the fish were not measured. Dexamethasone is a synthetic analog of the naturally occurring glucocorticoid hormones cortisol and hydrocortisone, and dexamethasone sodium phosphate is approximately three thousand times more soluble in water at 25°C than hydrocortisone (www.drugs.com).

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Table 1. Total mortality and *Ichthyophonus*-associated mortality of Yukon River Chinook salmon that were exposed to *Ichthyophonus* by feeding minced infected herring tissues at 2% body weight per day over a 6-day period at 15°C in freshwater, and retained at 15°C or transferred to new tanks 8 days after the beginning of the feeding exposure and held at 15°C or acclimated over a 3-day period to 10°C or 20°C for mortality monitoring. Equivalent control groups were fed minced specific-pathogen-free herring. Mortality was considered to be *Ichthyophonus*-associated if the parasite was detected in cultures of heart and liver tissue. CPM = cumulative percent mortality.

| Temperature | Treatment and replicate tank | Total fish | Total mortality (%) | <i>Ichthyophonus</i> -associated mortality (%) |
|-------------|--------------------------------------|------------|------------------------|--|
| 10°C | Control | 40 | 1 (3%) | 0 |
| | <i>Ichthyophonus</i> | | | |
| | Rep 1 | 40 | 2 (5%) | 2 (5%) |
| | Rep 2 | 40 | 5 (12%) | 5 (13%) |
| | Rep 3 | 40 | 8 (20%) | 8 (20%) |
| | <i>Ichthyophonus</i> average CPM ±SD | | | 13% ± 8% |
| 15°C | Control | 40 | 0 | --- |
| | <i>Ichthyophonus</i> | | | |
| | Rep 1 | 41 | 10 (24%) | 10 (24%) |
| | Rep 2 | 40 | 9 (23%) | 9 (23%) |
| | Rep 3 | 43 | 11 (26%) | 11 (26%) |
| | <i>Ichthyophonus</i> average CPM ±SD | | | 24% ± 2% |
| 20°C | Control | | | |
| | <i>Ichthyophonus</i> | | | |
| | Rep 1 | 39 | 3 (8%) | 3 (8%) |
| | Rep 2 | 40 | 40 (100%) ¹ | 9 (23%) |
| | Rep 3 | 38 | 43 (100%) ¹ | 11 (26%) |
| | <i>Ichthyophonus</i> average CPM ±SD | | | 8% ± 4% |

¹ Most mortality in tank associated with *Ichthyophthirius multifiliis* infection.

Table 2. *Ichthyophonus* prevalence as determined by heart and liver tissue culture in Yukon River Chinook salmon held in sampling tanks in the freshwater temperature experiment. The *Ichthyophonus* exposure and holding conditions for the experiment are described in Table 1. No *Ichthyophonus* was cultured from any control fish (data not shown).

| Treatment Group | Days post-exposure | | | |
|-----------------|--|------------|------------|------------|
| | Number of fish positive for <i>Ichthyophonus</i> /total cultured (%) | | | |
| | Day 7 | Day 20 | Day 34 | Day 62 |
| 10°C | --- | 3/10 (30%) | 2/10 (20%) | 3/15 (20%) |
| 15°C | 4/10 (40%) | 2/10 (20%) | 2/10 (20%) | 3/15 (20%) |
| 20°C | --- | 4/10 (40%) | 2/10 (20%) | 6/15 (40%) |

738

739 Table 3. Prevalence and mean parasite densities of *Ichthyophonus*, and occurrence and severity of host response to *Ichthyophonus*
 740 infections, observed in histological sections of tissues from Yukon River Chinook salmon in the freshwater temperature experiment.
 741 The sample day was the number of days after the date of the first feeding exposure. Slides were stained with periodic acid-Schiff
 742 (PAS) to facilitate detection of parasites. Slides examined for parasite detection were stained with periodic acid-Schiff (PAS), and
 743 slides examined for host response were stained with hematoxylin and eosin (H&E). Scores of lesion distribution (0-3) and degree (0-3)
 744 of inflammation, fibrosis, and necrosis were added together to obtain an overall host response severity measure for each tissue type
 745 (maximum severity score = 12). No *Ichthyophonus* or associated host response was detected by histopathology in fish in the day 7
 746 sample, when all fish were being held at 15°C, or in any control fish (data not shown).

