Salmon in a warming world: Impact of pathogen infections on juvenile Chinook salmon (*Oncorhynchus tshawytscha*)

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

INTEGRATIVE PATHOBIOLOGY

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2023

ACKNOWLEDGMENTS

First and foremost, I would like to praise and thank God, the Almighty, for granting me countless blessings, vast knowledge, and innumerable opportunities.

When alluding to the completion of a dissertation, the adage "it takes a village" could not be truer. Apart from my efforts, this accomplishment depended largely on the encouragement and guidance of many others. I would like to take this opportunity to express my sincere gratitude to everyone who has been instrumental in helping me complete this monumental task. While it would be impossible to list everyone, several people deserve my sincere and heartfelt gratitude.

My eternal gratitude goes to my family whose love and support made this possible. To my husband, Mohamed, and my two beloved children, Fares and Retaal, thank you for your unwavering support. Without your never-ending love, I would not have succeeded. You have given me the strength to persevere despite my doubts, and it was with your love, trust, and steadfast belief in me that I endured. Additionally, I would like to express my gratitude to my mom, brother, and sisters whose love and sacrifices from overseas were crucial during this journey.

I would like to sincerely thank my Ph.D. advisor and committee chair, Dr. Richard Connon. Richard, you are a true visionary who encouraged me to embark on endeavors I never would have otherwise attempted. You have not only allowed me to grow as a research scientist but also guided me through challenging times with incredible patience and compassion. I am completing my dissertation because of you, and I am proud of the work I have done and the person I have become under your guidance. Thank you for helping me with struggles big and small, I will always remember you when crossing literal and metaphorical bridges in my life. I would also like to thank Dr. Esteban Soto and Dr. Anne Todgham, members of my dissertation committee, for their expert advice. I will always be grateful for their interest in, guidance, and contributions to my dissertation. Esteban, thank you for helping shape my work throughout this endeavor. I will forever remember your words “Do not underestimate yourself”. Anne, thank you for your generosity as a mentor and collaborator, and your willingness to always help me. Every

interaction with you left me feeling inspired and stronger. Without a doubt, I'm in eternal debt to all of you.

I would like to express my gratitude to the members of my qualifying exam committee, Drs. Cecilia Giulivi (Chair), Barbra Byrne, Gary Cherr, Denise Imai, and Stuart Meyers. Your guidance encouraged my intellectual growth through thoughtful inquiries and discussion.

I would like to thank the many collaborators who made this dissertation possible. Thank you to Miles E. Daniels, Camilo Sanchez, Benjamin Atencio, Florian Mauduit, Brendan Lehman, Sascha L. Hallett, Stephen D. Atkinson, J. Scott Foott, Nermeen Raffat, and Jiangtian Guan. I truly could not have done it without you!

To my friends who have stood by me throughout the years, I will always be grateful for your companionship. To Cindy, Steve, Marie, Amira, and Aunt Ghazala—I don't know where I would be without you. I appreciate your guidance, the meals we shared, and the times we spent together. You've helped me get through the challenging moments of this wild Ph.D. ride. More importantly, though, you've celebrated all my accomplishments along the way. Without each of you, I'm not sure I could have done it. I look forward to new recipes and future adventures!

I cannot express how grateful I am to all my colleagues and lab mates at the Connon and Aquatic Animal Health labs at UC Davis. I would like to extend a special thanks to Dr. Taylor Heckman and Dr. Eva Marie Quijano Carde at the Aquatic Animal Health lab. You've helped me overcome challenges while caring for me during difficult times. The joy and passion for your work that both of you exude are inspirational. And to Dr. Khalid Shahin, thank you for your mentorship and for providing me with exciting research ideas.

Thank you to the Graduate Group in Integrative Pathobiology (GGIP) at UC Davis. I appreciate the dedication of Dr. Sara Thomasy and Dr. Erin Kent to the graduate students. Part of this dissertation was conducted at the Center for Aquatic Biology and Aquaculture (CABA) at UC Davis and would not have been as successful without the diligent efforts of Linda Deanovic and Matt Stone.

And while animals do not seem equipped to become formal authors, I was humbled to work with these incredible fish, who became ambassadors for the survival of their species. My thesis would have been impossible without their sacrifice. Being able to observe and appreciate the beauty, tenacity, and significance of Chinook salmon has been a privilege.

Dedication

In loving memory of my father, Mohamed Riyad, and my dear sister, Amal

No one could have predicted that my first year in graduate school at UC Davis would be their last in this life, but I will forever remember and cherish them

ABSTRACT

There is global concern over fish population declines, particularly for economically and culturally valuable salmonid species. Chinook salmon (*Oncorhynchus tshawytscha*), the largest in the genus *Oncorhynchus*, are anadromous salmonids native to the United States Pacific Northwest (PNW). Chinook salmon have declined in abundance and several populations are listed as threatened under the Endangered Species Act. Climate change is projected to have a further, significant impact on Chinook salmon populations, as water reservoir storage decreases, reducing river flows and increasing water temperatures. Sub-optimal water temperature has long been recognized as a key factor influencing disease progression in salmonids, by altering the nature or course of the immune response and increasing infectious agent abundance or virulence, to enhance the likelihood of disease progression. While many opportunistic pathogens may be endemic in a watershed, a disease often only manifests when other factors, such as poor environmental conditions, first compromise the host. *Flavobacterium columnare*, for example, is a re-emerging bacterial pathogen, especially during summer and early fall, and one of the etiological agents of columnaris disease. During the last two decades, columnaris has become one of the most serious threats to nearly all freshwater fishes including anadromous salmonids. Mitigating the effects of fish diseases requires a thorough understanding of the processes that drive host-pathogen interactions under various environmental conditions. There is a paucity of information on whether the outcomes of infections under various environmental conditions are caused by the environment's effects on the host, the pathogen, or on both organisms. To address this knowledge gap, the objective of my dissertation was to investigate the context-specific dynamics associated with infectious agents. I used juvenile fall-run Chinook salmon, a species of conservation concern in the California Central Valley, as a sentinel for other runs of Chinook

salmon listed as threatened or endangered. The overarching hypothesis of this dissertation was that the host physiological response is associated with the host immune- and stress-response, infection burden, and external stressors (e.g., temperature) that also mediate host-pathogen dynamics.

The emphasis of my dissertation was on evaluating host-related factors that influence the outcome of pathogen infections for juvenile Chinook salmon, including tissue damage, the host immune response to prevent, control and eliminate pathogens, and detrimental consequences caused by inflammation, during warming. Many tools have been developed to better understand disease progression mechanisms, which could indicate organismal health status. Insight into which aspects of immunity are recruited can be gained by measuring the direction and magnitude of transcriptional changes in host immune gene regulation and can be paired with the measurement of infection severities in the same tissues (Chapter 1). I examined salmon survival following challenge with *F. columnare* at two temperatures (14 °C and 18 °C), *F. columnare* abundance, and transcript abundance of pro-inflammatory, acute phase proteins, anti-inflammatory cytokines, immunoglobulins, and stress-related genes in gills over 16 days. I found that with a temperature rise of 4 °C, *F. columnare* abundance and prevalence increased. Additionally, data emphasize the significance of timely and balanced immune responses in challenged Chinook salmon. I demonstrated that immune activation at elevated water temperatures that Chinook salmon experience during outmigration (18 °C) appeared to elicit a strong response against infection, as demonstrated by higher transcription levels of immune-related genes. The strong immune response, however, did not result in increased salmon survival, as mortality rates were significantly higher in fish challenged with *F. columnare* at 18 °C compared to those kept at a cooler temperature, 14 °C.

Sub-lethal behavioral effects caused by bacterial infections can have long-lasting repercussions on fish ecological fitness through effects on predator avoidance and foraging success. I, therefore, conducted a study to determine whether *F. columnare* infection can alter the behavior of juvenile fall-run Chinook salmon. Specifically, I quantified locomotion and anxiety-like behavior. I utilized the open field test to assess locomotion followed by a novel object approach test to quantify anxiety. Additionally, I assessed *F. columnare* abundance in gills, and changes in expression of genes associated with behavior, in the brain tissue (Chapter 2). The results of this study indicated that: first, whereas challenged fish were less receptive to the presence of the novel object, control fish exhibited a range of behavioral responses. Second, there were significant differences in *F. columnare* abundance among challenged fish over time, with the pathogen abundance rapidly increasing but also showing potential for recovery over time. Lastly, *F. columnare* challenge elicited differential expression of behavior-related genes as the *F. columnare* infection progressed.

