Cumulative Effects of Thermal and Fisheries Stressors Reveal Sex-Specific Effects on Infection Development and Early Mortality of Adult Coho Salmon (*Oncorhynchus kisutch*)

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ABSTRACT

Multiple stressors are commonly encountered by wild animals, but their cumulative effects are poorly understood, especially regarding infection development. We conducted a holding study with repeated gill and blood sampling to characterize the effects of cumulative stressors on infection development in adult coho salmon. Treatments included chronic thermal stress (15°C vs. 10°C) and acute gill net entanglement with an air exposure (simulating fisheries bycatch release). The potential loadings of 35 infectious agents and the expression of 17 host immune genes were quantified using high-throughput quantitative polymerase chain reaction, while host physiology was characterized with chemical analysis of blood. Temporal increases in infectious agent richness and loads were concurrent with decreased expression of immune genes in fish sampled in the river. In the laboratory, mortality was minimal in cool water regardless of fishery treatment (<15%). Elevated water temperature under laboratory conditions increased mortality of males and females (8% and 28% mortality, respectively, delayed by >1 wk) and enhanced mortality associated with handling and biopsy (~40% both sexes). Experimental gillnetting at high temperature further enhanced female mortality (73%). Fish held at high temperature demonstrated heavier infectious agent loads, osmoregulatory impairment, suppressed female maturation, and upregulation of inflammatory and extracellular immune genes. At high temperature, heavy *Parvicapsula minibicornis* loads were associated with premature mortality. Females exhibited physiological impairment from both stressors after 1 wk, and infection burdens correlated poorly with immune gene regulation compared with males. Cumulative effects of multiple stressors on female mortality are likely a function of physiological impairment and enhanced infections at high temperature.

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Introduction

Evidence is mounting for a functional role of infectious agents in the ecology of all wild organisms, with influences on food webs (Selakovic et al. 2014; Tucker et al. 2016; Buck and Ripple 2017), migration behavior (Altizer et al. 2011; Johns and Shaw 2016), and other ecological facets that collectively have evolutionary implications (Vander Wal et al. 2014). As a result, interest has grown in how anthropogenic and climate change–associated stressors will alter current host-pathogen relationships in wild ecosystems. The stressors wild animals face throughout their lives are presumed to affect their resilience and resistance to naturally occurring infectious agents, thereby contributing to variation in disease development within and among populations (Johnson et al. 2015). Understanding how a species' ecology and relevant stressors influence infectious disease development and

survival is the cornerstone of disease ecology and crucial to effective natural resource management.

Physiological stress is known to alter immune competence (Glaser and Kiecolt-Glaser 1994), with stressor-specific immune responses (Bowers et al. 2008; Webster Marketon and Glaser 2008) and pathogen-dependent disease outcomes (e.g., viral and bacterial outcomes differ; Hori et al. 2013). Importantly, while concurrent stressors are commonly experienced by wild animals, they are rarely quantified for their cumulative effects, which may be additive, synergistic, or even antagonistic (Folt et al. 1999; Crain et al. 2008). Studies are especially rare that describe impacts of multiple stressors on disease development in the wild, which is inherently affected by dynamic environments and migrations that can span large geographic areas (Altizer et al. 2011; Miller et al. 2014). Given that climate change is expected to alter host-pathogen relationships directly and be compounded by other stressors (Altizer et al. 2013; Burge et al. 2014; Miller et al. 2014; Lohmus and Björklund 2015), evaluations of multiple stressors are increasingly needed to account for the context-specific nature of disease development, which comprises internal (genotype, immune portfolio, host condition) and external (environment) forces to influence survival outcomes (Mitchell et al. 2005).

Pacific salmonids (*Oncorhynchus* spp.) are frequently exposed to multiple stressors (Miller et al. 2014) and therefore are an excellent model for an evaluation of cumulative stressors and infection development given the comprehensive knowledge base describing their physiology (e.g., Farrell et al. 2008; Cooke et al. 2012; Patterson et al. 2016). Furthermore, novel genomic tools recently applied to Pacific salmon in British Columbia have vastly improved our understanding of the infectious agents carried by wild salmon (Miller et al. 2014; Bass et al. 2017; Tucker et al. 2018), the genomic and physiological responses that accompany those infections (Jeffries et al. 2014*a*; Miller et al. 2017; Teffer et al. 2017), and how host genomic profiles can predict migration success (Miller et al. 2011; Drenner et al. 2017). Juvenile Pacific salmon migrate to the marine environment to feed and grow until adults return to fresh water to reproduce (Groot and Margolis 1991). During the spawning migration, fasting adults are faced with increased energetic demands for reproduction and for swimming, while immune competence diminishes and infection burdens increase (Rand and Hinch 1998; Miller et al. 2009; Dolan et al. 2016; Bass et al. 2017). Although death occurs naturally after spawning, mortality before spawning—such as during the river migration period—eliminates any lifetime fitness through the total loss of potential offspring.

Two major stressors currently affecting adult Pacific salmon during river migration are high river temperature and fisheries nonretention (i.e., release after capture or escape from fishing gears), which are increasingly concurrent events. A prime example is the Fraser River in British Columbia, the largest salmon-producing river in Canada, which has shown significant climate-driven warming in recent decades (Patterson et al. 2007). Terminal fishing in the Fraser River (commercial, recreational, and ceremonial) targets certain salmon stocks and species using a variety of gear types, including gill nets, beach seines, hook and line, tangle nets, and others. The likelihood of nontarget species being caught and subsequently released as bycatch is high in this watershed because different Pacific salmon species comigrate through its main stem and tributaries toward spawning grounds. Indeed, all Pacific salmon species experience some degree of bycatch. For example, coho salmon (*Oncorhynchus kisutch*) can be captured by Fraser River fisheries targeting Chinook (*Oncorhynchus tshawytscha*) or sockeye (*Oncorhynchus nerka*), but a conservation mandate demands that they must be released, as is the case for threatened interior British Columbia coho populations (Decker and Irvine 2013; Raby et al. 2014). Stress and injury caused by fisheries capture (Davis 2002; Raby et al. 2015) can make released fish more susceptible to infections (Svendsen and Bøgwald 1997). Gill nets are a commonly used gear type in the Fraser River and, relative to other capture methods (e.g., beach seine, dip net), contribute to low postrelease survival of released catch, as well as enhanced pathogen loads and altered physiology and behavior (Baker and Schindler 2009; Raby et al. 2015; Teffer et al. 2017, 2018). Given that salmon released from fisheries are more likely to die when rivers are warm (Martins et al. 2012*b*) and carry severe infections of multiple pathogens (Teffer et al. 2017), we tested the hypothesis that thermal stress amplifies the impacts of capture on disease development and mortality because empirical evidence for such cumulative effects is absent.

The objectives of our study were to (1) characterize and quantify through repeated gill biopsy the development over time of pathogen communities and host immune responses within individual adult Chilliwack River coho salmon (British Columbia) during their river residency before spawning and (2) describe the individual and cumulative impacts of chronic high temperature following an acute gill net entanglement on the development of pathogenic infections and fish mortality. To ground-truth our laboratory results and provide baseline data on temporal shifts in infectious agent loads in the wild, we conducted concurrent lethal sampling of fish in the river over time. Pairing field and laboratory approaches provides a more comprehensive dynamics assessment of infections, both among surviving fish in the wild and within individual fish in the lab. We used high-throughput quantitative polymerase chain reaction (qPCR) applied to gill biopsies to simultaneously characterize pathogen community composition (prevalence) and structure (relative infection intensity) with host immune responses (gene expression) at weekly intervals, producing a trajectory of host-pathogen relationships. These genomic data were complemented by an array of physiological indices to provide a comprehensive characterization of the effects of multiple infections and stressors on host health before spawning.

Methods

Hatchery Sampling for Pathogen Community Composition and Structure

The protocols for this experiment were approved by the Animal Care Committees of Fisheries and Oceans Canada (Pacific Region), the University of British Columbia (certificate A11-0215), and the University of Victoria (certificate 2012-030). Tissues from wild

adult Chilliwack River coho salmon were obtained in 2012 by terminally sampling fish during their river residence at the Chilliwack River Hatchery, Chilliwack, British Columbia, during the "middle" burst of this population's normal migration (J. Mothus, hatchery manager, personal communication) on October 18 ($n = 9$), November 8 ($n = 10$), and November 26 ($n = 11$; table 1A). Fish were collected by dip net from raceways and euthanized via cerebral concussion before removing tissue samples from major organs, which included gill (5–6 gill filament tips), white muscle proximal to the lateral line just posterior to the dorsal fin, liver, spleen, heart ventricle, head kidney, and brain (every other fish brain alternated with histology; data not shown). Tissue samples (~0.5 mg) were preserved in 1.5 mL of RNA*later* (Ambion, Austin, TX) solution (whole brain 3 mL) at 4°C for 24 h and then at −80°C until analysis.

Infectious agent community composition and structure were evaluated in a pool of seven organ tissues collected from hatchery fish (including gill) and in each gill sample (separately). The qPCR screening panel included 34 assays targeting an array of viruses, bacteria, protozoa, and other microorganisms known to affect Pacific salmon (Miller et al. 2016; fig. S1, available online; see molecular methods below). Host immune gene expression was measured only in gill samples; this approach limits our scope of inference regarding genomic host responses to those occurring locally in the gills, which are likely primarily relevant to gill infections. Because tissues from fish at the hatchery were lethally sampled at discrete time points, the resulting data describe population-level temporal shifts in host responses and infectious agent communities among surviving fish in the river.

Table 1: Sample sizes by date and sex for coho salmon sacrificed at the Chilliwack River Hatchery, Chilliwack, British Columbia, in 2012 (A) and sample sizes by temperature, treatment, and sex for coho salmon transported, treated, and held at the Fisheries and Oceans Canada (DFO) Cultus Lake Salmon Research Lab, Cultus Lake, British Columbia (B)

A. Date	Female	Male
October 18	5	4
November 8	5	5
November 26	8	3

B. Temperature, treatment	Female		Male	
	N	Mortality (%)	N	Mortality (%)
10°C:				
Gill net	28	3.6	12	.0
Biopsy	26	11.5	14	14.3
Control	27	3.7	13	.0
15°C:				
Gill net	30	73.3	10	40.0
Biopsy	27	40.7	12	41.7
Control	29	27.6	12	8.3

Laboratory Study of the Cumulative Effects of Stressors on Pathogen Loads and Host Health

Over a 3-d period (Oct. 17–19), 240 coho salmon (silver with minimal scale loss) were transported in truck-mounted, aerated tanks (10°C; table 1B) from the raceways at the Chilliwack River Hatchery to the Cultus Lake Salmon Research Lab in Cultus Lake, British Columbia (a 30-min journey). Fish were sequentially distributed among 12 8,000-L holding tanks (10°C) at low densities ($n < 25$ tank^{-1}). Sex—which was unknown at the time of collection, as fish had not yet developed sex-specific morphologies—was subsequently determined when fish were sacrificed at the termination of the experiment; 60%–85% of the fish in each tank were female. Tanks were supplied with sand-filtered, UV-treated water from Cultus Lake in a flow-through system. A submerged pump produced a low-velocity current around the tank periphery to encourage fish to orientate during holding (~1 body length s^{-1}).