747

| Sample day | Group | Number fish positive for <i>Ichthyophonus</i> /total examined (%) | | | | | | | |
|---------------|-------|--|-------|-----------------------|-----------------------|---------------------|--------|------------------------|--------------------------|
| | | Mean number of parasites in 10 microscope fields at 200x magnification (range) | | | | | | | |
| | | Number of fish positive for host response/total examined (%) | | | | | | | |
| | | Mean host response severity score \pm SD | | | | | | | |
| | | Heart | Liver | GI tract ¹ | Pancreas ² | Kidney ³ | Spleen | Red skeletal muscle | White skeletal muscle |
| 20 | 10°C | 0/3 | 0/5 | 0/5 | 0/5 | 0/5 | 0/3 | 1/5 (20%) | 0/5 |
| | | --- | --- | --- | --- | --- | --- | 1 | --- |
| | | 0/3 | 0/5 | 0/5 | 0/5 | 0/5 | 0/3 | 1/5 (20%) | 0/5 |
| | | --- | --- | --- | --- | --- | --- | 5.0 | --- |
| | 15°C | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| | | --- | --- | --- | --- | --- | --- | --- | --- |
| | | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| | | --- | --- | --- | --- | --- | --- | --- | --- |
| | 20°C | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 |
| | | --- | --- | --- | --- | --- | --- | --- | --- |
| | | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 (20%) | 1/5 (20%) |
| | | --- | --- | --- | --- | --- | --- | 2.0 | 2.0 |

748 Table 3 continued next page

| | | | | | | | | | |
|----|------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------------------|
| 34 | 10°C | 0/4 | 0/5 | 0/5 | 0/5 | 0/5 | 0/3 | 0/5 | 0/5 |
| | | --- | --- | --- | --- | --- | --- | --- | --- |
| | | 0/4 | 0/5 | 0/5 | 0/5 | 0/5 | 0/3 | 0/5 | 0/5 |
| | | --- | --- | --- | --- | --- | --- | --- | --- |
| | 15°C | 2/5 (40%) | 2/5 (40%) | 0/5 | 1/5 (20%) | 1/5 (20%) | 0/4 | 2/5 (40%) | 3/5 (60%)⁴ |
| | | 35 (20–50) | (4–53) | | 60 | 2 | | 33 (20–47) | 6 (1–16) |
| | | 2/5 (40%) | 2/5 (40%) | 0/5 | 1/5 (20%) | 1/5 (20%) | 0/4 | 2/5 (40%) | 3/5 (60%) |
| | | 4.5 ± 0.7 | 3.0 ± 1.4 | --- | 6.0 | 7.0 | --- | 6.0 ± 1.4 | 3.3 ± 1.5 |
| | 20°C | 2/5 (40%) | 2/5 (40%) | 1/5 (20%) | 1/5 (20%) | 2/5 (40%) | 0/5 | 2/5 (40%) | 2/5 (40%) |
| | | 76 (26–127) | 32 (10–54) | 5 | 8 | 28 (6–50) | --- | 12 (4–20) | 6 (6–7) |
| | | 2/5 (40%) | 2/5 (40%) | 0/5 | 2/5 (40%) | 2/5 (40%) | 0/5 | 2/5 (40%) | 3/5 (60%) |
| | | 4.5 ± 3.5 | 4.0 ± 2.8 | --- | 2.0 ± 0.0 | 6.5 ± 0.7 | --- | 4.5 ± 0.7 | 3.7 ± 1.5 |
| 62 | 10°C | 1/5 (20%) | 1/5 (20%) | 0/3 | 0/3 | 1/5 (20%) | 1/5 (20%) | 1/5 (20%) | 1/5 (20%) |
| | | 192 | 6 | --- | --- | 105 | 3 | 5 | 11 |
| | | 1/5 (20%) | 0/5 | 0/3 | 0/3 | 1/5 (20%) | 0/5 | 1/5 (20%) | 1/5 (20%) |
| | | 8.0 | --- | --- | --- | 4.0 | --- | 3.0 | 3.0 |
| | 15°C | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 (20%)⁴ |
| | | --- | --- | --- | --- | --- | --- | --- | 1 |
| | | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 1/5 | 1/5 (20%) |
| | | --- | --- | --- | --- | --- | --- | 2.0 | 4.0 |
| | 20°C | 2/5 (40%) | 1/5 (20%) | 0/3 | 0/3 | 2/5 (40%) | 0/5 | 3/5 (60%) | 3/5 (60%) |
| | | 15 (2–29) | 1 | --- | --- | 6 (3–9) | --- | 8 (6–12) | 3 (2–3) |
| | | 2/5 (40%) | 2/5 (40%) | 0/3 | 1/3 (33%) | 2/5 (40%) | 0/5 | 3/5 (60%) | 3/5 (60%) |
| | | 5.0 ± 1.4 | 3.0 ± 1.5 | --- | 2.0 | 9.0 ± 0.0 | --- | 10.3 ± 0.6 | 8.7 ± 1.5 |