Lastly, research on host-environment-agent interactions has focused on single pathogen studies. In many systems, however, salmon are exposed to a variety of pathogens, and these multiple exposures may synergize and not result in simple additive effects on the host. Therefore, I determined the prevalence and abundance of pathogens in gill and kidney tissues of fish deployed in the Sacramento River (CA, USA). I further determined consequences of pathogen exposure on salmon histological and physiological responses: expression of immune, stress, and development-related genes (Chapter 3). I found that fall-run Chinook salmon were exposed to multiple potential pathogens when out-migrating from the Sacramento River, with *Ceratonova shasta* and *Parvicapsula minibicornis* being the two dominant pathogens. I noted that the presence of specific pathogens in fish tissue does not always imply disease establishment and

progression, especially for pathogens that are ubiquitous in the aquatic environment. I detected upregulation of immune and stress-related genes in gills before they were detected in kidneys, suggesting that gill biopsy may be useful for early-warning studies on what could potentially be a non-lethal target tissue. Furthermore, expression of investigated genes was altered following deployment; these responses could potentially represent irreversible impacts on salmon, thus demonstrating the threat of pathogen exposure and disease progression to the outmigrating Chinook salmon population.

My dissertation indicates that infectious diseases will likely remain and worsen as a conservation issue as climate change continues to reshape host-pathogen dynamics and will significantly influence fish physiology, behavior, and survival. Combining nonlethal sampling with molecular genetic-based identification techniques opens a wide range of possibilities for creative study plans that could potentially address the true complexity of the host-pathogen dynamic in a warming world. Predicting the trajectory of these dynamics will require the inclusion of greater knowledge and tools (e.g., molecular techniques, histopathology, and behavior testing) outlined in this dissertation and other studies. This dissertation research, however, provides a foundation for future avenues of research investigating host-environment-agent interactions and offers crucial knowledge for future conservation efforts.

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CHAPTER 1: Impact of elevated temperature on *Flavobacterium columnare* infection abundance and transcriptional response in juvenile Chinook salmon (*Oncorhynchus tshawytscha*)

A similar version of this chapter was submitted for publication by the journal of Fish and Shellfish Immunology

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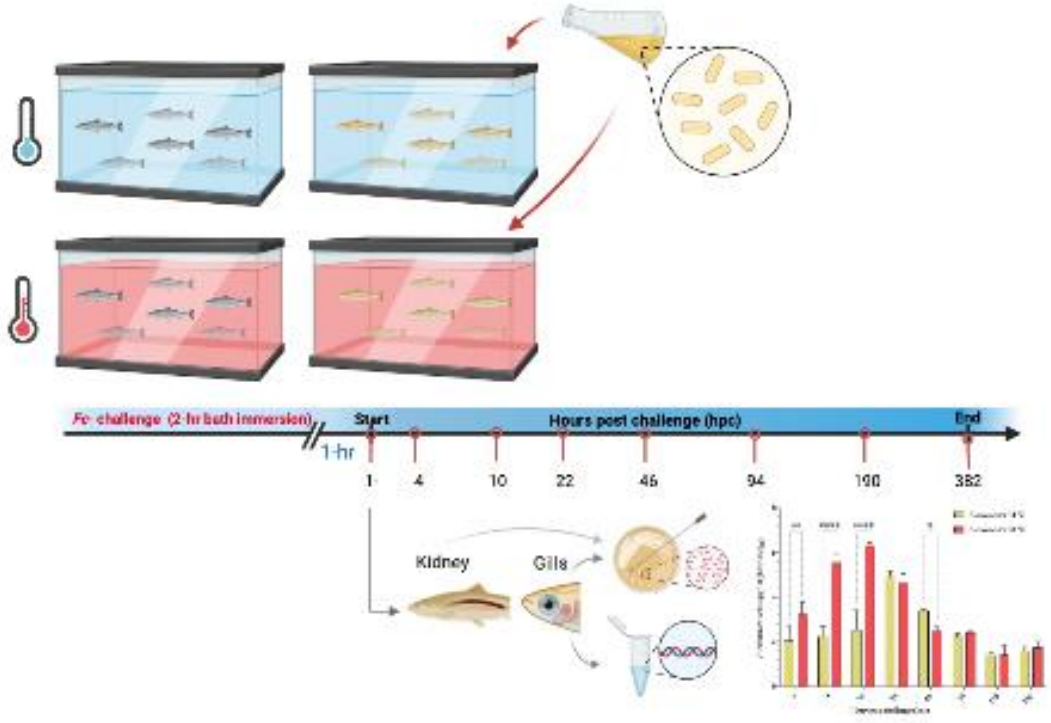
ABSTRACT

Infectious diseases are expected to remain a significant issue as climate change continues to influence host-pathogen dynamics. *Flavobacterium columnare* is a re-emerging bacterial pathogen and one of the etiological agents of columnaris disease. During the last two decades, columnaris has become one of the most serious threats to nearly all freshwater fishes including anadromous salmonids. Salmon populations are currently impacted by changes in water temperature and pathogen distribution driven by global climate change. Thus, we investigated the impact of elevated water temperature, *F. columnare* infection *via* bath immersion, and their potential interaction on juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Under laboratory-controlled conditions, fish were assigned to one of four treatments, designed as a full factorial of *F. columnare*: challenged or non-challenged, and water temperature: 14 °C or 18 °C. We examined salmon survival, the bacterium abundance, and transcript abundance of pro-inflammatory, acute phase proteins, anti-inflammatory cytokines, immunoglobulins, and stress-related genes in gills over 16 days. At 18 °C, challenged fish had an overall significantly lower survival rate compared to challenged fish maintained at 14 °C (53.6.0% and 88.2%, respectively). *Flavobacterium columnare* abundances were significantly higher in challenged fish at 18 °C compared to challenged fish at 14 °C as early as one hour post-challenge (hpc). Transcript abundance of *il1β*, *il8*, *il6*, and *saa* was significantly higher in gills of challenged fish maintained at 18 °C early in infection compared to challenged fish at 14 °C. However, *il10* transcript abundance was significantly lower in challenged fish at 18 °C at 4, 22, 94, and 190 hpc, and *igm* at 382 hpc when compared to challenged fish at 14 °C. Heat shock protein 47 was one of the most upregulated target genes, whose transcript abundance was influenced by temperature and *F. columnare* infection both singly and in combination. Our findings highlight

how environmentally relevant differences in water temperature resulted in diverse outcomes in terms of bacterial abundance as well as immune and general stress response, which may be indicative and/or prognostic of potential recovery or mortality. This study provides a foundation for future research assessing the effect of temperature on host-pathogen dynamics.

Keywords: *Flavobacterium columnare*; Columnaris; Temperature**;** Chinook salmon; Gene expression

Graphical abstract:



1. INTRODUCTION

Infectious diseases are among the most pressing risk factors linked to declining numbers of salmonid species and have been identified as an important contributor to fish mortality (Kent, 2011). While many opportunistic pathogens may be endemic in a watershed, a disease often only manifests when other factors, such as poor environmental conditions, first compromise the host (Lehman et al., 2020). The epidemiological triad is the core concept used to describe how pathogen exposure levels, host susceptibility, and the environment in which host and pathogen interact determine disease dynamics (Dicker et al., 2006). There is an extreme paucity of information on whether the outcomes of infections under various environmental conditions are caused by the environment's effects on the host, the pathogen, or both interacting organisms.

Ectothermic vertebrates' physiology is influenced by their environment, and ambient temperature variations impact biological systems at all scales, from molecule to organism, especially for those who inhabit thermally variable environments (Rebl et al., 2013). Sub-optimal water temperature has long been recognized as a key factor influencing disease progression in salmonids, by altering the nature or course of the immune response (Jeffries et al., 2012; Köllner and Kotterba, 2002) and increasing infectious agent abundance (Bailey et al., 2017) or virulence (Teffer et al., 2022), to enhance the likelihood of disease progression. Hence, when assessing impacts of environmental conditions like temperature, responses of both the infectious agent and host must be considered, as interactions between these three factors drive co-evolution of pathogens and host (Penczykowski et al., 2015).

Flavobacterium columnare, one of the causative agents of columnaris disease produces substantial mortality in a wide range of wild and cultured freshwater fish worldwide (reviewed by (Declercq et al., 2013)). Four genetic groups (GG) of *F. columnare* have recently been

recognized as four distinct species with the names *F. columnare*, *F. covae sp. nov*., *F. davisii sp. nov.*, and *F. oreochromis sp. nov*., representing genetic groups 1, 2, 3, and 4, respectively (LaFrentz et al., 2022). *Flavobacterium columnare* is ubiquitous in the aquatic environment and outbreaks are often triggered during the spring and summer months (Mohammed and Arias, 2014). *Flavobacterium columnare* can persist in subclinical carrier fish or environmental reservoirs (Kunttu et al., 2012; Suomalainen et al., 2005) and has been linked to mortality in hatchery and wild salmonid populations (Becker, 1978; Sebastião et al., 2021), posing a potential threat to out-migrating juvenile salmonids. Columnaris disease is notorious as it mostly affects the external mucosal surfaces of fry and fingerling, resulting in rapid and widespread destruction of gills, skin, and fins (Lafrentz et al., 2012) resulting in significant economic losses (Peterman and Posadas, 2019). Acute or chronic infections caused by *F. columnare* are mostly driven by the strain's virulence, the environment, and host-related factors (such as age and immune status; (Declercq et al., 2013)).