Twenty-four hours after the final transport, the temperature of half of the tanks was increased over a 2-d period from the normal migration temperature (10°C; low) to 15°C (high). The 15°C treatment is above the temperature range currently experienced by Chilliwack River coho salmon (e.g., ~12°C maximum in Chilliwack River; Barnes and Magnusson 2000) but is within the range currently encountered by wild interior coho stocks migrating through the main stem of the Fraser River in late September (Patterson et al. 2007; Raby et al. 2014) and reflects projected temperature increases given climate-driven changes to Fraser River hydrology (Morrison et al. 2002). Water temperature was controlled by varying the proportion of water from above or below the thermocline in the lake and by adding boiler-heated water, if necessary. Boiler-heated water was incorporated at a minimum relative volume necessary to achieve experimental temperatures and was dispensed into tanks from above the water line to disperse gasses via aeration and prevent supersaturation. Both temperature groups were divided into three treatment groups (each replicated in two tanks): gill net treated with biopsy, biopsied controls (a control for gill net treatment), and nonbiopsied controls (a control for biopsy), which generated genomic, physiological, and mortality data in a longitudinal design over 14 d with biopsy sampling (e.g., Cooke et al. 2005; Teffer et al. 2017) at days 0, 7, and 14.

The gill net treatment was a standardized representation of capture and release from a gill net fishery in the Fraser River watershed and progressed as follows: each fish was removed from its holding tank using a dip net and quickly submerged in a smaller flow-through tank where the opening of the dip net faced a wide frame with a 5.25-inch (13.3-cm) mesh gill net mounted within it. Upon exiting the bag of the dip net, the fish was caught in the gill net. If the fish escaped, the timer was stopped until entanglement had been achieved. After 20 s of sustained underwater entanglement, the fish and gill net were pulled from the water and into a dip net while the fish was detangled from the net in air for a standardized 1-min period (simulating release by fishers). The fish was then submerged in a foam-lined, flow-through sampling trough, where it was sampled

for blood (∼2 mL from the caudal vasculature; 21-gauge needle with lithium heparinized Vacutainer; Becton-Dickson, NJ) and a small gill biopsy (2–3 gill filament tips, ∼0.5 mg tissue, preserved in 1.5 mL RNA*later*), implanted intraperitoneally with a small (12 mm) passive integrated transponder (Biomark, Boise, ID) and examined for injuries. Total time in the trough was ∼2 min, after which the fish was placed into a recovery tank until all individuals in the holding tank had been treated and sampled, and then all fish were returned to holding. Biopsied controls proceeded directly from holding tanks to the sampling trough and followed the same biopsy procedure as gill net–treated fish. After 7 d of holding, gill net–treated fish and biopsied controls were biopsied again, following the protocols described above (no gill net treatment). Nonbiopsied controls were left untouched until morbidity or the termination of the experiment. Any fish that showed signs of morbidity during the experiment (gulping, loss of equilibrium) were euthanized by cerebral concussion and biopsy sampled according to the protocols described above for hatchery-sampled fish. Additionally, blood was taken and various morphological measurements—including length (postorbital hypural ± 1.0 mm), organ weights, and gross pathology (e.g., fungus cover, lesions, macroparasites)—were recorded. After 14 d, surviving fish in all treatment groups (excluding half of the nonbiopsied controls) were euthanized and processed according to sampling protocols outlined for moribund fish. A subset of nonbiopsied controls were excluded from our terminal pathogen analysis for use in a pilot study examining long-term effects of thermal stress on infection development (see Miller et al. 2014).

Molecular Analysis

Host gene expression and pathogen prevalence and intensity were measured in gill biopsies and in a pool of seven terminally sampled tissues using high-throughput qPCR on the BioMark platform (for details regarding validation and methodology, see Miller et al. 2016; assay primer and probe sequences, efficiencies, and host biomarker functions are listed in tables 2, 3). Although gills are exposed to the environment and an infectious agent load therefore reflects contributions from the exterior and interior of the fish, infectious agent loads measured in blood are similar to those in gill for most agents, except those that are typically gill residents (i.e., not likely to be detected in blood; Teffer and Miller, forthcoming). Furthermore, gill infectious loads have been correlated with migration success and survival as well as physiological indexes of wild salmon (Jeffries et al. 2014a; Miller et al. 2014; Bass et al. 2017; Teffer et al. 2017), demonstrating gill as an effective infectious agent screening tissue, especially when paired with host gene expression data.

We used RNA transcripts rather than DNA to measure infectious agent loads not only to include RNA viruses in our screening panel but also to describe relative productivity of infectious agents via collective transcription levels. These data are limited in that they do not enable us to compare load levels across agents, only within agent species, and they do not represent counts of individuals but rather the transcription activity and RNA maintenance of all individuals within each tissue

sample. This RNA screening approach has been shown previously to correlate well with physiological impairment and mortality of adult salmon in the lab and field (Bass et al. 2017; Teffer et al. 2017), and high loads of viral genetic material have demonstrated association with histopathologically and genomically characterized disease development (Di Cicco et al. 2017, 2018; Miller et al. 2017). However, our intent is not to assign specific disease states to qPCR load levels because the relevance of genetic material to disease development will inevitably vary across agent and host species, which can be misleading. We are instead investigating how infectious agent loads as determined by qPCR relate to host responses at local (gill gene expression), systemic (blood chemistry), and whole organism (mortality) levels. These relationships then provide a better understanding of how wild adult salmon survive and respond to various migratory conditions.

For molecular analyses of hatchery-sampled fish, both gill, and a multitissue pool were screened for infectious agents at discrete lethal sampling dates. For laboratory holding analyses, gill was nonlethally sampled on three occasions to measure infection development over time (i.e., within individuals) and a multitissue pool was also evaluated from held fish at death. As this experiment was performed before full validation of the platform, some aspects of the analysis differed slightly from those described in Miller et al. (2016), including several primer and probe sequences (see table 2) and minor aspects of the protocol described below (for more specific protocol descriptions, see Teffer et al. 2017).

Briefly, tissue samples were thawed and trimmed to normalize weights (∼0.5 mg), transferred into sterile microtubes with stainless steel beads, and homogenized in trireagent (600 μL; Ambion, Austin, TX) and 1-Bromo-3-chloropropane (75 μL) using a MM301 mixer mill (Retsch, Newtown, PA). After centrifugation (1,500 g, 6.5 min), the aqueous phase (100 μL) was transferred into 96-well plates for RNA purification using MagMAX-96 for Microarrays Kits (Ambion), following manufacturer protocols for the spin method, with a DNase treatment after the first wash. To create a tissue pool, organs were homogenized separately, and aliquots of aqueous phase from each tissue in equal volumes were pooled for each individual before RNA purification (water was substituted where brain was absent). RNA quantity (A_{260}) and quality ($A_{260/280}$) were assessed using spectrophotometry, and samples were normalized to 1 μg RNA per well before cDNA synthesis using SuperScript VILO (Invitrogen, Carlsbad, CA) cDNA Synthesis Kits under PCR cycling conditions of 25°C for 10 min, 42°C for 60 min, and 85°C for 5 min. As recommended by the manufacturer (Biomark), a preamplification step consisting of a multiplex PCR including all primers to be used in the qPCR was completed before qPCR. A mixture of 200 nM primer mix, TaqMan PreAmp Master Mix (Applied Biosystems, Foster City, CA), and cDNA was cycled at 95°C for 10 min followed by 15 cycles of 95°C for 10 s and 60°C for 4 min, followed by treatment with ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, CA; 37°C for 15 min, 80°C for 15 min) and fivefold dilution (TEKnova suspension buffer, Hollister, CA). Negative controls were included from homogenization forward,

Table 2: Infectious agent assay information

Pathogen	Abbreviation	Type	Primer F	Primer R	Probe	E	Reference
Aeromonas hydrophila	ae_hyd	Bacterium	ACCGCTGCTC-ATTACTCTG-ATG	CCAACCCAGA-CGGGAAGAA	TGATGGTGAG-CTGGTTG	.97	Lee et al. 2006
Aeromonas salmonicida	ae_sal	Bacterium	TAAAGCACTG-TCTGTTACC	GCTACTTCACC-CTGATTGG	ACATCAGCAG-GCTTCAGA-GTCACTG	1.19	Keeling et al. 2013 (modified)
Renibacterium salmoninarum	re_sal	Bacterium	CAACAGGGTG-GTTATTCTG-CTTTC	CTATAAGAGCC-ACCAGCT-GCAA	CTCCAGCGCCG-CAGGAGGAC	1.00	Powell et al. 2005
Piscichlamydia salmonis	pch_sal	Bacterium	TCACCCCCAG-GCTGCTT	GAATTCCATTT-CCCCCTCTTG	CAAAACTGCTA-GACTAGAGT	.81	Nylund et al. 2008
Piscirickettsia salmonis	pisck_sal	Bacterium	TCTGGGAAGT-GTGGCGAT-AGA	TCCCGACCTAC-TCTTGTTT-CATC	TGATAGCCCCG-TACACGAAA-CGGCATA	.94	Corbeil et al. 2003
Rickettsia-like organism	rlo	Bacterium	GGCTCAACCC-AAGAACTG-CTT	GTGCAACAGC-GTCAGTGACT	CCCAGATAACC-GCCTTCGCC-TCCG	.96	Lloyd et al. 2011
Gill chlamydia	sch	Bacterium	GGGTAGCCCG-ATATCTTCA-AAGT	CCCATGAGCC-GCTCTCTCT	TCCTTCGGGAC-CTTAC	.95	Duesund et al. 2010
Vibrio anguillarum	vi_ang	Bacterium	CCGTCATGCT-ATCTAGAG-ATGTAT-TTGA	CCATACGCAGC-CAAAAATCA	TCATTTCGACG-AGCGTCTTG-TTCAGC	1.07	MGL
Atlantic salmon paramyxovirus	aspv	Virus	CCCATATTAG-CAAATGAG-CTCTATCTT	CGTTAAGGAA-CTCATCATT-GAGCTT	AGCCCTTTTGT-TCTGC	.97	Nylund et al. 2008
Infectious hematopoi-etic necrosis virus	ihnv	Virus	AGAGCCAAG-GCACTGT-GCG	TTCTTTGCGGC-TTGGTTGA	TGAGACTGAG-CGGGACA	.88	Purcell et al. 2013
Piscine orthoreovirus	prv	Virus	TGCTAACACT-CCAGGAGT-CATTG	TGAATCCGCTG-CAGATGAGTA	CGCCGGTAGC-TCT	1.04	Wiik-Nielson et al. 2012
Pacific salmon parvovirus	pspv	Virus	CCCTCAGGCT-CCGATTTT-TAT	CGAAGACAAC-ATGGAGGTG-ACA	CAATTGGAGG-CAACTGTA	.97	MGL

Table 2 (*Continued*)