749 ¹GI tract = gastrointestinal tract

750 ²Includes inter-pancreatic connective tissue

751 ³Parasites and host response in hematopoietic tissue

752 ⁴Degenerated schizont(s) only parasite(s) observed in white skeletal muscle of one fish

Table 4. *Ichthyophonus* prevalence as determined by heart and liver tissue culture in Yukon River Chinook salmon that were exposed to *Ichthyophonus* by feeding minced infected herring tissues at 4% of body weight per day four times over a 6-day period at 7°C in seawater. One group of fish was held at 7°C for 72 days after the first feeding exposure. Subgroups of fish were transferred from the 7°C tank at 7 and 28 days after the first feeding exposure, acclimated over a 1-week period to 17°C water, and held at 17°C until sampling 44 days after the transfer dates. Equivalent control groups were fed minced specific-pathogen-free herring and sampled at the same time points as the treatment groups; no *Ichthyophonus* was cultured from any control fish (data not shown). NS = no sample.

| Treatment Group | Days post-exposure | | | |
|---------------------|--|--------------------|------------|------------|
| | Number of fish positive for <i>Ichthyophonus</i> /total cultured (%) | | | |
| | Day 7 | Day 28 | Day 51 | Day 72 |
| 7°C | 4/21 (19%) | 7/20 (35%) | 8/17 (47%) | 8/19 (42%) |
| 17°C early transfer | NS (transfer date) | NS | 6/17 (35%) | --- |
| 17°C late transfer | --- | NS (transfer date) | --- | 1/16 (6%) |

764 Table 5. Prevalence and mean parasite densities of *Ichthyophonus* observed in histological sections of tissues from Yukon River
765 Chinook salmon in the seawater temperature experiment. The *Ichthyophonus* exposure and holding conditions for the experiment are
766 described in Table 4. The sample day was the number of days after the date of the first feeding exposure. Slides were stained with
767 periodic acid-Schiff (PAS) to facilitate detection of parasites. No parasites were detected in any control fish (data not shown).
768

| Sample day | Treatment group | Number of positive fish/total examined (%) | | | | | |
|------------|-----------------------------|--|-----------|-----------------------------|-----------|---------------------|-----------------------|
| | | Mean number of parasites in 10 microscope fields at 200x magnification (range) | | | | | |
| | | Heart | Liver | Kidney hematopoietic tissue | Spleen | Red skeletal muscle | White skeletal muscle |
| 51 | Cold (7°C) | 3/8 (38%) | 1/8 (13%) | 2/8 (25%) | 1/8 (13%) | 3/7 (43%) | 2/7 (29%) |
| | | 46 (2–171) | 1 | 1 (<1–2) | 2 | 8 (<1–24) | 7 (<1–13) |
| | Early (7d) transfer to 17°C | 4/7 (57%) | 1/7 (14%) | 2/8 (25%) | 1/6 (17%) | 5/8 (63%) | 3/8 (38%) |
| | | 6 (3–12) | < 1 | 5 (1–9) | 1 | 2 (≤1–3) | 1 (<1–1) |
| 72 | Cold (7°C) | 6/7 (86%) | 2/8 (25%) | 4/8 (50%) | 0/8 | 7/8 (88%) | 5/8 (63%) |
| | | 4 (2–7) | 1 (1–2) | 2 (1–2) | --- | 5 (1–23) | 1 (<1–3) |
| | Late (28d) transfer to 17°C | 3/8 (38%) | 2/8 (25%) | 1/8 (13%) | 0/8 | 4/8 (50%) | 3/8 (38%) |
| | | 5 (1–11) | 2 (<1–3) | 1 | --- | 2 (1–3) | < 1 (<1) |