Pacific salmon, *Oncorhynchus spp.,* is a genus of iconic fish with significant cultural, economic, and recreational value (Lichatowich and Lichatowich, 2001; National Research Council et al., 1996). Chinook salmon (*Oncorhynchus tshawytscha*), the largest in the genus *Oncorhynchus*, are anadromous salmonids native to the United States Pacific Northwest (PNW) (Lichatowich and Lichatowich, 2001); and are especially sensitive to climate change since they live in a variety of environments during migration. Chinook salmon populations have declined in abundance and several populations are listed as threatened under the Endangered Species Act (NOAA, 2022). Chinook salmon are projected to be further impacted by climate change as reservoir storage decreases, reducing river flows and increasing water temperatures (Moyle et al., 2017).

While the field of fish immunology is still in its infancy when compared to terrestrial immunology, many tools have been developed to better understand mechanisms associated with disease progression, which could be indicative of organismal health status (i.e., biomarkers of fish health). Measurements of gene expression patterns determined from salmon gill biopsies can be used to indicate whether a fish is responding physiologically to pathogen infections, as well as the magnitude of that response (Connon et al., 2018; Jeffries et al., 2014). Insight into which aspects of immunity are recruited can be gained by measuring the direction and magnitude of transcriptional changes in host immune gene regulation and can be paired with the measurement of infection severities in the same tissues (Jeffries et al., 2014).

Mitigating the effects of fish diseases requires a thorough understanding of the processes that drive host-pathogen interactions under various environmental conditions. How increases in surface water temperature may benefit *F. columnare* and its potential to cause disease in Chinook salmon remains unclear. Therefore, we investigated how water temperature increases influence *F. columnare* infection and abundance in juvenile fall-run Chinook salmon, a "species of conservation concern" according to the National Oceanic and Atmospheric Administration (NOAA) Fisheries (Buchanan et al., 2018), and as a sentinel for other runs of Chinook salmon. Additionally, we evaluated immune- and general stress-related host responses at the molecular level. We hypothesized that increases in thermal exposure and duration result in a higher *F. columnare* burden and elicit an increase in transcriptional response of immune- and general stress-related genes, which can be predictive of risk of mortality.

2. MATERIALS & METHODS

2.1 Experimental fish and acclimation

All fish care and use protocols were reviewed and approved by the University of California Davis (UC Davis) Institutional Animal Care and Use Committee (IACUC Protocol # 19499). Juvenile fall-run Chinook salmon were transported from the Mokelumne River Hatchery (California Department of Fish and Wildlife, Clements, CA, USA) to the UC Davis Center for Aquatic Biology and Aquaculture (CABA). Fish were maintained in a flow-through ultraviolet-filtered well water 980-L tank with continuous aeration at 11 °C under natural photoperiod conditions for Davis, CA, USA (38.5 N, 121.7 W). Fish were fed with a pelleted salmon diet (Salmon Sink; Skretting, Toole, UT, USA) *ad libitum* once daily.

Fish (*n* = 380; mean fork length ± SD, 10.41 ± 1 cm; mean mass ± SD, 9.11 ± 0.6 g) were haphazardly assigned to two experimental groups (6 tanks group^{-1}; 32 fish tank^{-1}) receiving flow-through well water at a rate of three liters minute^{-1}, with dissolved oxygen (DO) levels kept at ~9 mg O_2 L^{-1}. One group was acclimated to 14 °C ± 1.0 °C and the other group was acclimated to 18 °C ± 1.0 °C, with temperatures adjusted at a rate of 1 °C day^{-1}. A temperature of 18 °C was chosen since this is an elevated temperature, typically recorded in PNW watersheds (Billman et al., 2014) when conditions (high temperature, low flow) are favorable to *F. columnare* epizootics. After target temperatures were reached, fish were acclimated for a further 14 days. Ten fish per temperature were sampled at the end of acclimation and euthanized with tricaine methanesulfonate (MS-222; 500 mg L^{-1}, buffered with sodium bicarbonate), for necropsy and posterior kidney bacterial culture to confirm fish were disease-free prior to bacterial challenge.

2.2 Bacterial isolate and growth conditions

Flavobacterium columnare (117/MK981921) used in this study was isolated from a Chinook salmon's kidney from a hatchery in California during a 2017 outbreak (Sebastião et al., 2021).

Flavobacterium columnare was sourced from frozen stock, streaked onto modified Shieh agar (MSA) supplemented with tobramycin (1 μg mL^{-1}), and incubated at 28 °C for 48 h. Two flat, rhizoid, yellow colonies with irregular margins tightly adherent to the agar were selected and inoculated into 5 mL of modified Shieh broth (MSB) supplemented with tobramycin and incubated at 28 °C for 17–18 h with shaking at 180 rpm (Declercq et al., 2013). This initial culture was then introduced into 110 mL of MSB in a 500 mL Erlenmeyer flask and incubated at 28 °C for ~17–18 h with shaking at 180 rpm. Cultures were then adjusted using MSB to an optical density of 1 read on a UV/Vis photometer (BioPhotometer Plus, Eppendorf AG, Hamburg, Germany) at 600 nm [OD_{600}] to produce ~10^9 colony-forming units [CFU] mL^{-1}.

2.3 Laboratory-controlled challenges and tissue collection

Fish were assigned to triplicate tanks (30 fish $tank^{-1}$) into one of four mutually exclusive treatments: *F. columnare* challenged or non-challenged, at water temperatures of 14 °C or 18 °C. *Flavobacterium columnare* challenge was carried out as in Soto et al. (2008) with some modifications. On the day of the challenge, water levels were reduced from 100 to 50-L, water flow was suspended, and constant aeration was maintained. To achieve a final dose of ~6.4×10^6 CFU mL^{-1}, 150 mL of cultivated MSB was diluted in 1-L of water before adding this solution directly to the tank. Fish were exposed *via* bath immersion for 2 h before water flow was restored. Comparable volumes of MSB without bacteria were used in mock immersion challenges for fish in control groups. Two fish from each replicate (n = 3 $treatment^{-1}$) were arbitrarily sampled at 1, 4, 10, 22, 46, 94, 190, and 382-h post-challenge (hpc) and euthanized. Gill and posterior kidney swabs were streaked on MSA plates and incubated at 28 °C for 48 h. Presumptive identification of isolates was based on colony color and morphology. Right and left

gill arches were then excised and immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analyses in terms of *F. columnare* copy number ng^{-1} genomic DNA and gene expression analyses, respectively. Mortality was recorded daily for a period of 16 days.

2.4 Genomic DNA extraction, total RNA extraction, and cDNA synthesis

A QIAcube system 230 V (Qiagen, Redwood City, CA) was used to extract genomic DNA (gDNA) and total RNA from gills using the DNeasy Blood and Tissue Kit (Qiagen) and RNeasy Plus Mini Kit (Qiagen) respectively, according to the manufacturer's protocol. Extracted gDNA and RNA were assessed for quality (by measuring the A260:280 and A260:230 ratios) and concentration (ng μL^{-1}) using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The integrity of the RNA was assessed by electrophoresis on a 1% w/v agarose gel. QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA from 1 μg of total RNA following the manufacturer's protocol, with the exception that reaction volumes were scaled to a final volume of 40 μL. Generated cDNA template was diluted 1:10 with DEPC-treated water and then stored at −20 °C until analysis.

2.5 Detection and quantification of *F. columnare* in gill tissue

Probe-based quantitative PCR (qPCR) was used for the detection and quantitation of *F. columnare* in gills targeting a 113 bp nucleotide region of the chondroitin AC lyase gene of *F. columnare* (GenBank accession number AY912281). *Flavobacterium columnare*-specific primers (forward 5′-CCTGTACCTAATTGGGGAAAAGAGG-3′ and reverse 5′-GCGGTTATGCCTTGTTTATCATAGA-3′) and a dual-labeled probe (5′-ACAACAATGATTTTGCAGGAGGAGTATCTGATGGG-3′) were used for specific detection

of *F. columnare* (Panangala et al., 2007). Quantitative PCR reactions consisted of 6 µL of TaqMan™ Environmental Master Mix 2.0 (ThermoFisher Scientific), 0.4 µM of each primer, 0.08 µM of the probe, 5 µL of template gDNA, and nuclease-free water to a total volume of 12 µL. Plasmid DNA with specific sequences corresponding to *F. columnare* (Eurofins Genomics LLC, Louisville, KY, USA) was used to create a standard curve of known copy numbers, as well as for assay quality control and quality assurance. All DNA samples along with 10-fold serially diluted plasmid standards were amplified in 384-well plates in a QuantStudio7 Flex Real-Time PCR System (Real-time PCR Research and Diagnostics Core Facility, UC Davis). All reactions were tested in triplicate under standard amplification conditions: 50 °C for 2 min; 95 °C for 10 min; followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Fluorescent signals were analyzed using QuantStudio™ Design and Analysis Software v2 (Thermo Fisher Scientific). *Flavobacterium columnare* presence in gills was calculated as copy number ng^{-1} of gDNA, hereafter referred to as abundance, and presented as log-transformed.