Pathogen	Abbreviation	Type	Primer F	Primer R	Probe	E	Reference
Piscine totivirus (CMS)	cms	Virus	TTCCAAACAA-TTCGAGAA-GCG	ACCTGCCATTT-TCCCCT CTT	CCGGGTAAAG-TATTTGC GTC	.98	Løvoll et al. 2010
Salmon alphavirus 1, 2, and 3	sav	Virus	CCGGCCCTGA-ACCAGTT	GTAGCCAAGT-GGGAGAAA-GCT	TCGAAGTGGT-GGCCAG	.88	Andersen et al. 2007
Viral encephalopathy and retinopathy virus	ver	Virus	TTCCAGCGAT-ACGCTGT TGA	CACCGCCCGTG-TTTGC	AAATTCAGCCA-ATGTGC CCC	1.01	Korsnes et al. 2005
Viral hemorrhagic septicemia virus	vhsv	Virus	ATGAGGCAGG-TGTCGGAGG	TGTAGTAGGA-CTCTCCCAG-CATCC	TACGCCATCAT-GATGAGT	.84	Garver et al. 2011
Ceratonova shasta	ce_sha	Myxozoan	CCAGCTTGAG-ATTAGCTC-GGTAA	CCCCGGAACCC-GAAAG	CGAGCCAAGTT-GGTCTCTCC-GTGAAAAC	1.07	Hallett and Bartholomew 2006
Kudoa thyrsites	ku_thy	Myxozoan	TGGCGGCCAA-ATCTAGGTT	GACCGCACAC-AAGAAGTTA-ATCC	TATCGCGGAGA-GCCGC	.93	Funk et al. 2007
Myxobolus arcticus	my_arc	Myxozoan	TGGTAGATAC-TGAATATC-CGGGTTT	AACTGCGCGG-TCAAAGTTG	CGTTGATTGTG-AGGTTGG	.85	MGL
Myxobolus cerebralis	my_cer	Myxozoan	GCCATTGAAT-TTGACTTTG-GATTA	ACCATTCATGT-AAGCCCGA-ACT	TCGAAGCCTTG-ACCATCTTT-TGGCC	1.02	Kelley et al. 2004
Parvicapsula pseudobranchicola	pa_pse	Myxozoan	CAGCTCCAGT-AGTGTATT TCA	TTGAGCACTCT-GCTTTATT-CAA	CGTATTGCTGT-CTTTGACAT-GCAGT	.93	Jørgensen et al. 2011
Tetracapsuloides bryosalmonae	te_bry	Myxozoan	GCGAGATTTG-TTTGCATTTA-AAAAG	GCACATGCAGT-GTCCAA TCG	CAAAATTGTGG-AACCGTCCG-ACTACGA	1.00	Bettge et al. 2009
Parvicapsula minibicornis	pa_min	Myxozoan	AATAGTTGTT-TGTCGTGC-ACTCTGT	CCGATAGGCTA-TCCAGTACC-TAGTAAG	TGTCCACCTAG-TAAGGC	.87	Hallett and Bartholomew 2009
Parvicapsula kabatai	pa_kab	Myxozoan	GTCGGATGAT-AAGTGCAT-CTGATT	ACACCACAACT-CTGCCTT CCA	TGCGACCATCT-GCACGGTAC-TGC	.94*	MGL

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Facilispora margolisi	fa_mar	Microsporidian	AGGAAGGAG-CACGCAAG-AAC	CGGGTGCAGC-CCAGTAC	TCAGTGATGCC-CTCAGA	.92	MGL
Nucleospora salmonis	nu_sal	Microsporidian	GCCGCAGATC-ATTACTAA-AAACCT	CGATCGCCGC-ATCTAAACA	CCCGCGCATC-CAGAAAT-ACGC	1.06	Foltz et al. 2009
Paranucleospora theridion	pa_ther	Microsporidian	CGGACAGGG-AGCATGGT-ATAG	GGTCCAGGTTG-GGTCTT GAG	TTGGCGAAGA-ATGAAA	.91	Nylund et al. 2010
Heterosigma akashiwo	he_aka	Flagellate	GCTTCTGCAT-TGCCGTT TCT	CCCAACTTCCT-TCGGTTAGT-CA	CTTCTTAGAGG-GACTTTC	NA*	MGL
Spironucleus salmonicida	sp_sal	Flagellate	AACCGGTTAT-TCGTGGGA-AAG	TTAACTGCAGC-AACACAATA-GAATA CTC	TGCCAGCAGC-CGCGGTA-ATTC	.97*	MGL
Nanophyetus salmincola	na_sal	Fluke	CGATCTGCAT-TTGGTTCTG-TAACA	CCAACGCCAC-AATGATAGC-TATAC	TGAGGCGTGTT-TTATG	1.04	MGL
Gyrodactylus salaris	gy_sal	Fluke	CGATCGTCAC-TCGGAATCG	GGTGGCGCAC-CTATTCT ACA	TCTTATTAACC-AGTTCTGC	.98	Collins et al. 2010
Ichthyophonus hoferi	ic_hof	Protozoan	AGTGGGTGCT-CTTAATTGA-GTGTCT	GCCTGCTTTGA-ACACTCTAA-TTTTC	CTGAGTTCGGG-ACTTT	.88*	MGL
Sphaerothecum destruens	sp_des	Protozoan	GCCGCGAGGT-GTTTGC	CTCGACGCACA-CTCAATTAA-GC	CGAGGGTATC-CTTCCTCTC-GAAATTGGC	.84*	MGL
Ichthyophthirius multifiliis	ic_mul	Ciliate	AAATGGGCAT-ACGTTTGC-AAA	AACCTGCCTGA-AACACTCTA-ATTTTT	ACTCGGCCTTC-ACTGGTTCG-ACTTGG	1.05	MGL

Note. Assays referenced as "MGL" were developed at the Molecular Genetics Lab, Fisheries and Oceans Canada (DFO) Pacific Biological Station, Nanaimo, British Columbia. Assay efficiencies (*E*) are reported from the validation by Miller et al. (2016); an asterisk indicates they used alternate primers and probes but were similarly calculated using artificial constructs and standard curves. No artificial constructs were developed for the he_aka assay.

Table 3: Immune gene biomarker assay information

Assay	Gene information	Assay type	EST/accession no.	Primer and probe sequences	E	Reference
b2m	Beta 2-microglobulin	Immune	AF180490	F - TTTACAGCGCGGTGGAGTC R - TGCCAGGGTTACGGCTGTAC P - AAAGAATCTCCCCCAAGGTGCAGG	.87	Haugland et al. 2005
C3	Complement factor	Immune	U61753, AF271080	F - ATTGGCCTGTCCAAAACACA R - AGCTTCAGATCAAGGAAGAAGTTC P - TGGAATCTGTGTGTCTGAACCCC	1.01	Raida and Buchmann 2009
CD4	Cell receptor	Immune	AY973028	F - CATTAGCCTGGGTGGTCAAT R - CCCTTTCTTTGACAGGGAGA P - CAGAAGAGAGAGCTGGATGTCTCCG	.94	Raida and Buchmann 2008
CD83	Cell receptor	Immune	AY263794	F - GATGCACCCCTTGAGAAGAA R - GAACCCTGTCTCGACCAGTT P - AATGTTGATTTACACTCTGGGGCCA	.95	Raida et al. 2011
Hep	Hepcidin	Immune	AF281354.1	F - GAGGAGGTTGGAAGCATTGA R - TGACGCTTGAACCTGAAATG P - AGTCCAGTTGGGGAACATCAACAG	.96	Raida and Buchmann 2009
IFNa	Interferon-α	Immune	AY216595	F - CGTCATCTGCAAAGATTGGA R - GGGCGTAGCTTCTGAAATGA P – TGCAGCACAGATGTACTGATCATCCA	.94	Ingerslev et al. 2009
IgMs	Immunoglobulin	Immune	S63348, AB044939	F - CTTGGCTTGTTGACGATGAG R - GGCTAGTGGTGTTGAATTGG P - TGGAGAGAACGAGCAGTTCAGCA	1.00	Raida et al. 2011
IL-11	Cytokine	Immune	AJ535687	F - GCAATCTCTTGCCTCCACTC R - TTGTCACGTGCTCCAGTTTC P - TCGCGGAGTGTGAAAGGCAGA	.97	Raida and Buchmann 2008
IL-15	Cytokine	Immune	AJ555868.1	F - TTGGATTTTGCCCTAACTGC R - CTGCGCTCCAATAAACGAAT P - CGAACAAGCGTGATGACAGGTTTTT	1.06	Raida et al. 2011
IL-1R	Cytokine	Immune	AJ295296	F - ATCATCCTGTCAGCCCAGAG R - TCTGGTGCAGTGGTAACTGG P - TGCATCCCCTCTACACCCCAAA	.94	Raida et al. 2011

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				E	
IRF1	Interferon regulatory factor 1	CB511515	F - CAAACCGCAAGAGTTCCTCATT R - AGTTTGGTTGTGTTTTTGCATGTAG P - CTGGCGCAGCAGATA	.85	MGL
MHCI	Major histocompatibility complex I		F - GCGACAGGTTTCTACCCCAGT R - TGTCAGGTGGGAGCTTTTCTG P - TGGTGTCCTGGCAGAAAGACGG	1.00	Ingerslev et al. 2009
MHCIIb	Major histocompatibility complex IIβ	AF115533	F - TGCCATGCTGATGTGCAG R - GTCCCTCAGCCAGGTCACT P - CGCCTATGACTTCTACCCCAAAACAAAT	.95	Raida and Buchmann 2008
MMP13	Matrix metalloproteinase	213514499	F - GCCAGCGGAGCAGGAA R - AGTCACCTGGAGGCCAAAGA P - TCAGCGAGATGCAAAG	.86	Tadiso et al. 2011
Mx	Antiviral protein		F - AGATGATGCTGCACCTCAAGTC R - CTGCAGCTGGGAAGCAAAC P - ATTCCCATGGTGATCCGCTACCTGG	.94	Eder et al. 2009
RIG-I	Retinoic acid inducible gene I	NM_001163699	F - ACAGCTGTTACACAGACGACATCA R - TTTAGGGTGAGGTTCTGTCCGA P - TCGTGTTGGACCCCACTCTGTTCTCTC	.96	Larsen et al. 2012
TF	Transferin	D89083	F - TTCACTGCTGGAAAATGTGG R - GCTGCACTGAACTGCATCAT P - TGGTCCCTGTCATGGTGGAGCA	.94	Raida and Buchmann 2009
78d16.1		CA056739	F - GTCAAGACTGGAGGCTCAGAG R - GATCAAGCCCCAGAAGTGTTTG P - AAGGTGATTCCCTCGCCGTCCGA	.85	MGL
COIL-P84-2		CA053789	F - GCTCATTTGAGGAGGAAGGAGGATG R - CTGGCGATGCTGTTCCTGAG P - TTATCAAGCAGCAAGCC	.81	MGL

Note. Biomarkers referenced as "MGL" were developed at the Molecular Genetics Lab, Fisheries and Oceans Canada (DFO) Pacific Biological Station, Nanaimo, British Columbia. E = assay efficiency.

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and serial dilutions of host and pathogen preamplified cDNA were included in the qPCR (artificial constructs available for most infectious agent assays, subsequently validated by Miller et al. 2016; table 2). Sample (TaqMan Gene Expression Master Mix, GE Sample Loading Reagent, and preamplified cDNA) and assay (primer pair [9 μM], probe [2 μM], Assay Loading Reagent) mixes were loaded into reaction chambers using the integrated fluidics circuit controller, and qPCR cycling was completed following the GE 96 × 96 Standard v1.pcl (TaqMan) protocol. Passive reference dye (ROX) confirmed the presence of sample in each well, one probe (with VIC dye) was included to detect artificial constructs in samples (i.e., lab contamination), and a second probe (with FAM dye) quantified target amplicons. Pathogen assays with detections of the VIC-labeled probe or those not detected in duplicate (with FAM-labeled probe) were considered failed. Quantification cycle (Cq) is reported as the average of assay duplicates; host genes are reported as relative expression derived following Pfaffl (2001) using two reference genes, which showed stable expression throughout the experiment. Pathogens are reported as relative load, calculated by subtracting the Cq from 40 (maximum Cq).