Table 6. Total mortality and *Ichthyophonus*-associated mortality of Yukon River Chinook salmon in mortality monitoring tanks in the cortisol experiment (single replicate tank of 20 fish for each treatment or control group). Fish were exposed to *Ichthyophonus* by feeding minced infected herring tissues at 6% body weight per day over a 6-day period at 15°C in freshwater. Thirteen days after the beginning of the feeding exposure, one subgroup of fish was injected intraperitoneally (IP) with hydrocortisone (50 µg/10 g body weight) emulsified in a vegetable shortening:vegetable oil vehicle, a second subgroups was injected with the vehicle only (50 µL/10 g body weight), and a third subgroup was not injected. Equivalent control groups were fed minced specific-pathogen-free herring.

| <i>Ichthyophonus</i> exposure | Cortisol treatment | Total mortality (%) | <i>Ichthyophonus</i> -associated mortality (%) |
|-------------------------------|--------------------------|-----------------------|--|
| <i>Ichthyophonus</i> | Hydrocortisone + vehicle | 11 (55%) ¹ | 10 (50%) |
| | Vehicle only | 0 | --- |
| | No injection | 1 (5%) | 1 (5%) |
| Control | Hydrocortisone + vehicle | 0 | --- |
| | Vehicle only | 0 | --- |
| | No injection | 0 | --- |

¹Fin and body fungus observed on all mortalities

Table 7. *Ichthyophonus* prevalence as determined by heart and liver tissue culture in Yukon River Chinook salmon sampled at various times after exposure in the cortisol experiment. The *Ichthyophonus* exposure and holding conditions for the experiment are described in Table 6. Equivalent control groups fed minced specific-pathogen-free herring were sampled at the same time points as the treatment groups; no *Ichthyophonus* was cultured from any control fish (data not shown).

| Treatment Group | Days post-exposure | | | |
|--------------------------|--|-------------|-------------|---------------------|
| | Number of fish positive for <i>Ichthyophonus</i> /total cultured (%) | | | |
| | Day 7 | Day 23 | Day 49 | Day 51 ¹ |
| Hydrocortisone + vehicle | --- | 10/15 (67%) | 2/4 (50%) | 6/9 (67%) |
| Vehicle only | --- | 13/15 (87%) | 8/15 (53%) | 7/9 (78%) |
| No injection | 12/15 (80%) | 13/15 (87%) | 11/15 (73%) | 6/9 (67%) |

¹Fish sampled from mortality monitoring tanks at the termination of the experiment. All other fish were taken from sample tanks.

Table 8. Prevalence and mean parasite densities of *Ichthyophonus*, and occurrence and severity of host response to *Ichthyophonus* infections, observed in histological sections of tissues from Yukon River Chinook salmon in the cortisol experiment. The *Ichthyophonus* exposure and holding conditions for the experiment are described in Table 6. Data shown are from samples taken 23 days after the beginning of the *Ichthyophonus* exposure (10 days after cortisol or vehicle injection). Slides examined for parasite detection were stained with periodic acid-Schiff (PAS), and slides examined for host response were stained with hematoxylin and eosin (H&E). Scores of lesion distribution (0-3) and degree (0-3) of inflammation, fibrosis, and necrosis were added together to obtain an overall host response severity measure for each tissue type (maximum severity score = 12). No *Ichthyophonus* or host response associated with hydrocortisone or vehicle injection was detected in any control fish (data not shown).