2.6 Gene expression analysis

Quantitative PCR was used to examine the expression profile for 11 target genes associated with immune function and general stress responses in gills. Primer pairs (Table 1.1) for each gene were either designed using Primer3 software from NCBI sequences, or retrieved from published literature, and were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). Primer efficiency was tested using 10-fold serial dilutions of cDNA from pooled RNA samples. Each qPCR reaction was carried out in a final volume of 12 µL; 6 µL of 2x PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 µM of the respective forward and reverse primers, and 5 µL of cDNA, and RNase free water to volume. All

measurements were performed in triplicate on 384-well plates using the QuantStudio7 Flex Real-Time PCR Systems together with QuantStudio Design and Analysis Software v2. The cycling conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min. A dissociation curve was performed at 95 °C for 15s, 60 °C for 1 min, and 95 °C for 15 s with each run to confirm specificity. Transcript abundance for investigated genes was normalized to three reference genes: 60S ribosomal protein L7, β-Actin, and glyceraldehyde-3-phosphate dehydrogenase. Consequently, normalized values were calibrated to respective controls at 14 °C or 18 °C, at the corresponding sampling time. Relative expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and is presented as $\log_2$-transformed fold-change.

2.7 Statistical analyses

All statistical tests were performed in GraphPad Prism v9.4.1(GraphPad Software, USA). Survival curves were compared with Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. Statistical differences in *F. columnare* abundance were determined by two-way ANOVA and Tukey's multiple comparisons tests with time and temperature as main effects. For gene expression data, the differences between respective control and *F. columnare* challenged groups and between challenged groups were tested for significant differences using a t-test and Holm-Šídák multiple comparisons. A p-value of < 0.05 was considered statistically significant.

3. RESULTS

3.1 Clinical signs and survival of juvenile Chinook salmon following challenge with *F. columnare*

Juvenile fall-run Chinook salmon survival rate was statistically different between respective controls and *F. columnare*-challenged fish, and between *F. columnare*-challenged fish at 14 °C and 18 °C for a period of 16 days. Challenged fish at 14 °C and 18 °C had an overall significantly lower survival rate ($p < 0.01$, and $p < 0.001$, respectively) when compared to the respective control group (Fig. 1.1). Challenged fish at 18 °C had an overall significantly lower survival rate compared to challenged fish maintained at 14 °C ($p < 0.001$; 53.6.0% and 88.2% respectively). At 14 °C and 18 °C, the onset of mortality in challenged fish was at 46 and 10 hours post-challenge (hpc), respectively. Mortalities were recorded up to 142 hpc (14 °C) and 262 hpc (18 °C) in challenged fish (Fig. 1.1). No mortalities or morbidities were observed in control fish maintained at 14 °C or 18 °C. By 22 hpc, challenged fish presented behavioral changes such as lethargy, anorexia, and swimming near the surface, yet fish often died without exhibiting signs. The main gross findings observed in challenged fish at 18 °C were pale gills with yellow-white patches (Fig. 1.2A); discolorations of the skin, mostly initiated at the base of the dorsal fin (Fig. 1.2B); and frayed fins and erythematous skin (Fig. 1.2C).

3.2 *Flavobacterium columnare* infection prevalence and bacterial abundance

In challenged fish maintained at 14 °C and 18 °C, pure *F. columnare* cultures were re-isolated from the gills as early as one hpc at 100% prevalence (Table 1.2). However, re-isolation of *F. columnare* from kidney was confirmed at 100% prevalence in challenged fish at 22 and 10 hpc maintained at 14 °C and 18 °C, respectively (Table 1.2). *Flavobacterium columnare* was not detected from any of the control fish at either temperature at any sampling point or *F. columnare*-challenged fish at 190 and 382 hpc.

Changes in *F. columnare* abundance under different temperature treatments were evident (Fig. 1.3), with the higher temperature resulting in rapid bacterial proliferation and an earlier peak of bacterial abundance. Specifically, *F. columnare* abundance was significantly higher in challenged fish maintained at 18 °C compared to the 14 °C group at 1, 4, and 10 hpc ($p < 0.01$, $p < 0.0001$, and $p < 0.0001$, respectively; Fig. 1.3). *Flavobacterium columnare* abundance reached a peak in copy numbers $\log_{10} \mu g^{-1} \pm$ SEM at 22 and 10 hpc in challenged fish maintained at 14 °C (4.95 ± 0.11) and 18 °C (6.03 ± 0.09), respectively. Over the course of the study, a decrease in *F. columnare* abundance was observed, with *F. columnare* abundance at 18 °C significantly lower than that at 14 °C at 46 hpc ($p < 0.05$; Fig. 1.3).

3.3 Immune and general stress gene response to *F. columnare*

3.3.1 Effects of an increase in temperature only (controls 14 °C vs. 18 °C)

Increased water temperature (18 °C) alone elicited increases in transcript abundance of immune genes when compared to fish maintained at 14 °C (Supplementary Table S1.1). Control fish maintained at 18 °C had significantly higher transcript abundance of pro-inflammatory genes: tumor necrosis factorα (*tnfα*; $p < 0.05$), interleukin1β (*il1β*; $p < 0.001$), and *il8* ($p < 0.01$) as early as 1 hpc compared to control fish maintained at 14 °C. Transcript abundance of the genes was then downregulated, either significantly as seen in *tnfα* ($p < 0.05$) and *il8* ($p < 0.01$) at 22 hpc, *il6* ($p < 0.05$) at 10 hpc and ($p < 0.01$) at 22 hpc, or non-significantly ($p > 0.05$) as in *il1β*. Furthermore, serum amyloid A (*saa*) transcript abundance was significantly upregulated at 4, 10, 22, and 46 hpc ($p < 0.05$ for all time points). Anti-inflammatory cytokine, *il10* transcript abundance was significantly upregulated at 4, 10, and 190 hpc ($p < 0.001$, $p < 0.05$, and $p < 0.05$, respectively). Transcript abundance of adaptive immune genes immunoglobulin T (*igt*) and

immunoglobulin M (*igm*) showed a significant downregulation ($p < 0.05$ and $p < 0.05$, respectively) at 10 hpc. Heat shock protein 47 (*hsp47*) transcript abundance was significantly upregulated ($p < 0.001$ for all time points) except for 94 hpc.

3.3.2 Effects of *F. columnare* challenge and potential interactions of *F. columnare* challenge and temperature on gene expression

Significantly increased transcript abundance of investigated genes was observed in challenged fish maintained at 14 °C and 18 °C compared to their respective non-challenged control groups, but the pattern of changes in transcript abundance was different (Supplementary Table S1.1). *Flavobacterium columnare* challenge at 14 °C compared to its respective control elicited an immune response with significant upregulation of pro-inflammatory immune gene transcription of *il1β* ($p < 0.001$) and *il8* ($p < 0.001$) at both 10 and 22 hpc**,** as well as *saa* ($p < 0.05$) at 94 hpc. Concerning the transcript abundance of anti-inflammatory cytokine, *il10* was significantly upregulated ($p < 0.05$) at 22, 46, 94, and 190 hpc. The transcript abundance of *hsp47* was significantly upregulated at 1, 4, 10, 46, and 190 hpc ($p < 0.05$).

At the higher temperature (18 °C), challenge with *F. columnare* elicited an immune response (Supplementary Table S1.1) compared to its respective control as early as 4 hpc. There was a significant upregulation in transcript abundance of *il1β* ($p < 0.001$, $p < 0.001$, and $p < 0.001$) and *il8* ($p < 0.01$, $p < 0.001$, and $p < 0.05$) at 4, 10, and 22 hpc, *tnfα* ($p < 0.001$ and $p < 0.001$) and *il6* ($p < 0.001$ and $p < 0.001$) at 10 and 22 hpc, as well as *saa* ($p < 0.001$) at 10 hpc. Transcript Abundance of *il10* was significantly upregulated ($p < 0.01$) at 10 hpc, and *igm* ($p < 0.05$) at 190 hpc, whereas *hsp47* was significantly upregulated ($p < 0.001$ and $p < 0.05$) at 10 and 190 hpc.