Blood Properties

Hematocrit and leucocrit were measured in the field following blood sampling via centrifugation (2 min at 10,000 g; LW Scientific ZIPocrit; Lawrenceville, GA) in heparinized microcapillary tubes (Drummond Scientific, Broomall, PA). Plasma was extracted and flash frozen in liquid nitrogen after centrifugation at 7,000 g for 7 min (Clay Adams Compact II centrifuge; Becton-Dickson, Sparks, MD). Ions (chloride, sodium, potassium), metabolites (lactate, glucose), and osmolality were measured according to Farrell et al. (2001), while hormones (cortisol, estradiol, testosterone) were measured using enzyme-linked immunosorbent assay (ELISA) kits (Neogen, Lansing, MI) following manufacturer protocols.

Statistical Analysis

All statistical tests were completed using R statistical software (R Core Team 2015). Results from qPCR analysis of gill and pooled tissues from fish sacrificed at the Chilliwack River Hatchery were used to identify temporal and sex-specific differences in infectious agent richness (number of unique pathogens), relative loads of individual pathogens (40 − Cq), and relative infection burden (RIB; Bass et al. 2019), which is a composite score incorporating aspects of pathogen richness and relative loads via the equation

$$\text{RIB} = \sum_{i \in m}^{m} \frac{L_i}{L \max_i}, \tag{1}$$

where for a given fish, the relative load of the ith infectious agent (L_i) is divided by the maximum load within the population for the ith infectious agent ($L\max_i$) and then summed across all agents (m) infecting the given fish. Analysis of variance (ANOVA) was used to test for significant differences in each disease metric

between sampling dates and sexes (with an interaction term) and post hoc Tukey tests for individual differences between groups. To examine how overall host immune gene expression of hatchery fish was influenced by date and sex (i.e., expression of 17 immune genes as multivariate response), we used permutational multivariate analysis of variance (PERMANOVA) with sampling date, sex, and an interaction term as predictors of host immunity; principal components analysis (PCA) and environmental fitting (envfit function, vegan library) were then used to graphically represent the data and describe the relationships of gene biomarkers to predictor variables (Oksanen et al. 2016).

For laboratory data (held fish), impacts of temperature, treatment, and sex on survival were evaluated using survival analysis and Cox proportional hazards (survival package; Therneau 2014), including and excluding nonbiopsied controls to account for possible impacts of nonlethal biopsy on survival. Following an initial model that included both sexes to test for overall sex, treatment, and temperature effects, males and females were evaluated separately to determine hazard ratios associated with stressors within sexes. Exponents of coefficients (e^β) represent the relative daily hazard of mortality.

Effects of thermal and fisheries stressors, sex, and time on disease metrics measured in gills were assessed using linear mixed effects (LME) models (nlme package; Zuur et al. 2009; Pinheiro et al. 2017). Three discrete gill sampling events (T0: Oct. 22–23, T1: Oct. 29–30, T2: Nov. 5–6) were included in the analysis, as well as samples taken from moribund fish that died in the interim between sampling events (T0.5, T1.5); for all time-dependent analyses (LME, survival analysis), data from samples taken from fish that died before the termination of the study were grouped into T1 if the fish died before T1 or into T2 if the fish died after T1. Inclusion of data from moribund fish improved the comprehensiveness of our analysis beyond just survivors. Missing values in the response variable (e.g., negative detections) were excluded from each analysis. Differences between treatments, temperatures, and sexes over time were tested for each disease metric in a repeated measures framework. Equation (2) describes the full mixed effects model:

$$\begin{aligned} D_i = {} & \beta_1 H + \beta_2 G + \beta_3 S_i + \beta_4 T \\ & + \beta_5(H \times G) + \beta_6(H \times S_i) + \beta_7(H \times T) + \beta_8(G \times S_i) \\ & + \beta_9(G \times T) + \beta_{10}(S_i \times T) + (\alpha + T|\mu_i) \\ & + \varepsilon_i \mu_i {\sim} N(0, \sigma^2), \varepsilon_i {\sim} N(0, \sigma^2), \end{aligned} \tag{2}$$

where D_i is the disease metric (i.e., richness, RIB, or relative load of an individual agent) for individual i; β_1 is the coefficient of the fixed effect of high temperature (H); β_2 is the coefficient of the fixed effect of gill net treatment (G); β_3 is the coefficient of the fixed effect of the sex of individual i (S_i); β_4 is the coefficient of the fixed effect of time (T); β_5 is the coefficient of the interaction between high temperature and gill net treatment; β_6 is the coefficient of the interaction between high temperature and sex; β_7 is the coefficient of the interaction between high temperature and time; β_8 is the coefficient of the interaction between gill net treatment and sex; β_9 is the coefficient of the interaction between

gill net treatment and time; and β_{10} is the coefficient of the interaction between sex and time. A random intercept (α) and slope ($T|\mu_i$) describe individual variability (μ_i) in the response variable and its relationship to time; μ_i and the residual error (ε_i) are normally distributed with a mean of zero and variance σ^2. Random intercepts and/or slopes were included to control for individual variation and autocorrelation across sampling events within individuals. Optimal random effects (intercept, slope, or both) were identified using top-down model comparison and Akaike's information criterion (AIC), varying the random component of each full fixed effects model (Zuur et al. 2009). Fixed effects (treatment, temperature, sex, and time) and all two-way interactions were then evaluated, beginning with the most complex model and then removing noninfluential fixed effects (low t-value and high P value). Stepwise reduction was repeated sequentially until the reduced model contained only significant factors and interactions (and nonsignificant main effects of components of significant interactions) with reduced AIC. AIC values, β estimates for fixed effects with standard errors, and P values are reported from the most parsimonious models. Where significant interactions prevented further model reduction to test the significance of main effects, P values were derived from t-values. Intraclass correlation coefficients (ICC) are also reported, which describe the proportion of total variance due to individual differences.

Effects of stressors and sex on infection intensities in pooled tissues of survivors at T2 were evaluated using ANOVA on richness, RIB, and relative loads for highly prevalent agents (>70%) and logistic regression on presence-absence data for agents with lower prevalence (<70%). Temperature, treatment, and sex with all possible interactions were included as predictor variables in ANOVAs, while only main effects were examined for logistic regressions due to low power (few positive samples). The influence of pathogens on survival was quantified using survival analysis with time-dependent covariates. Because few fish died at 10°C, this analysis was conducted only using high-temperature fish (biopsied controls and gill net treated at 15°C). Sex and treatment were included as constant covariates, while time-dependent covariates included RIB and load information from highly prevalent agents. Because nonlethal biopsy was conducted weekly, survival times were grouped by week (i.e., survival to T1 or T2). Relative loads were transformed into a binary response: 0 for negative or low loads (less than the average load of held population) or 1 for high loads (greater than the average load).

The gene expression and blood analyses included only samples taken from live fish at the first and second sampling times (T0, T1); this approach targeted changes occurring in the first week of holding that prefaced and may have influenced delayed mortality (occurring mostly after T1). Changes in host immune responses were evaluated using PERMANOVA to evaluate the relative influences of temperature, treatment (and their interaction), fate (survival 14 d), and RIB at T0 and T1. PCA and environmental fitting (envfit) were used to visually represent gene biomarker relationships to predictor variables (temperature, treatment, fate, RIB), while generalized linear models (GLMs) related predictor variables to the first 2–3 PC axes (cumulative variance explained

>50%). Tests were run for males and females separately to account for sex-specific differences, thereby characterizing treatment effects within each sex. To assess immediate and delayed physiological responses to stressors, ANOVA was used to test for significant differences in blood properties due to thermal and fishery stressors (with an interaction) at T0 and at T1. Analyses were performed for males and females separately to account for sex-specific differences in blood chemistry, and each time point was evaluated independently. Cortisol, lactate, testosterone, and glucose were log transformed to meet normality assumptions.

Results

Pathogen Community Trajectories and Host Responses of Coho Salmon Sampled in the River

Of the 34 infectious agents evaluated in the pooled tissues from 30 coho salmon sampled at the Chilliwack River Hatchery, 11 infectious agents were positively detected. *Ceratonova shasta* and *Parvicapsula minibicornis* were found in all fish, while *Ichthyophthirius multifiliis* was found in nearly all fish ($n = 27$ fish). *Kudoa thyrsites* ($n = 22$) and *Rickettsia*-like organism (RLO; 15) had at least 50% prevalence, while the remaining seven infectious agents ranged between 3% and 27% prevalence: *Paranucleospora theridion* ($n = 8$), *Tetracapsuloides bryosalmonae* ($n = 5$), piscine orthoreovirus (PRV; $n = 2$), *Aeromonas salmonicida* ($n = 1$), *Ichthyophonus hoferi* ($n = 1$), and *Sphaerothecum destruens* ($n = 1$). Total positive detections for each agent as a function of total positive detections of all agents on each sampling date suggested a relatively static community composition over time in pooled tissues (fig. 1). Richness per fish ranged between 3 and 6 agents, and RIB ranged between 1.69 and 5.80 across sampling events, but neither changed significantly over time or between sexes (pooled tissues; $P > 0.05$). The relative loads of two freshwater myxozoans (*P. minibicornis* and *C. shasta*) increased over time in pooled tissues ($P < 0.001$ and $P = 0.006$, respectively) due to significant increases only in females (Tukey tests for Nov. 8 vs. Nov. 26: *C. shasta*, $P = 0.030$; *P. minibicornis*, $P = 0.001$; Tukey tests for Oct. 18 vs. Nov. 26: *C. shasta*, $P = 0.056$; *P. minibicornis*, $P = 0.002$). Females also carried higher loads of another myxozoan (*K. thyrsites*; $P = 0.001$) and at a higher prevalence than males (F = 57%, M = 17%).

In gill biopsies from hatchery fish, the only significant temporal difference detected in infection burdens was an increase in *P. minibicornis* loads ($P = 0.019$), primarily between November 8 and November 26, but with no sex-specific effect ($P = 0.473$). In these same fish, PERMANOVA identified distinct immune profiles in gill depending on collection date ($r^2 = 0.161$, $P = 0.003$) and sex-specific differences ($r^2 = 0.118$, $P = 0.020$) that demonstrated high variability among females relative to males, especially at later sampling dates. RIB and relative loads of highly prevalent agents (*C. shasta* and *P. minibicornis*) in gill explained little variation in immune gene expression patterns in gill ($r^2 < 0.060$, $P > 0.05$). Immune genes were generally grouped by their functions in the PCA (PC1: 22% variance explained; PC2: 11%), with biomarkers of adaptive immunity
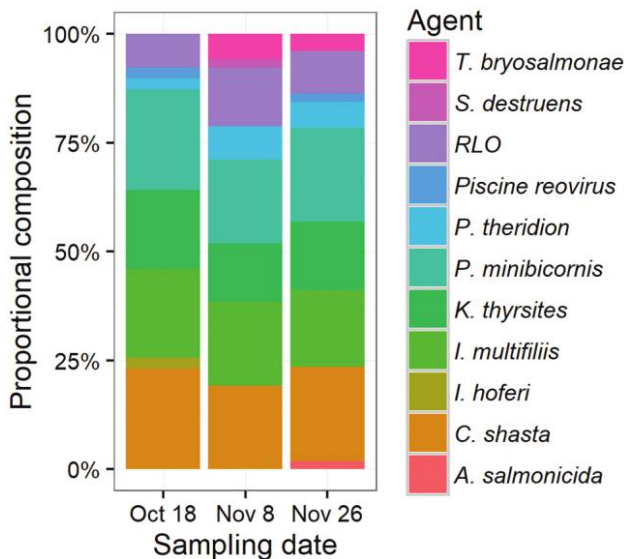
Figure 1. Proportional prevalence (total positive detections for each agent divided by the total positive detections of all agents detected on each sampling date) measured in a pool of seven tissues from coho salmon sampled on October 18 ($n = 9$), November 8 ($n = 10$), and November 26 ($n = 11$) of 2012 at the Chilliwack River Hatchery. Agents were measured using quantitative polymerase chain reaction in a multitissue pool of gill, muscle, liver, spleen, head kidney, heart ventricle, and brain (every other individual).