| Treatment (<i>Ichthyophonus</i> - exposed fish) | Number fish positive for <i>Ichthyophonus</i> /total examined (%) | | | | | | | |
|--|--|--|--|---|--|--|---|---|
| | Mean number of parasites in 10 microscope fields at 200x magnification (range) | | | | | | | |
| | Number of fish positive for host response/total examined (%) | | | | | | | |
| | Mean host response severity score \pm SD | | | | | | | |
| | Heart | Liver | GI tract ¹ | Pancreas ² | Kidney ³ | Spleen | Red skeletal muscle ⁴ | White skeletal muscle |
| Hydrocortisone + vehicle | 6/6 (100%) 17 (2–70) 5/6 (83%) 4.4 \pm 2.6 | 2/6 (33%) 4 (2–5) 2/6 (33%) 6.0 \pm 0.0 | 1/6 (17%) 1 3/6 (50%) 2.0 \pm 0.0 | 3/6 (50%) 5 (1–11) 2/6 (33%) 4.0 \pm 0.0 | 5/6 (83%) 4 (<1–6) 6/6 (100%) 5.6 \pm 2.5 | 4/6 (67%) 2 (1–3) 4/6 (67%) 4.5 \pm 1.0 | 6/6 (100%) 5 (1–12) 6/6 (100%) 5.2 \pm 1.2 | 6/6 (100%) 6 (2–13) 6/6 (100%) 5.2 \pm 1.5 |
| Vehicle only | 4/6 (67%) 5 (2–8) 5/6 (83%) 2.8 \pm 1.1 | 1/6 (17%) 2 3/6 (50%) 2.0 \pm 0.0 | 1/6 (17%) 1 1/6 (17%) 2.0 | 2/6 (33%) 1 (1) 2/6 (33%) 3.0 \pm 1.4 | 3/6 (50%) 3 (1–7) 4/6 (67%) 5.8 \pm 1.5 | 2/6 (33%) 4 (2–6) 2/6 (33%) 4.0 \pm 0.0 | 5/6 (83%) 10 (2–20) 6/6 (100%) 7.8 \pm 3.1 | 5/6 (83%) 9 (<1–26) 6/6 (100%) 5.5 \pm 2.1 |
| No injection | 5/6 (83%) 24 (1–65) 5/6 (83%) 5.6 \pm 2.3 | 1/6 (17%) 1 2/6 (33%) 3.0 \pm 1.4 | 2/6 (33%) 2 (1–3) 2/6 (33%) 3.5 \pm 2.1 | 3/6 (50%) 2 (1–4) 3/6 (50%) 4.0 \pm 0.0 | 4/6 (67%) 3 (<1–7) 4/6 (67%) 6.3 \pm 2.6 | 2/6 (33%) 2 (1–3) 2/6 (33%) 4.0 \pm 0.0 | 5/6 (83%) 10 (2–30) 5/6 (83%) 7.4 \pm 2.3 | 5/6 (83%) 4 (1–10) 5/6 (83%) 6.4 \pm 2.7 |

¹GI tract = gastrointestinal tract

800 Table 8. contd.

801

802 ²Includes inter-pancreatic connective tissue

803 ³Parasites and associated host response in hematopoietic tissue

804 ⁴Some parasites and associated host response observed in dermis of skin external to red skeletal muscle

805

List of Figures

Figure 1. Percentage of degenerated schizonts among total *Ichthyophonus* parasites observed in histological sections of tissues of Yukon River Chinook salmon sampled from the freshwater (A) and seawater (B) temperature experiments. The total number of *Ichthyophonus* parasites detected in all tissues examined from all *Ichthyophonus*-positive fish in a treatment group for a given sample day (days after the beginning of exposure) is shown above each bar. The *Ichthyophonus* exposure and holding conditions for the freshwater and seawater temperature experiments are described in Tables 1 and 4, respectively, and the tissue types examined are shown in Tables 3 and 5, respectively. Sections were stained with periodic acid-Schiff (PAS). No *Ichthyophonus* was detected in any control fish in either experiment (data not shown). NS = no sample; 17°C early = 17°C early transfer group; 17°C late = 17°C late transfer group.

Figure 2. Measured and mean plasma cortisol levels (pg/mL) in treatment groups of Yukon River Chinook salmon that were exposed to *Ichthyophonus* by feeding minced tissues from *Ichthyophonus*-infected herring, and control groups were fed minced SPF herring. Subgroups of fish in the treatment and control groups were injected IP 13 days after the beginning of the exposure with either hydrocortisone (50 µg/10 g body weight) in a vegetable oil:vegetable shortening vehicle, vehicle alone (50 µL/10 g body weight), or were not injected (no inj.). Fish were sampled for cortisol analysis 7, 23, and 49 days after the beginning of the feeding exposure; samples taken on days 23 and 49 were obtained 10 and 36 days, respectively, after cortisol or vehicle injection. Mean plasma

828 cortisol values were calculated from measurements from 10 individual fish per subgroup
829 at each sample time except for the day 49 *Ichthyophonus* cortisol subgroup (n = 4 fish).
830 Note differences in vertical axis scales.