In general, fish challenged at 18 °C exhibited significantly higher transcript abundance than fish challenged at 14 °C, particularly during the acute stage of infection (Supplementary Table S1.1). Specifically, transcript abundance of pro-inflammatory genes: *tnfα* ($p < 0.01$; Fig. 1.4A) at 22 hpc, *il1β* ($p < 0.001$; Fig. 1.4B) at 10 hpc, *il8* ($p < 0.01$, $p < 0.001$, and $p < 0.05$; Fig. 1.4C) at 4, 10, and 22 hpc, *il6* ($p < 0.01$ and $p < 0.01$; Fig. 1.4D) at 10 and 22 hpc, and *saa* ($p < 0.05$; Fig. 1.4E) at 10 hpc. Of note, *il1β* and *il8* transcript abundance peaks approximated 5- and 10-fold changes, respectively, at 10 hpc, in stark contrast to challenged fish at 14 °C (Fig. 1.4B and 1.4C). After 10 hpc, there was a decline in all investigated genes in challenged fish at 18 °C, with a significant decrease in *il1β* ($p < 0.01$) and *saa* ($p < 0.05$) transcript abundance at 94 hpc compared to challenged fish at 14 °C (Fig. 1.4B and 1.4E). Although the magnitude of transcript abundance between *F. columnare* challenges at 14 °C and 18 °C differed among genes, the timing of peak for many genes was considerably earlier in challenged fish at 18 °C compared to challenged fish at 14 °C, particularly as seen in pro-inflammatory genes. At 4, 22, 94, and 190 hpc, the anti-inflammatory cytokine, *il10* transcript abundance was significantly lower ($p < 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.001$) in challenged fish at 18 °C, and significantly higher ($p < 0.05$) at 382 hpc compared to challenged fish at 14 °C (Fig. 1.4F). Immunoglobulin M, *igm*, was significantly higher in challenged fish at 18 °C ($p < 0.01$) at 190 and exhibited a significantly lower transcript abundance ($p < 0.01$) at 382 hpc compared to challenged fish at 14 °C (Fig. 1.4I). Lastly, at 1, 4, and 46 hpc, challenged fish at 18 °C exhibited significantly lower *hsp47* transcript abundance ($p < 0.001$, $p < 0.05$ and $p < 0.05$) than fish challenged at 14 °C (Fig. 1.4J). However, at 10 hpc, challenged fish at 18 °C exhibited significantly higher *hsp47* transcript abundance ($p < 0.05$) than fish challenged at 14 °C. All other comparisons did not show significant differences.

4. DISCUSSION

In the current study, we determined that with a temperature rise of 4 °C, *F. columnare* abundance and prevalence increased. Substantial variations and changes in gene expression were seen for immune (pro-inflammatory, anti-inflammatory, and adaptive) and general stress-related genes, likely impacting the observed differences in the survival rate of juvenile fall-run Chinook salmon. This 4 °C temperature difference is a common change experienced by out-migrating juvenile salmonids that may impact their capacity to fend off an invasive pathogen (Zillig et al., 2021). These findings reflect a distinctive pattern of disease progression in the warmed environment, suggesting the potential for a shift in disease phenology resulting from rising water temperatures.

Our findings support our hypothesis that warmer water temperature, within the ranges that Chinook salmon experience during outmigration, would influence infection progression and bacterial abundance in fish, as evidenced by higher *F. columnare*-associated mortality in fish maintained at temperatures near their thermal tolerance limit (18 °C; (Poletto et al., 2017)) relative to 14 °C. Water temperature is one of the key factors influencing the onset of columnaris disease. Columnaris epizootic occurs primarily in warm summer months since *F. columnare* is a quintessential pathogen that prospers under high temperatures (Mohammed and Arias, 2014), providing compelling evidence of the disease's concurrent seasonal occurrence. When water temperatures are between 18 °C and 22 °C in the spring, summer, and fall, the majority of fish species are vulnerable to columnaris (Amend and Ross, 1970). Nevertheless, it is not unusual to diagnose this disease in fish, including salmonid species, even in water as cold as 12 °C –14 °C (Starliper and Schill, 2011). Full epidemics may even occur in water temperatures below 25 °C to as low as 15 °C, though mortality rate and severity of the disease are significantly higher in

warmer temperatures (Stoskopf, 1993). In a previous study, when steelhead trout (*O. mykiss*) and coho salmon (*O. kisutch*) were experimentally infected with *F. columnare* and maintained in water at 12 °C to 20 °C, the mortality rate increased with increasing temperature (Holt et al., 1975). In the current study, clinical signs, such as lethargy, anorexia, swimming near the surface and characteristic lesions (e.g., skin lesions, fin erosion, and gill lesions) as described by (Declercq et al., 2013) were observed in challenged fish at 18 °C that suggest mortality due to respiratory malfunction. Additionally, high mortality observed in challenged fish at 18 °C may be explained by the observed increases of bacterial abundance at 18 °C, or the combined amount of energy required for physiological homeostasis and the consequences of the immune response may have increased mortality.

In the current study, we exposed fish at both temperatures to the same infectious dose of *F. columnare*. While initial *F. columnare* dosage was the same at 14 °C and 18 °C, qPCR verified that the bacterium abundance in gills at 4 and 10 hpc was approximately three times higher at the warmer temperature (18 °C), compared to challenged fish maintained at 14 °C. There are two general, and not mutually exclusive explanations for why warmer temperatures increased abundance. One: a high temperature may render fish more susceptible to infections. Two: it may enhance the adhesion, proliferation, or pathogenicity of *F. columnare* (Declercq et al., 2013). Given this, equal consideration must be given to the effect of temperature on virulence and pathogenicity in terms of pathogen burden, as well as the capabilities of the host immune response, which equally contribute to the stable, yet tolerable system we have observed in the cooler temperature. Over the course of the infection, *F. columnare* abundance in gills of challenged fish at 14 °C generally remained lower than that of challenged fish at 18 °C. The exception to this was at 46 hpc, which coincided with the peak inflammatory immune response

in fish at higher temperatures. Possibly, the immune mechanisms reduced bacterial load at this time point.

In the kidney, *F. columnare* was re-isolated at 100% at 22 and 10 hpc from challenged fish at 14 °C and 18 °C, respectively, indicating that the systemic infection happened faster at the warmer temperature. Highly virulent strains of *F. columnare* have been isolated from the kidneys of infected fish (Decostere and Haesebrouck, 1999; Hawke and Thune, 1992). Hence, prolonged presence of pathogens in the kidney may contribute to the development of long-lasting immunity, as the presence of the pathogen in this tissue may stimulate continuous antibody production and greater avidity by increasing the specificity of the adaptive immune response to specific pathogen types (Randall and Griffin, 2017). Since no *F. columnare* was re-isolated from gills or kidneys of challenged fish at 190 or 382 hpc at either temperature, we suggest that the immune response in the fish that survived resulted in efficient bacterial clearance.

Flavobacterium columnare in challenged Chinook salmon elicited a modulated immune response that was strongly influenced by temperature. A panel of immune and stress genes was measured and clustered into groupings of their functional pathways (pro-inflammatory, anti-inflammatory, adaptive, and general stress). Differences between the timing and magnitude of the immune response to *F. columnare* infection were evident between challenged fish at 14 °C and 18 °C. Specifically, results showed that temperature had an impact on the expression of *tnfα*, *il1β*, *saa*, and *il8*, both independently and in combination with *F. columnare* infection. Specifically in this study, greater abundance of these transcripts were frequently observed in higher magnitudes at the higher temperature (18 °C), whereas lower abundance was generally observed at the lower temperature (14 °C).

In mammals and fish, *tnfα* is often the first cytokine released during the pro-inflammatory signaling cascade, which subsequently causes *il1β* and further chemokines like *il8* to be released (Holopainen et al., 2012). Upregulation of *il1β* in the early stages of infection is crucial for initiating the inflammatory response (Long et al., 2019). While, *il8* displays chemotactic activity for monocytes, lymphocytes, basophils, and eosinophils (Wang et al., 2019). Our findings support that the primary immune genes involved in reacting to *F. columnare* infection were *il1β* and *il8* and were the first to be significantly upregulated in challenged fish compared to their respective controls. Specifically, *il1β* in gills showed the highest fold change among pro-inflammatory cytokines. These findings are consistent with earlier research when *F. columnare*-challenged rainbow trout (*O. mykiss*) had strongly expressed levels of *il1β* and *il8* along with other adaptive immune genes (Xu et al., 2020). *Il8* and *tnfα* were among genes that were constitutively expressed at higher levels in the gills of resistance compared to susceptible catfish (*Ictalurus punctatus*) following infection with *F. columnare* (Peatman et al., 2015). Following challenge with *F. columnare, il1β* and *il8* were highly up-regulated in gills and kidney as early as 3 hpc in Indian major carp (*Catla catla*) (Ravindra et al., 2019), and in Mandarin fish (*Siniperca chuatsi*) (Zhou et al., 2015).

Interleukin-6 functions early in the infection in a pro-inflammatory capacity, since it is produced before the emergence of an adaptive response (Bjork et al., 2014). In our study, *il6* was significantly upregulated in fish challenged at 18 °C at 10 and 22 hpc, but not in fish challenged at 14 °C when compared to the respective control groups. This upregulation could make it particularly important as a marker of activation of pro-inflammatory cytokines in the presence of multiple factors (i.e. pathogens and warming). *Il6* has both pro- and anti-inflammatory properties since it attenuates the synthesis of other pro-inflammatory cytokines (Eggestøl et al., 2020) while

having little effect on the synthesis of anti-inflammatory cytokines such as *il10* and *tgfβ* (Opal and DePalo, 2000).