(CD83, CD4, MHCI, MHCIIb) and cytokines (IL1R, IL15, IL11) associated with the first and second samplings (Oct. 18 and Nov. 8), while biomarkers of antiviral activity (Mx) and iron metabolism (Hep) clustered with the third sampling (Nov. 26). Most immunity biomarkers and several other antiviral indicators (IRF1, IFNa) were negatively associated with the last sampling

date, suggesting an overall downregulation of these genes with time.

Cumulative Effects of Multiple Stressors on Survival, Infections, and Host Health

Fish Survival. At 10°C, 85%–100% of both sexes survived 14 d in all treatment groups (table 1B; fig. 2), with no significant effect of either biopsy or fishery simulation. In contrast, survival at 15°C was differentially affected by treatment and sex. While nonbiopsied control male survival remained high (92%), gill net treatment and biopsy significantly similarly reduced male survival to ~60%. We interpret this similarity as a common effect of handling and biopsy without a fishery simulation effect. For females, however, survival of nonbiopsied controls was significantly reduced at 15°C (72%), further reduced by biopsy (59%), and reduced again by gill net treatment (27%). Thus, temperature alone and biopsy and handling affected survival of females, and gill net treatment was an additive effect. Overall, mortality was delayed by more than 1 wk after study start, with most fish (~98%) surviving to the second sampling occasion (T1).

The significance of treatment and sex effects on survival were assessed in a two-stage survival analysis (likelihood ratio test $P < 0.050$; fig. 2). The first set of models evaluated survival relative to nonbiopsied controls, which indicated significant effects of high temperature ($P < 0.001$), gill net treatment ($P = 0.001$), and sex-specific differences that showed a greater overall daily hazard (of mortality) for females relative to males ($e^{\beta} = 2.0$, $P = 0.040$). Among females, high temperature increased the daily mortality risk to 15.6 times that at low temperature ($P < 0.001$). Gill net–treated females experienced 3.7 times the daily hazard of nonbiopsied controls ($P = 0.005$), but biopsied and nonbiopsied controls did not significantly differ ($e^{\beta} = 1.5$, $P = 0.396$). Mortality was minimal among males, which reduced our power to
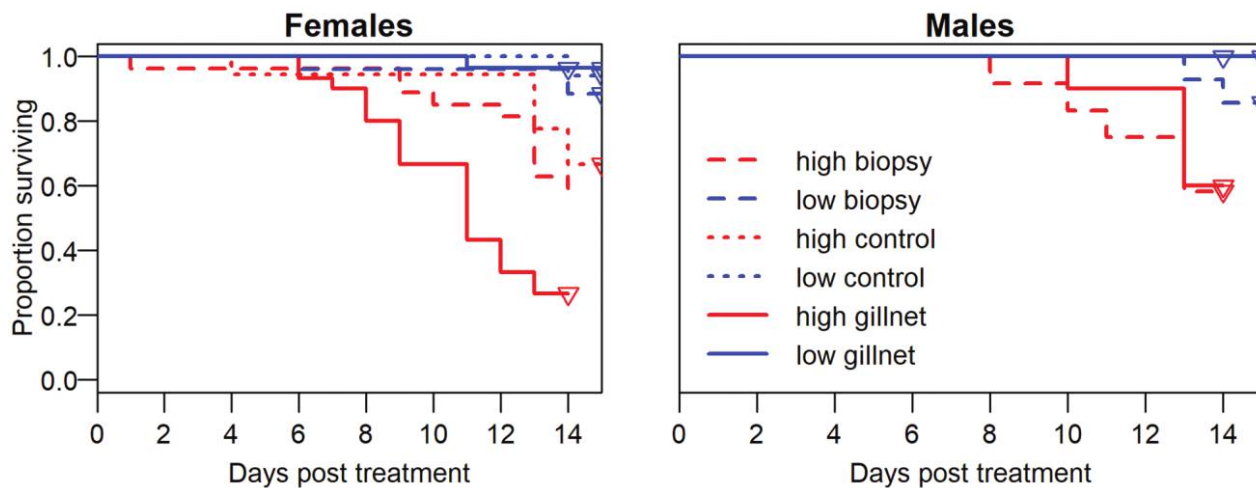

Figure 2. Kaplan-Meier curves showing the survival of female and male Chilliwack River coho salmon (*Oncorhynchus kisutch*). Solid lines represent fish exposed to a standardized gill net treatment (20 s entanglement in water plus 1 min air exposure) and a biopsy; dashed lines represent controls that were biopsied; and dotted lines represent controls that were not biopsied. Colors depict holding temperatures corresponding to cool (10°C = blue) or warm (15°C = red) thermal experiences during migration.
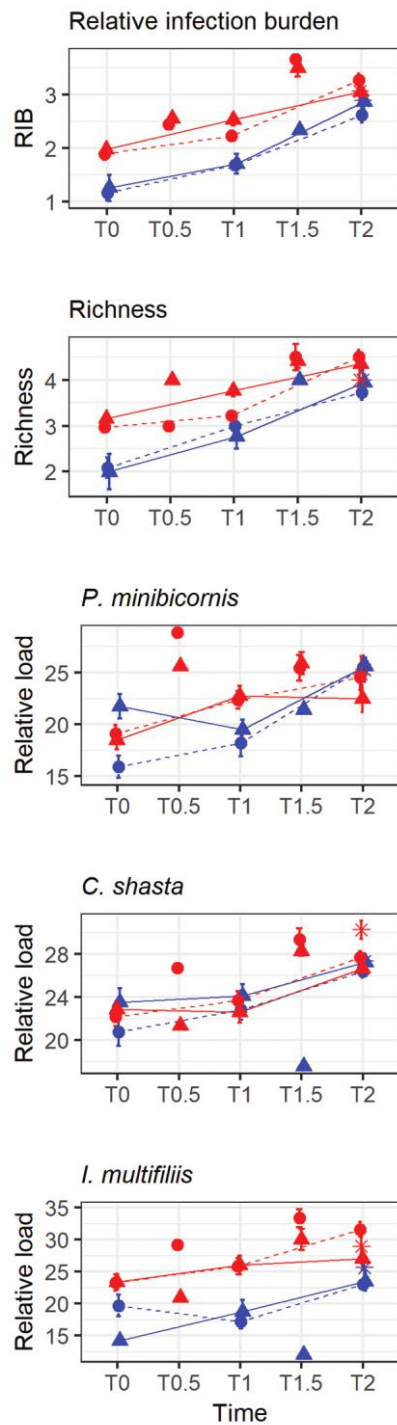
Figure 3. Relative infection burden (RIB), infectious agent richness, and loads of three prevalent infectious agents (*Parvicapsula minibicornis*, *Ceratonova shasta*, and *Ichthyophthirius multifiliis*) measured in gill biopsies from Chilliwack River coho salmon (*Oncorhynchus kisutch*) using quantitative polymerase chain reaction. Nonlethal gill biopsies were taken at the start of the study (T0) and after 7 d (T1); all surviving fish were sacrificed and sampled after 14 d (T2), and fish that died prematurely were sampled at morbidity in the interim between live-sampling events (T0.5, T1.5). Color represents holding temperature (blue = 10°C, red = 15°C), while symbols and line types indicate treatment groups (triangles and solid line = gill net treatment; circles and

characterize differences using survival analysis, and a marginal violation of the proportional hazards assumption ($P = 0.050$) was evident, wherein a significant negative effect of high temperature ($e^{\beta} = 6.9, P = 0.013$) likely decreased with time among males.

In the second set of models, which excluded nonbiopsied controls and included a temperature-treatment interaction term, temperature significantly affected survival ($P = 0.008$) and enhanced the effect of gill net treatment (interaction: $P = 0.028$), but no significant overall sex effect was detected ($P = 0.109$). However, females consistently demonstrated more severe responses to stressors than males. High temperature significantly reduced female survival ($e^{\beta} = 3.9, P = 0.008$), and thermal enhancement of the impact of gill net treatment almost reached statistical significance (interaction: $e^{\beta} = 10.0, P = 0.058$). Excluding the temperature-treatment interaction from the model, females at high temperature experienced 13.1 times the daily hazard of females at low temperature ($P < 0.001$), and gill netting increased the daily hazard to 2.3 times that of biopsied controls ($P = 0.014$). Differences in hazard ratios of females depending on the inclusion of nonbiopsied controls suggests an additive effect of handling (biopsy) on female survival in the presence of additional stressors. No interaction term was included in the male survival model due to minimal male mortality, and only temperature significantly reduced male survival ($e^{\beta} = 6.9, P = 0.013$).

Pathogen Dynamics during Holding Relative to Host Fate. Results from LME analysis of relative loads and composite infection metrics in repeated gill biopsies showed impacts of temperature, treatment, and sex, but the strength and nature of these relationships varied depending on the metric and time (fig. 3; model coefficients in table 4). The delay in mortality (by >1 wk) meant that most fish (98%) survived to be biopsied on the second sampling occasion. Sex-specific differences were identified only in *I. multifiliis*, with higher loads overall in females. All metrics increased with time, and most were enhanced at high temperature (excluding *C. shasta*). Furthermore, *P. minibicornis* and richness reached maximum levels sooner at high temperature (i.e., significant negative temperature-time interactions, suggesting greater temperature-driven differences at T0 and T1 than at T2), which means that changes in the pathogen dynamics preceded fish mortality. Survival analysis with time-dependent covariates identified significant effects of sex ($e^{\beta} = 2.59, P = 0.016$) and *P. minibicornis* loads ($e^{\beta} = 2.48, P = 0.021$; model concordance = 0.73, likelihood ratio test $P = 0.013$) on survival at high temperature, with greater mortality risk among females and individuals with higher *P. minibicornis* loads in gill.

In pooled tissues of survivors at T2 (study termination), 11 agents were positively detected (excludes *Myxobolus arcticus* due to intermittent brain inclusion in qPCR analysis that can bias detection probability; Mahony et al. 2015), with a wide

dashed line = biopsied controls; star = nonbiopsied controls, T2 only). Mean ± SEM.

Table 4: Parameters ($\beta \pm$ SEM) from linear mixed effects models describing changes in infection metrics over time including loads, richness (total unique agents), and relative infection burden (RIB) in gill

Infection metric	ΔAIC	ICC	Sex	Time	H	Time $\times$ H
Parvicapsula						
minibicornis	9.49	.35		$\beta = 4.56 \pm .61^{***}$	$\beta = 5.01 \pm 1.77^{**}$	$\beta = -1.59 \pm .74^{*}$
Ceratonova shasta	5.55	.14		$\beta = 2.37 \pm .31^{***}$		
Ichthyophthirius						
multifiliis	7.69	.14	$\beta = 2.54 \pm .92^{**}$	$\beta = 3.18 \pm .50^{***}$	$\beta = 6.44 \pm .96^{***}$	
Richness	5.72	.20		$\beta = .90 \pm .08^{***}$	$\beta = 1.23 \pm .23^{***}$	$\beta = -.25 \pm .11^{*}$
RIB	9.30	.18		$\beta = .71 \pm .04^{***}$	$\beta = .66 \pm .08^{***}$	

Note. Only significant parameters for time, high temperature (H), and interactions between terms are shown with change in Akaike's information criterion (ΔAIC) and intraclass correlation coefficients (ICC).