831

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Figure 1.

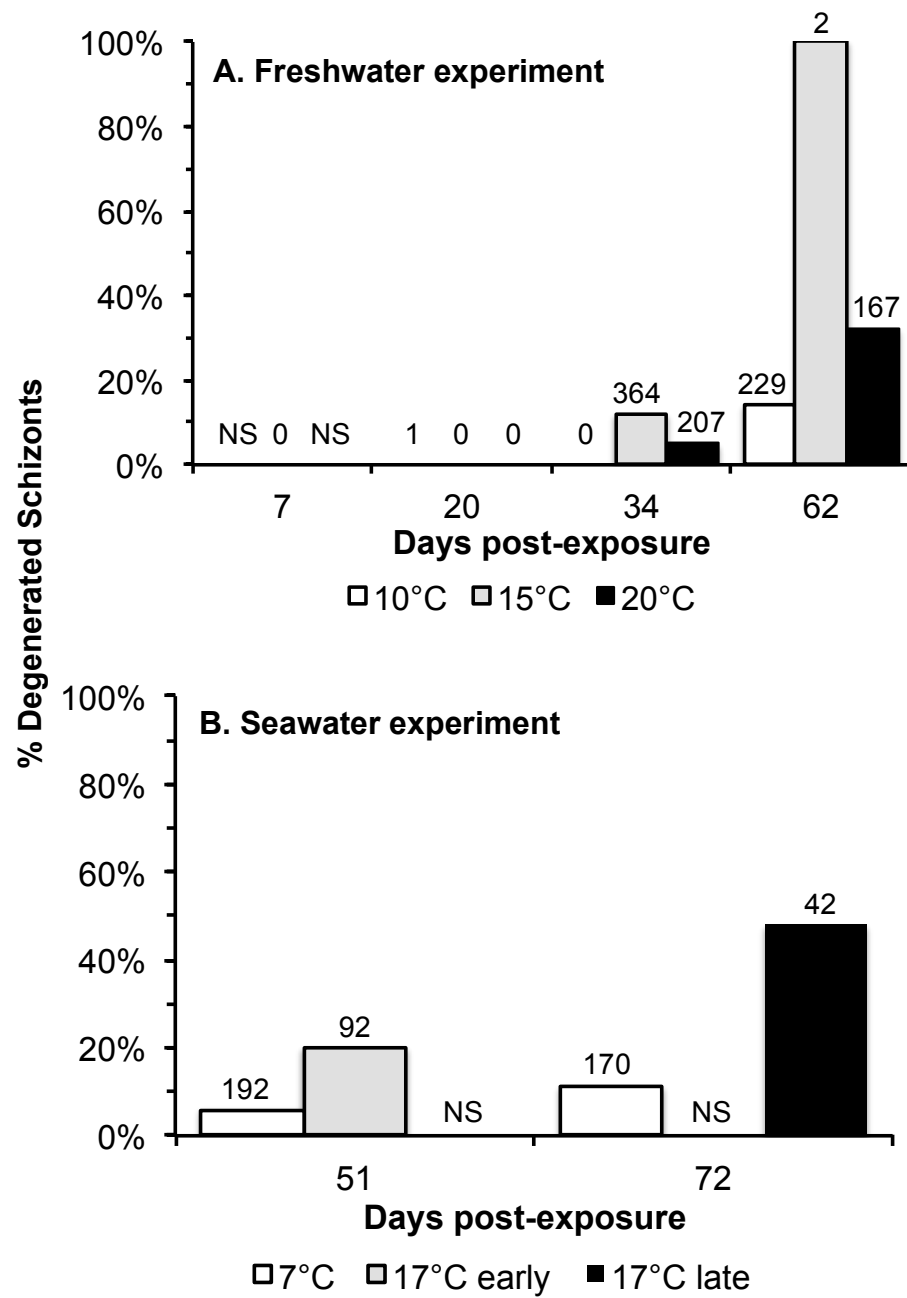


Figure 2.