Acute phase proteins (APPs), triggered by stressors, infection, and other factors, such as serum amyloid A, help recognize, eliminate, and resolve pathogen invasions (Cray et al., 2009). Transcript abundance of *saa* in gills was upregulated in control fish at 18 °C; these findings demonstrate that *saa* transcripts increase in fish not only in the context of disease but also in response to warming. In *F. columnare*-challenged fish, the levels of *saa* reached their maximal fold change at 46 and 10 hpc at 14 °C and 18 °C, respectively, potentially indicating time-dependent inflammatory responses. Overall, this dataset provides evidence that salmon exposed to *F. columnare* had a prolonged elevation in inflammatory response, which could have led to a detrimental effect on salmon because of inflammation.

Upregulation of the anti-inflammatory cytokines allows for modulation of the pro-inflammatory response during infection, limiting the potentially detrimental effects elicited by an excessive inflammatory response (Opal and DePalo, 2000). *Il10* transcript abundance levels were significantly higher in challenged fish at 14 °C compared to challenged fish at 18 °C, indicating enhanced activation of anti-inflammatory mechanisms to minimize potential tissue damage following immune activation. *Il10* is a key biomarker of poor prognosis when highly expressed in fish with pathogen infections, whereas moderately modulated *il10* mRNA expression levels suggest recovery (Bailey et al., 2017; Gorgoglione et al., 2013). *Il10* and *tgfβ* suppress pro-inflammatory cytokine genes (such as *il-1* and *tnf*) while also directing adaptive immune pathways involving B lymphocytes (D'Amico et al., 2000; Zou and Secombes, 2016).

Adaptive immune response (*igt* and *igm*) did not differ between challenged fish and their corresponding controls, except *igm* in challenged fish at 18 °C at 190 hpc. One explanation for

the lack of expression changes of *igt* and *igm* after challenge with *F. columnare* may be because cold-water species like Chinook salmon have been shown to take six to twelve weeks after pathogen exposure before developing adaptive immunity (Sitjà-Bobadilla, 2008). *Flavobacterium columnare* has been utilized as a pathogen model to assess gill adaptive immune responses. For instance, when rainbow trout were infected with *F. columnare*, *igt* and *igm* mRNA expression levels increase at 28- and 75-days post-challenge, respectively (Tongsri et al., 2020). There is still much to understand about teleost mucosal tissues, notably in gills, and adaptive immunological responses against *F. columnare* infection.

Heat shock protein 47 (*hsp47*, encoding Serpin H1) is a chaperone protein that facilitates protein stability against heat stress and is considered a reliable indicator of general and thermal cellular stress (Akbarzadeh et al., 2018). In our study, *hsp47* was one of the most upregulated target genes, whose transcript abundance was influenced by temperature and *F. columnare* infection both independently and in combination. This upregulation was most obvious in control fish at 18 °C and challenged fish at 14 °C. Our findings imply that *hsp47* is involved in the response to bacterial infection and thermal stress. There also appears to be an interesting non-linear, antagonistic interaction between warming and infection and reduced levels of expression of *hsp47* in challenged fish at 18 °C that requires further investigation. Hsps are crucial for the cellular defense against pathogens and are found to initiate innate immune system's inflammatory responses, support extracellular antigen presentation, and participate in the initial phases of the immunological response to bacterial infection (Elibol-Flemming et al., 2009; Srivastava, 2002). Caution in interpreting gene expression data must be taken, in that transcription is just one stage in a process that also includes translation, protein modification, and multiple levels of regulation.

Future Chinook salmon management will depend on the ability to recognize possible additive, synergistic, or antagonistic health impacts brought on by environmental stressors. Mortality and allostatic overload can come from an unsuccessful response to persistent stressors. When assessing the likelihood of success, managers must strike a balance between the requirement to reduce the risk of disease transmission and the necessity to provide suitable environmental conditions (e.g., cold water refugia). Monitoring variables like water temperature is more straightforward, but precisely determining and reducing the risk of disease transmission is more challenging. Given current and projected climate change, it is of utmost importance to deepen our understanding of how an elevated temperature affects the energetic cost of immune activation in ectotherms as well as how infection and its consequences are regulated by adverse environmental conditions.

The findings discussed here emphasize the significance of timely and balanced immune responses in Chinook salmon. Here, we demonstrate that immune activation at elevated water temperatures near the species' thermal maximum appeared to elicit a strong response against infection, as demonstrated by higher transcription levels of immune-related genes. This escalated response, however, did not result in increased salmon survival, as mortality rates were significantly higher in fish challenged with *F. columnare* at 18 °C compared to those kept at 14 °C. To maintain internal homeostasis and minimize collateral tissue damage, host survival during infection necessitates a delicate balance between host defense, which is necessary for identifying and eradicating pathogens, and host tolerance.

Funding

This study was funded by the Delta Water Quality and Ecosystem Restoration Grant Program from the California Department of Fish and Wildlife (CDFW; Proposition 1 grant #P1896051 to REC, ES, and collaborators).

Acknowledgments

We would like to thank the Graduate Group in Integrative Pathobiology at UC Davis. We especially thank Dr. Anne Todgham for reviewing and enhancing the manuscript prior to submission.

5. FIGURES & TABLES

Table 1.1: Primer sequences for genes used in quantitative polymerase chain reaction (qPCR) analyses to assay host biomarkers of immunity, general stress, and reference in juvenile Chinook salmon (*Oncorhynchus tshawytscha*).

Gene	Function		Forward primer sequence (5′–3′) Reverse primer sequence (5′–3′)	Primer efficiency	Reference
Tumor necrosis factor α	Pro-inflammatory	F R	CCC TGT GTA CCC AGC AAC G TGC AGC TGA GAC ACA CAA TGC	101.38	Mauduit et al. (2022)
Chemokine interleukin 1β	Pro-inflammatory	F R	GAC ACA TGT CCA CCG GTT TG CCT GGA GTC TGC CAG GTT CA	108.58	Mauduit et al. (2022)
Chemokine interleukin 8	Pro-inflammatory	F R	GCG ATG TCG CTG CAT TGA CGA GCT GGG AGG GAA CAT C	94.26	Mauduit et al. (2022)
Chemokine interleukin 6	Pro-inflammatory	F R	CAG TTT GTG GAG GAG TTT CAG A TGT TGT AGT TTG AGG TGG AGC A	104.68	Bjork et al. (2014)
Serum amyloid protein A	Pro-inflammatory	F R	CTC GGG GCA ACT ATG ATG CT CAT CTC CCG GCC ATT ACT GAT	91.54	Mauduit et al. (2022)
Interleukin-10	Anti-inflammatory	F R	CTA CGA GGC TAA TGA CGA GC GAT GCT GTC CAT AGC GTG AC	112.82	Bjork et al. (2014)
Transforming growth factor β	Anti-inflammatory	F R	AGA TAA ATC GGA GAG TTG CTG TG CCT GCT CCA CCT TGT GTT GT	101.63	Bjork et al. (2014)
Immunoglobulin T	Adaptive	F R	AGC TCC GCG TGG CTA AGA AT CAG GTA CTG GGT GGT GCC AA	109.09	This study
Classical immunoglobulin	Adaptive	F R	CAG CTG CCT CCT GTG TTC ACT GAC CTG GCT GGA CAG TCA TAG AG	96.44	Mauduit et al. (2022)
Heat shock protein serpin H1	General stress	F R	TCC CTT CAT CTT CCT GGT GAA CCT TTG GGT CGC ACC ATT C	108.96	This study
60S Ribosomal gene 7l	Reference gene	F	GCA AGG TCG GGA ACT TCT ACG	98.93	

		R	CCC CTG ATC CTG ATG ACG AA		Mauduit et al. (2022)
β-actin	Reference gene	F R	GGA CTT TGA GCA GGA GAT GG ATG ATG GAG TTG TAG GTG GTC T	100.87	Bjork et al. (2014)
Glyceraldehyde-3-phosphate	Reference gene	F R	CGC GTC GCT GAC CTG TT TGT GAC ACT GGA GGG TGT GAG T	99.35	This study