$^{*}P < 0.05$.

$^{**}P < 0.01$.

$^{***}P < 0.001$.

range in prevalence (2%–100%; fig. S1; P values of factor effects in table 5). Richness, RIB, and loads of *C. shasta*, *I. multifiliis*, RLO, and *K. thyrsites* were increased in the pooled tissues of survivors after 2 wk of chronic thermal stress ($P \leq 0.016$). Females carried higher loads of *P. minibicornis*, *I. multifiliis*, RLO, and *K. thyrsites* in pooled tissues, as well as higher richness and RIB ($P \leq 0.001$). Sex-specific treatment effects were apparent in *I. multifiliis* and RLO loads, with a positive effect of biopsy sampling in males only (i.e., sex-treatment interactions). Logistic regression identified a positive influence of high temperature on the prevalence of *T. bryosalmonae*, while biopsy sampling and gillnetting increased the prevalence of *A. salmonicida*, and biopsy decreased the prevalence of *P. theridion*. Low prevalence agents

with too few detections to assess statistically included PRV (2%), *Nucleospora salmonis* (2%), *S. destruens* (2%), and *I. hoferi* (3%).

Host Immune Responses over Time. High temperature was the primary factor influencing gill immune gene expression in both sexes (fig. 4; model coefficients in table 6). Among females at T0, PERMANOVA identified temperature, RIB, and fate (survival 14 d) as significant explanatory variables describing immune gene expression patterns ($P < 0.05$), though coefficients of variation were low ($r^2 < 0.10$). The GLM identified PC1 as negatively associated with high temperature and RIB in females at T0. Most immune biomarkers loaded negatively on PC1, suggesting an overall upregulation of these genes in females with high RIB

Table 5: Prevalence and loads (mean $\pm$ SEM) of positively detected infectious agents in a multitissue pool from 132 Chilliwack River coho salmon after laboratory holding and experimentation

Infectious agent	Total detections	Prevalence (%)	Mean load ($\pm$SEM)	S	H	G	$S \times G$
Ceratonova shasta	132	100	27.86 ± 2.85		***		
Parvicapsula minibicornis	132	100	22.80 ± 2.95	***			
Ichthyophthirius multifiliis	128	97	22.59 ± 5.85	**	***	*	**
Rickettsia-like organism	104	79	19.24 ± 4.69	***	***		**
Kudoa thyrsites	93	70	18.00 ± 3.89	***	***		
Tetracapsuloides bryosalmonae	43	33	18.04 ± 3.22		***		
Paranucleospora theridion	35	27	12.99 ± 4.45			*	
Aeromonas salmonicida	30	23	14.11 ± 4.96			*	*
Ichthyophonus hoferi	4	3	16.88 ± 8.65				
Sphaerothecum destruens	3	2	17.49 ± 4.22				
Nucleospora salmonis	2	2	25.03 ± 3.55				
Piscine orthoreovirus	2	2	16.98 ± 5.91				

Note. Multitissue pools included gill, liver, spleen, heart, head kidney, white muscle, and brain (alternated every other fish). Significance values ($P < 0.05$) pertaining to the influence of sex (S), high temperature (H), gill net treatment (G), and interaction terms on infectious loads (agents with $\geq$70% prevalence) or presence-absence (agents with $\geq$20% prevalence) were derived using analysis of variance and generalized linear models, respectively, on surviving fish after 14 d.

$^{*}P < 0.05$.

$^{**}P < 0.01$.

$^{***}P < 0.001$.

and/or exposed to increasing temperature for 48 h (fig. 4). At T1 (1 wk later), the influence of thermal stress on immune gene regulation in females increased ($r^2 = 0.18$), and a weak association with fate was still apparent ($r^2 = 0.03$), but with no significant association with RIB. High temperature, early mortality, and the expression of MMP13, IL11, and Hep all negatively associated with PC1, while most immune biomarkers loaded positively, suggesting primarily iron sequestration, inflammation, and tissue repair responses in dying and thermally stressed females. High temperature was also associated with the loadings of immunoglobulin (IgMs) and cellular immune components (CD4, MHCI,

b2m) on PC3, possibly signifying thermal stress responses that are independent of fate.

Among males at T0, temperature was the only significant factor associated with overall gene expression, but the GLM identified negative relationships of both high temperature and gill net treatment with PC1 (table 6). Like females, most immune biomarkers negatively loaded on PC1, indicating enhanced immune gene expression in males exposed to chronic thermal stress and immediately after gill net entanglement. At T1, the influence of temperature on immune gene expression in males increased ($r^2 = 0.28$), and a weak influence of RIB
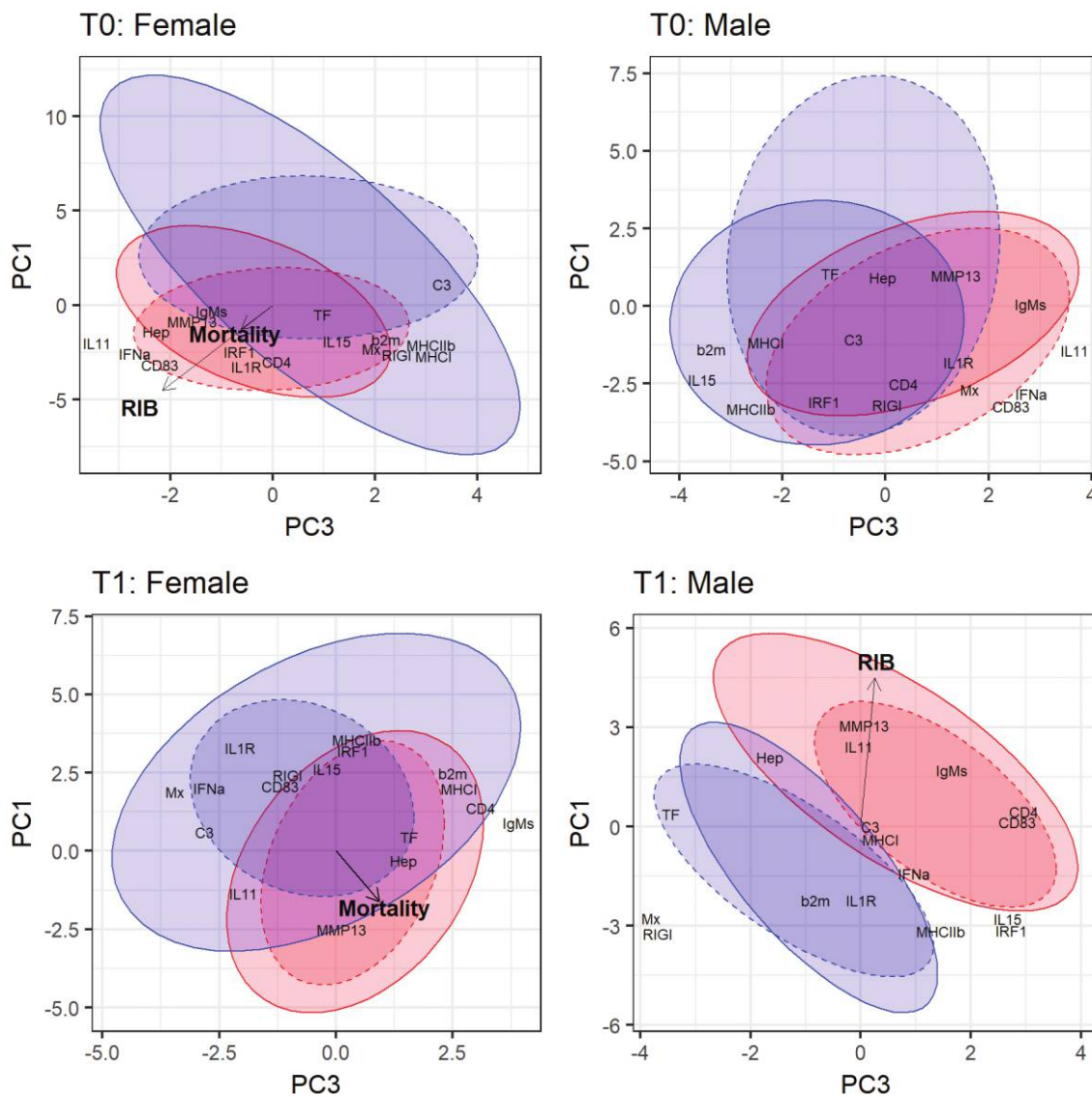

Figure 4. Principal component analysis (PCA) of the relative expression of 17 immune-related genes measured in the gill of Chilliwack River coho salmon (*Oncorhynchus kisutch*). Females and males are plotted separately and were sampled at the start of the study (T0) and after 7 d (T1). Ellipses depict 95% confidence intervals for each temperature and treatment group (blue = 10°C, red = 15°C; solid line = gill net treated, dashed line = biopsied control). Vectors represent directionality and strength of significant correlations ($P < 0.05$) between relative infection burden (RIB) and fate (mortality) with gene expression profiles; temperature significantly influenced gene expression in all analyses.

Table 6: Results from permutational multivariate analysis of variance (PERMANOVA) and generalized linear models (GLM) describing the relationships of stressors, survival, and relative infection burden (RIB) with immune gene regulation in male and female coho salmon

	Study start (T0)		After 7 d (T1)			
	PERMANOVA	PC1	PERMANOVA	PC1	PC2	PC3
Female:		(28%)		(27%)	(18%)	(11%)
H	$r^2 = .08$**	$\beta = -2.68 \pm .78$**	$r^2 = .18$***	$\beta = -1.64 \pm .68$*		$\beta = 1.51 \pm .47$**
RIB	$r^2 = .04$*	$\beta = -.91 \pm .38$*				
Fate	$r^2 = .03$*		$r^2 = .03$*	$\beta = -1.18 \pm .50$*		
Male:		(27%)		(23%)	(18%)	(14%)
H	$r^2 = .12$***	$\beta = -2.13 \pm .88$*	$r^2 = .28$***			$\beta = 3.23 \pm .48$***
G		$\beta = -2.01 \pm .91$*				
RIB			$r^2 = .05$*	$\beta = .97 \pm .41$*	$\beta = -1.26 \pm .47$*	$\beta = -.81 \pm .29$**
H × G						$\text{\ss} = -1.61 \pm .67$*

Note. Gill biopsies were taken at the start of the study (T0) and after 1 wk (T1). GLMs used high temperature (H) and gill net treatment (G) with an interaction ($H \times G$), RIB, and fate (survival 14 d) as predictors of principal component (PC) analysis axes describing host immune gene expression. Percentages are variance explained by each PC; gene loadings in the PCA are shown in fig. 5. Only significant parameters and coefficients ($\beta \pm$ SEM) are shown.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

was also apparent ($r^2 = 0.05$). RIB correlated with PC1, PC2, and PC3, associating high RIB with iron regulation (Hep, TF), humoral immunity (IgMs, C3), and tissue repair (MMP13), while cellular immune components (e.g., receptors) were generally characteristic of low RIB. Temperature was positively associated with gene expression loadings for cell receptors (CD4, CD83, MHCIIb), interferon response regulators (IRF1, IFNa), and IgMs on PC3 but negatively associated with gene expression loadings for antiviral (Mx, RIG1) and iron (TF, Hep) metabolism. Variation along PC3 also supported a temperature-treatment interaction in males ($\beta = -1.61 \pm 0.67$, $P = 0.021$), suggesting antagonistic treatment effects (i.e., the thermal response signal was reduced and more variable for males that were gillnetted).

Initial and Sustained Stress Responses over Time. Thermal and fishery stressors caused an immediate stress response detectable in the blood of both sexes, but females demonstrated more severe physiological disturbances and minimal capacity to resolve the stress after 1 wk (fig. 5; coefficients in table 7). Again, changes in the blood physiology preceded fish mortality. At T0, evidence of thermal stress in females at 15°C (after 48 h of increasing temperature from 10°C) included elevated levels for cortisol, hematocrit, lactate, leucocrit, chloride, sodium, potassium, and osmolality, as well as depressed estradiol and testosterone compared with females at 10°C. Immediate responses to gill net treatment were temperature dependent in females: gillnetting at 10°C increased hematocrit, lactate, and potassium, while gillnetting at 15°C increased hematocrit and sodium relative to biopsied controls and muted thermally driven decreases in estradiol. After 1 wk, females maintained a significant stress response at high temperature and showed signs of osmoregulatory impairment, with elevated cortisol, glucose, lactate, and potas-

sium, as well as depressed estradiol, testosterone, osmolality, chloride, and sodium compared with fish at 10°C. Delayed effects of gill net treatment in females were apparent after 1 wk, which were mostly independent of temperature and included elevated cortisol, glucose, and lactate and depressed leucocrit, estradiol, and testosterone (especially at 15°C) relative to biopsied controls.

Males at T0 had relatively mild responses to thermal stress that included increased lactate and potassium and decreased testosterone at 15°C. Gill net treatment immediately increased hematocrit and lactate in males, regardless of temperature. Males showed an enhanced thermal stress response after 1 wk at 15°C, with elevated cortisol, glucose, lactate, and potassium and depressed testosterone, osmolality, and sodium relative to fish at 10°C. The only significant response to gill net treatment detected in males at T1 was elevated lactate and only at 15°C.

Discussion

This study experimentally explored how cumulative thermal and fishery stressors influence the survival of adult coho salmon by evaluating the development of natural coinfections concurrently with host immune gene regulation and physiology. Overall, survival was high at low temperature, suggesting that both sexes were resilient to capture stress at a normal migration temperature of 10°C. By elevating the temperature by just 5°C, however, we confirmed earlier laboratory findings where warming similarly reduced the survival of sockeye and pink salmon (*Oncorhynchus gorbuscha*; Jeffries et al. 2012, 2014*b*) and exacerbated the impacts of gill net entanglement, especially among females (Teffer et al. 2017). Stressor-induced mortality was typically delayed by more than a week, which is also consistent with previous studies of
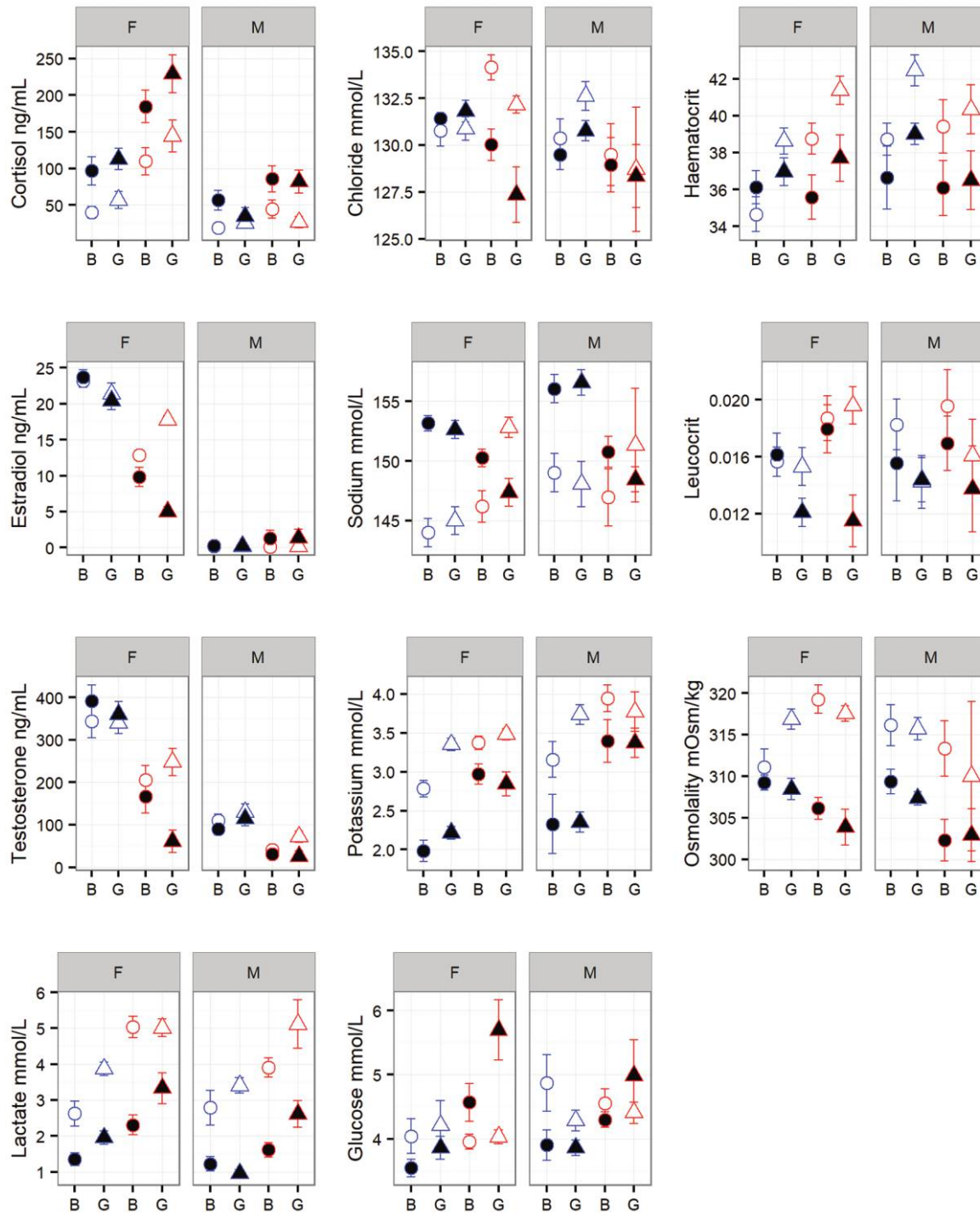
Figure 5. Hormones (cortisol, estradiol, testosterone), ions (chloride, sodium, potassium), metabolites (glucose, lactate), relative blood cell volumes (hematocrit, leucocrit), and osmolality measured in the blood of Chilliwack River coho salmon (*Oncorhynchus kisutch*). Females (F) and males (M) were sampled at the start of the study (T0; white symbols) and again after 7 d (T1; black symbols). Colors indicate holding temperature (blue = 10 °C, red = 15 °C), and symbols indicate treatment (triangles = gill net treated [G]; circles = biopsied control [B]). Mean ± SEM.

Pacific salmon in fresh water (Donaldson et al. 2012; Jeffries et al. 2014*b*; Patterson et al. 2017; Teffer et al. 2017). Sex-specific differences in pathogen loads, immune profiles in gills, and blood characteristics indicated poor recovery of females from thermal and capture stress compared with males. Females also exhibited lower defenses against multiple infections, with suppressed maturation indexes and circulating blood leukocytes that preceded any appreciable mortality.

Disease-associated mechanisms of mortality at high temperature were indicated by a higher prevalence and loading of most infectious agents in multiple tissues and evidence of osmoregulatory impairment and chronic stress. Infection development was enhanced by thermal stress, while host physiology was impaired by both thermal and fishery stressors. Although mortality patterns would predict further increases in pathogen loads among gillnetted females at high temperature, no further increase was detected. Infection intensity was therefore not the sole contributor to mortality but more likely due to reduced host infection thresholds under chronic stress. Combined impacts of severe physiological impairment, immune modulation, and enhanced infection intensities likely contributed to the increased mortality of gill net–treated females at high temperature. Our results demonstrate relevant roles of pathogen dynamics and host response patterns of coho salmon during freshwater residence that were predictive of early mortality, influenced by multiple stressors, and sex specific.

Sex-Specific Differences in Infection Patterns and Host Responses

Sex-specific differences observed in the present study demonstrate alternate responses of females to stressors and infections compared with males. Female salmon have lower cardiac and metabolic capacity relative to males (Clark et al. 2009; Sandblom et al. 2009), which are potential mechanisms contributing to reduced survival under adverse migratory conditions (e.g., high temperature, hydraulic challenges; Roscoe et al. 2011; Martins et al. 2012a), as well as reduced disease resistance during spawning migration. Furthermore, female sex hormones were initially depressed at high temperature (after 2 d of increasing temperature) and continued to drop, especially after gillnetting. This phenomenon, which has been demonstrated in wild sockeye salmon following gill net entanglement (Baker et al. 2013; Teffer et al. 2017) and during chronic high temperature exposure (Mathes et al. 2010), may indicate suppressed or inhibited maturation of females. Though captivity can inhibit maturation (Patterson et al. 2004), decreases were not uniform across treatment groups. Cumulative stressors therefore would result in prespawn mortality of females if maturation, ovulation, and spawning were not achieved before natural senescence or infectious disease development.

The physiological shifts imposed by chronic thermal stress in females may have overwhelmed or masked their immediate responses to gill net treatment, suggesting that coping strategies for acute stress are deficient or delayed in females at high temperature. Indeed, female responses to gill net treatment were stronger after 1 wk and just before the onset of mortality. Our results indicated that high temperature generally enhanced components of adaptive immunity (cell surface receptors, antibodies) and reduced antiviral activity (Mx) in gill regardless of survival, whereas high temperature effects in fish that would die also included biomarkers of iron sequestration and tissue repair. Increased infection intensities and altered immunity at high temperature combined with an inability to resolve

physiological stress may serve as possible mechanisms for the additive effects of multiple stressors on female mortality. Indeed, sex-specific differences in glucose metabolism previously identified in sockeye salmon (Teffer et al. 2017) were also found in the present study, with higher plasma glucose levels after 1 wk in females at high temperature, suggesting enhanced energy mobilization relative to males. These findings support a limited capacity of female Pacific salmon to cope with multiple stressors during spawning migration and demonstrate links between energy needs, infection intensities, maturation indices, and mortality. Whether physiological impairment results from or contributes to infection development and immune modulation is unknown and warrants further examination.

Pathogen Communities and Immune Responses at the Hatchery

Multiple infections were a common finding in gill and pooled tissues of hatchery-sampled fish, a common trait of wild animals with inherent complexity for characterizing pathogen virulence (Sofonea et al. 2015; Kinnula et al. 2017). The pathogen community (i.e., RIB) among fish in the river was rather static over time, despite increased loading by several agents (myxozoans). Hatchery data were produced from independent lethal sampling events that do not encapsulate infection development within individuals but rather population-level shifts among surviving fish. Given that laboratory-held fish increased gill RIB with time, it is unlikely that gill RIB was maintained at low levels in hatchery fish by targeted immune responses. Rather, removal of ripe fish by hatchery staff for spawning may artificially reduce RIB among surviving fish because RIB is temporally confounded with maturity. Sex-specific differences in pathogen dynamics were primarily restricted to myxozoan parasites in pooled tissues, with consistently higher loads carried by females (*Kudoa thyrsites*, a marine and freshwater nonpathogenic myxozoan) or increasing loads with time at the population level in females only (*Ceratonova shasta* and *Parvicapsula minibicornis*, freshwater myxozoan pathogens). These findings are similar to those described for Chinook salmon in southwestern British Columbia (Bass et al. 2017).