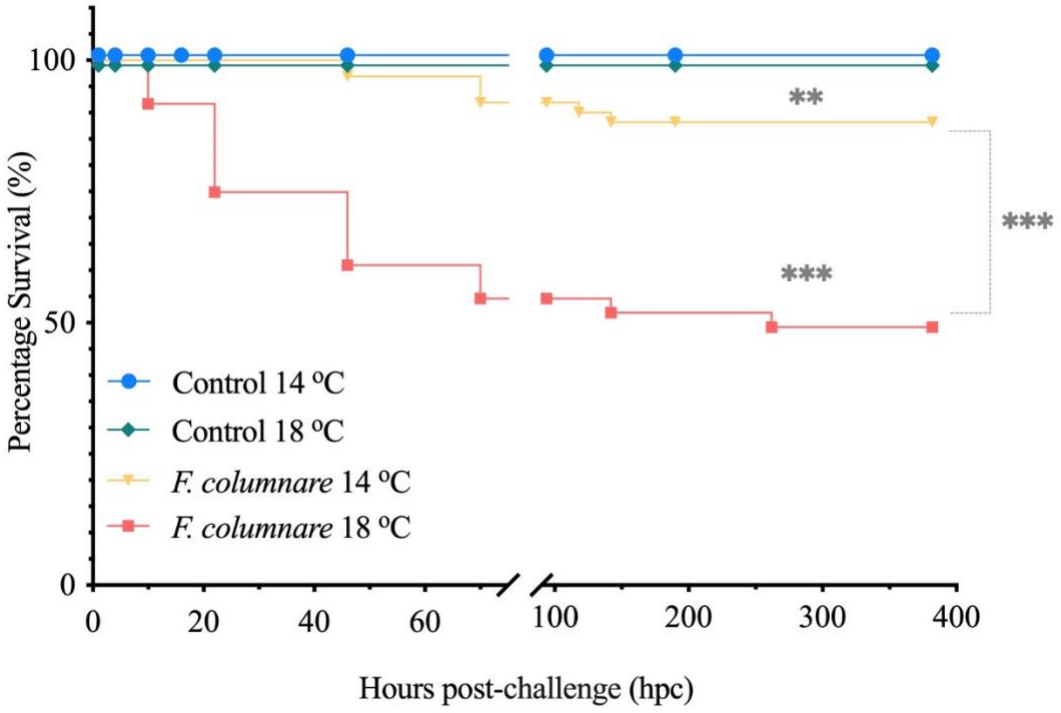


Figure 1.1: Mean survival for juvenile Chinook salmon (*Oncorhynchus tshawytscha*) challenged with modified-Shieh broth (MSB; control) or ~6.4 x 10^6 CFU mL^{-1} *Flavobacterium columnare via* bath immersion for 2h at 14 °C or 18 °C and monitored for 16 days. Asterisks indicate statistical significance (** $p < 0.01$, and *** $p < 0.001$), as determined by log-rank Mantel-Cox and Gehan-Breslow-Wilcoxon tests.

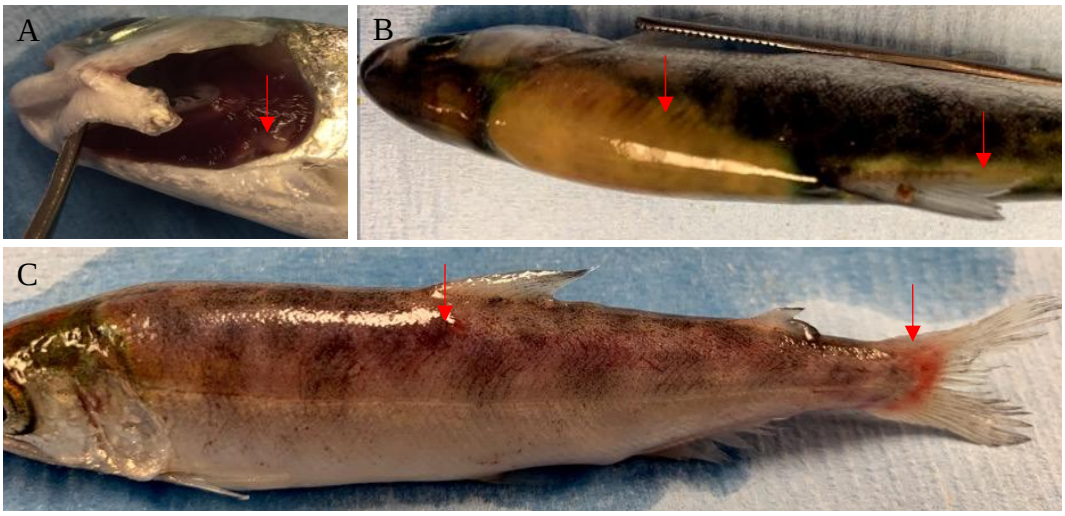


Figure 1.2: Morphological findings in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) collected following challenge with *Flavobacterium columnare* at 18 °C **A)** pale gills with yellow-white patches (arrow), **B)** discolorations of the skin (mostly initiated at the base of the dorsal fin, arrows), **C)** frayed fins and erythematous skin (arrows).

Table 1.2: Re-isolation of *Flavobacterium columnare* from gills and kidney of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) at different hours post-challenge.

	Hours post-challenge (hpc)											
	1		4		10		22		46		94	
	Gills	Kidney	Gills	Kidney	Gills	Kidney	Gills	Kidney	Gills	Kidney	Gills	Kidney
F. columnare 14 °C*	6/6	0/6	6/6	0/6	6/6	0/6	6/6	6/6	6/6	2/6	6/6	0/6
F. columnare 18 °C*	6/6	0/6	6/6	0/6	6/6	6/6	6/6	2/6	6/6	5/6	5/6	0/6

*No *Flavobacterium columnare* was detected in control fish, nor at 190, or 382 hpc from challenged fish at 14 °C or 18 °C.

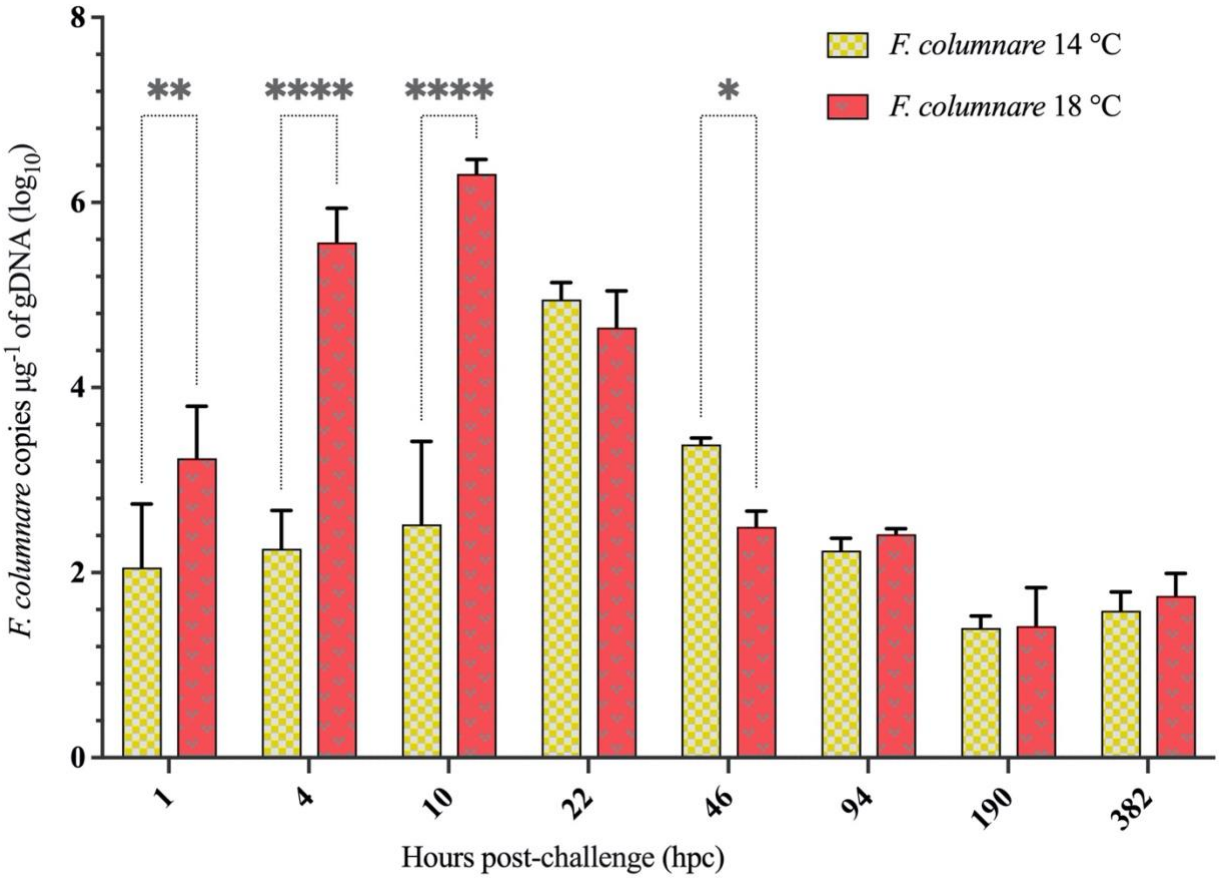


Figure 1.3: *Flavobacterium columnare* abundance in challenged juvenile Chinook salmon (*Oncorhynchus tshawytscha*) presented in $\log_{10}$-transformed copy number μg^{-1} of gDNA in gills at 14 °C or 18 °C from 1- to 382-hours post-challenge. Data represent mean ± SEM (n = 3 treatment^{-1}). Asterisks indicate statistical significance (***** $p < 0.05$, ****** $p < 0.01$, and ******** $p < 0.0001$) as determined by two-way ANOVA and Tukey's multiple comparisons tests.