The absence of significant temporal or sex-specific differences in gill infection burdens among hatchery fish supports an incomplete or alternate microbe community profile in gill relative to other tissues, as described previously in sockeye and Chinook salmon (Teffer et al. 2017; Teffer and Miller, forthcoming). Differences in infection metrics derived from gill versus other tissues should be incorporated into data interpretations and assessed according to the ecology of each agent (e.g., tissues of primary infection). More importantly, our approach for hatchery sampling (discrete lethal sampling events) neglected temporal changes in gill loads within individuals, which were evident in laboratory-held fish; these results emphasize the need to account for how hatchery practices (mature fish removal), predation, and other means of mortality influence population-level infectious agent communities in wild animals. Loss of high-load individuals from wild animal populations would reduce disease likelihood

among survivors and may be an evolutionary driver for the strenuous migrations undertaken by salmon (e.g., migratory culling; Altizer et al. 2011). The stressors encountered during migration would also influence the effectiveness of this life-history strategy with respect to disease development.

Cumulative Effects of Thermal and Capture Stressors on Pathogen Dynamics

By taking repeated biopsies from the same laboratory-held fish over time, we detected temporal shifts in the gill pathogen community structure, characterized by increases in richness and loads that differed depending on temperature and, in the case of *Ichthyophthirius multifiliis*, sex of the host. Significant increases in RIB were apparent at high temperature and with time, but impacts of acute gill net capture stress on RIB were not observed, which may require more than 2 wk or a more severe stressor to manifest (Teffer et al. 2017). The degree to which a stressor affects host immune responses can differ depending on the pathogen (Hori et al. 2013), suggesting that infectious agent community composition as well as the types of stressors encountered are relevant to disease-induced mortality of wild animals (Sofonea et al. 2015). Notably, both *I. multifiliis* and *C. shasta* levels in gills at high temperature remained slightly lower for gillnetted fish, which may signify effective host responses targeted toward these parasites following acute stress, despite the immunosuppressive effects of chronic thermal stress (Wendelaar Bonga 1997; Campisi et al. 2002; Dhabhar 2002; Mateus et al. 2017). As these fish were also more likely to die, successfully maintaining lower infection levels may prove deleterious for semelparous fish by causing epithelial damage during the immune response (e.g., inflammation hypertrophy, hyperplasia, lamellar fusion in gill) or facilitating enhancement of other pathogenic infections by redirecting immunity (*P. minibicornis*; Buchmann et al. 2001; Bradford et al. 2010a; Sofonea et al. 2017). Thermal enhancement of *C. shasta* in pooled tissues but not in gill may signify accelerated migration of spores from gills to the gut (via the blood) at high temperature (Bartholomew et al. 1997; Okamura et al. 2015), thereby contributing to pathogenesis, but this hypothesis requires further study. Load increases of several agents in response to stressors differed between gill and pooled tissues, again suggesting that gill biopsies alone may be inadequate to characterize infection development of some agents, such as *C. shasta*, that do not mature in the gill.

Importantly, our analysis identified a positive association of *P. minibicornis* loads with mortality at high temperature, where survival was contingent on maintaining low loads in gill. *Parvicapsula minibicornis* is a myxozoan parasite endemic to the Fraser River that has been previously linked to premature mortality of adult sockeye salmon (Jones et al. 2003; Bradford et al. 2010b). Our findings provide further evidence for a role of *P. minibicornis* in premature mortality of stressed salmon by confirming temperature as a driver of *P. minibicornis* infection intensity in salmon (Wagner et al. 2005) and lending support to the idea that high pathogen loadings during the late stage of freshwater residence lead to mortality of adult coho salmon (Miller

et al. 2014). Despite its association with kidney disease, *P. minibicornis* has been documented in the gill of sockeye salmon (Bradford et al. 2010b), and diseased fish show respiratory stress (Bradford et al. 2010a) with negative impacts on exercise recovery (Wagner et al. 2005). Given that high loads of *P. minibicornis* in gill were associated with high temperature and mortality in the present study but loads in pooled tissues (including kidney) of survivors showed no temperature effects, gill infections of *P. minibicornis* may be more relevant to survival (Bradford et al. 2010b), emphasizing the importance of infection locale within the body. Alternatively, as only survivors were included in pooled tissue analyses, infection thresholds may be reduced for *P. minibicornis* at high temperature (i.e., high-load fish died before study termination).

Shifts in osmoregulatory and stress indexes and reduced aspects of immune gene expression in gill demonstrated the highest level of physiological impairment in gillnetted females held at high temperature. RIB in gill was more strongly associated with the immune profiles of males after 1 wk than that of females, which may signify more targeted responses of males to infections. Because RIB was not strongly associated with female immunity after 1 wk and was not the primary predictor of mortality, it is likely that female mortality was not solely pathogen-load driven but rather a function of both enhanced infections and impaired physiological resilience. Additionally, not all infectious agents contributing to mortality were comprised by our metric, as our evaluation did not comprise the full array of agents impacting wild Pacific salmon populations (Miller et al. 2016) and most notably does not include some newly discovered viruses in British Columbia salmon (K. Miller, unpublished data). Furthermore, our experiment was not designed to identify the nature of interpathogen interactions that may have influenced survival but instead provides crucial insight for future challenge work to include a range of environmental conditions and coinfections.

Immune and Physiological Responses to Pathogens and Multiple Stressors

Our results add to growing evidence for decreased immune activity of Pacific salmonids late in freshwater residence, suggested to be due to senescence processes in advance of mortality rather than immune suppression by pathogens (Dolan et al. 2016). Demonstration of this phenomenon in Chinook salmon in the upper Willamette River Basin (Dolan et al. 2016), Early Stuart sockeye salmon from the Fraser River (Teffer et al. 2017), and Chilliwack River coho in the present study provides evidence for conservation of this trait across river basins, latitudes, and species. Stressor effects on immune gene expression in gill were apparent and primarily attributable to thermal stress, characterized by increased expression of most immune genes after just 1–2 d of rising temperature followed by a decrease in most aspects of immunity after 1 wk (i.e., just before the onset of mortality). Pink and sockeye salmon showed similar increases in immune gene expression that were maintained after 5–7 d of chronic thermal stress (Jeffries et al. 2014b), suggesting species-specific

or season-dependent differences in the timing of immune modulation. Most immune biomarkers were decreased in coho salmon gills after 1 wk at high temperature, likely due to immunosuppressive effects of chronic thermal stress (Barton and Iwama 1991; Dittmar et al. 2014) and included the interferon-induced antiviral protein Mx. However, Mx was positively associated with premature mortality and gillnetting, consistent with previous work on sockeye salmon (Teffer et al. 2017). The only virus positively detected in the current study was PRV and then in only two individuals. The observed expression of Mx (and RIG-1) could be a relic of a cleared infection or a component of a conserved response to acute stress (Zwollo 2012) or possibly senescence, or may be in response to one of the novel viruses recently identified in British Columbia Chinook and sockeye salmon (K. Miller, unpublished data).

Differences in immune gene expression in gill between gillnetted and nongillnetted females at high temperature indicate alternate responses to enhanced infectious loads. After 1 wk, gillnetted fish showed positive association with biomarkers of iron regulation (Hep, TF), antiviral and intracellular responses (Mx, RIG-1), and inflammation (MMP13, IL11) but lower and more variable expression of most other aspects of immunity including extracellular pathogen recognition (e.g., IgMs, MHCIIb). Parasites associated with mortality (e.g., *P. minibicornis*) would contribute to an inflammatory response but not iron metabolism or intracellular responses (Buchmann et al. 2001; Okamura et al. 2015). RLO is an alphaproteobacteria associated with strawberry disease (Metselaar et al. 2010), but RLO is not considered pathogenic in culture settings (Olson et al. 1985). Modulation of iron metabolism and inflammation are characteristic of bacterial infection (Raida and Buchmann 2009), but it is unknown whether RLO can recruit the intracellular immune components observed. RLO was not evaluated in gill, but as it is an endosymbiont of *I. multifiliis* (Sun et al. 2009) with highly correlated loads in Pacific salmon (Bass et al. 2017), the responses and prevalence of these agents were likely similar. Finally, our analysis, though comprehensive, did not include several bacterial assays such as *Flavobacterium psychrophilum* and *Candidatus* Branchiomonas cysticola. Both of these bacteria are highly prevalent among Chilliwack River Hatchery Chinook salmon (Bass et al. 2017) and very likely contributed to the bacterial responses we observed in coho salmon.

The physiological effects of thermal and fishery stressors impacting wild adult salmon have been well studied (Farrell et al. 2008; Cooke et al. 2012; Hinch et al. 2012; Martins et al. 2012*a*; Raby et al. 2015; Patterson et al. 2017), but data showing linkages between physiological variables and disease development are scarce (but see Miller et al. 2014; Bass et al. 2017; Teffer et al. 2017, 2018). High temperature effects on plasma ions were apparent, causing decreases in chloride and sodium, especially among gillnetted fish. These shifts indicate different osmoregulatory responses to individual and combined stressors that can influence longevity (Hruska et al. 2010; Jeffries et al. 2011) and are very likely pathogen driven (Buchmann et al. 2001; Bradford et al. 2010*a*; Bass et al. 2017). A stress response was apparent in fish at high temperature, with cortisol, lactate, glucose, and hematocrit all significantly increased at the start of the study and well before

changes in mortality and pathogen loadings. Gillnetting enhanced muscle activity, with initial elevation and subsequent clearance of metabolites (e.g., lactate) that are consistent with previous studies (Farrell et al. 2001; Raby et al. 2012; Teffer et al. 2017). Temporal changes in glucose were temperature dependent, increasing only at high temperature and especially among gillnetted females. Impaired exercise recovery of infected fish relative to healthy fish (Wagner et al. 2005) and the potential for thermal influences on this relationship (Kocan et al. 2009) have implications for migration success, especially if rivers are warm.

Our results provide novel experimental data describing the disease ecology of an ecologically, culturally, and economically valued fish species. Fisheries bycatch is a prevalent phenomenon, with discards estimated to comprise between 10% and 40% of global marine catches (Davies et al. 2009; Zeller et al. 2018), and the effects of climate change, especially regarding temperature, are expected to impact the range and resilience of wild animals all over the world (Root et al. 2003; Poloczanska et al. 2013). The context-specific nature of animal responses to multiple stressors warrants continued research into their cumulative effects and associated disease development, especially in the face of climate change (Crozier et al. 2008; Altizer et al. 2013). The necessity of such studies to ensure effective management of resources and ecosystem services affected by multiple stressors, especially given variability in their cumulative effects, is accentuated by the rarity of experiments that comprise this complexity (Folt et al. 1999; Crain et al. 2008). Although infectious agents are inherent components of wild ecosystems, anthropogenic changes to climates and habitats can accelerate disease development by pushing animals to physiological extremes in addition to shifting the geographic ranges of hosts and pathogens (Walther et al. 2002; Pörtner and Knust 2007; Altizer et al. 2013). Our application of ecologically relevant multiple stressors combined with examination of infection development and stress and immune responses of Pacific salmon over time is a necessary step toward comprising the complexity of disease dynamics in wild ecosystems.

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