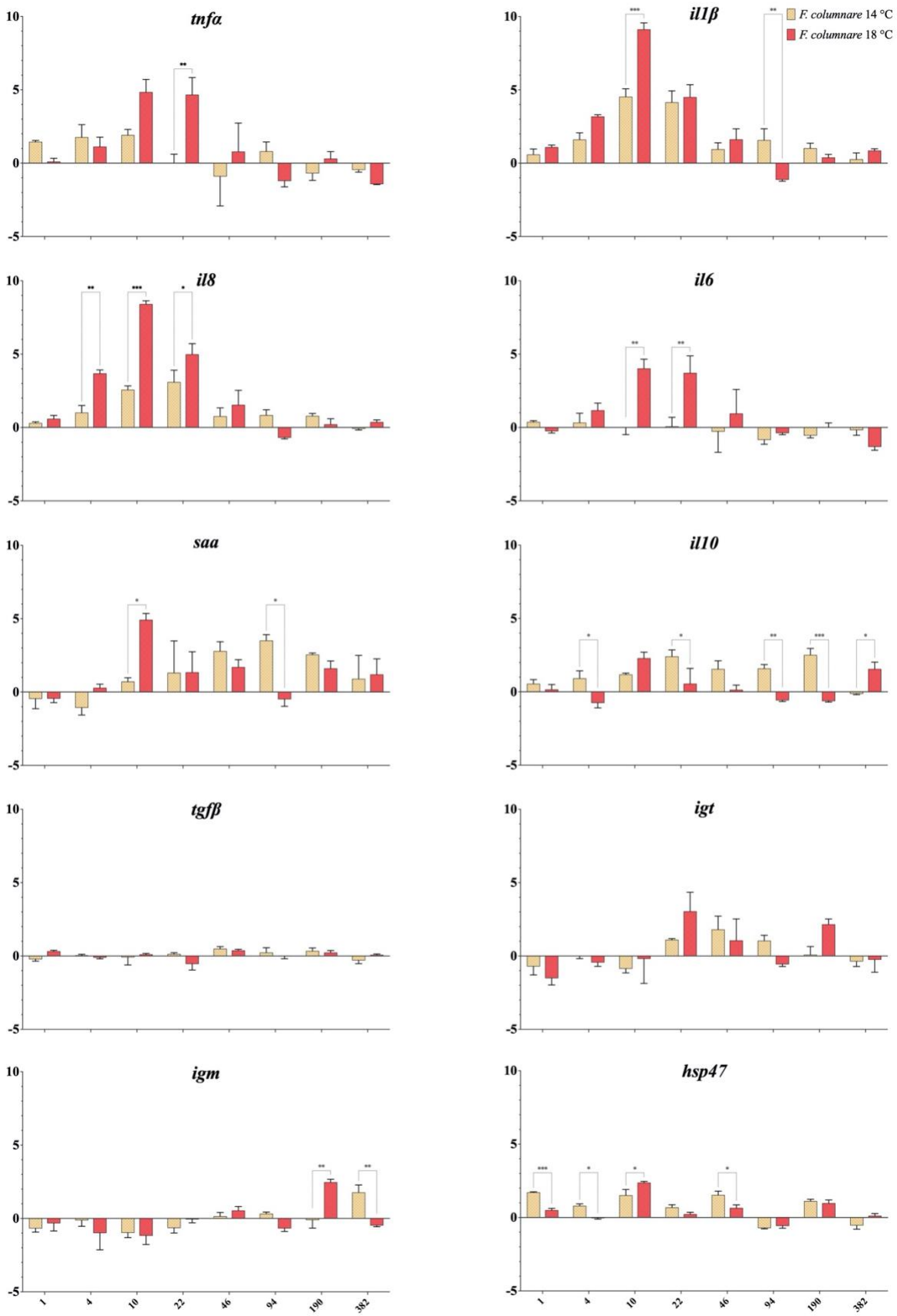

tnfα
il1β
il8
il6
saa
il10
tgfβ
igt
igm
hsp47
F. columnare 14 °C
F. columnare 18 °C
10
5
0
-5
1
4
10
22
46
94
190
382

Figure 1.4: Gene expression in gills of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) post-challenge to modified Shieh broth (MSB; controls) or *Flavobacterium columnare* at 14 °C or 18 °C. Transcript abundance of pro-inflammatory genes: **A)** tumor necrosis factor α (*tnfα*), **B)** interleukin-1β (*il1β*), **C)** interleukin-8 (*il8*), **D)** interleukin-6 (*il6*), **E)** acute phase protein serum amyloid A (*saa*), Anti-inflammatory genes: **F)** interleukin-10 (*il10*), **G)** transforming growth factor β (*tgfβ*), Adaptive immune genes: **H)** immunoglobulin T (*igt*), **I)** immunoglobulin M (*igm*), General stress gene **J)** heat shock protein 47 (*hsp47*), are presented as a $\log_2$-fold change. Gene expression was normalized against the housekeeping genes *rpl*7, act*β*, and *gapdh* and subsequently expressed as fold change ($\log_2$) relative to transcript abundance of respective control fish (Mean ± SEM). Asterisks indicate statistical significance (***** $p < 0.05$, ****** $p < 0.01$, and ******* $p < 0.001$) as determined by t-test and Holm-Šídák multiple comparisons.

Supplementary Table S1.1: Gene expression in gills of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) post-challenge to *Flavobacterium columnare* or modified Shieh broth (MSB; controls) at 14 °C or 18 °C. Transcript abundance of pro-inflammatory genes: tumor necrosis factorα (*tnfα*), interleukin1β (*il1β*), interleukin 8 (*il8*), interleukin 6 (*il6*), acute phase protein serum amyloid A (*saa*), Anti-inflammatory genes: interleukin 10 (*il10*), transforming growth factor β (*tgfβ*), Adaptive immune genes: immunoglobulin T (*igt*), immunoglobulin M (*igm*), General stress gene: heat shock protein 47 (*hsp47*). Gene expression was normalized against the housekeeping genes *rpl7*, *actβ*, and *gapdh* and subsequently expressed as fold change (log_2) relative to [a] transcript expression of control fish at 14 °C, [b] transcript abundance of respective control (Mean ± SEM). Bold text highlights significance (*p*-value of < 0.05) as determined by t-test and Holm-Šídák multiple comparisons.

		1 hpc			4 hpc			10 hpc			22 hpc			46 hpc			94 hpc			190 hpc			382 hpc		
		Mean	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**	**Mean**	**SEM**	***p*-value**
tnfα	Control 14 °C	0.00	0.56	**0.019**	0.00	0.27	0.129	0.00	0.29	0.899	0.00	0.46	**<.001**	0.00	0.27	**0.040**	0.00	0.62	0.729	0.00	0.55	0.967	0.00	0.60	0.576
	Control 18 °C [a]	2.22	0.48		1.58	0.27		-0.28	0.34		-3.15	0.75		-1.98	0.27		-0.64	0.61		0.03	0.45		0.91	0.55	
	Control 14 °C	0.00	0.56	0.622	0.00	0.27	0.447	0.00	0.29	0.394	0.00	0.46	0.989	0.00	0.27	0.895	0.00	0.62	0.895	0.00	0.55	0.895	0.00	0.60	0.895
	F. columnare 14 °C [b]	1.45	0.10		1.77	0.85		1.91	0.39		0.01	0.60		-0.91	2.01		0.81	0.64		-0.69	0.48		-0.46	0.16	
	Control 18 °C	0.08	0.48	0.979	0.00	0.27	0.769	0.00	0.34	**<.001**	-0.11	0.75	**<.001**	0.00	0.27	0.842	0.00	0.61	0.769	0.00	0.45	0.948	0.00	0.55	0.70
	F. columnare 18 °C [b]	0.11	0.22		1.12	0.65		4.84	0.86		4.66	1.17		0.78	1.95		-1.21	0.40		0.31	0.48		-1.43	0.03	
	F. columnare 14 °C [b]	1.45	0.10	0.752	1.77	0.85	0.819	1.91	0.39	0.172	0.01	0.60	**0.007**	-0.91	2.01	0.650	0.81	0.64	0.534	-0.69	0.48	0.819	-0.46	0.16	0.819
	F. columnare 18 °C [b]	0.11	0.22		1.12	0.65		4.84	0.86		4.66	1.17		0.78	1.95		-1.21	0.40		0.31	0.48		-1.43	0.03	
il1β	Control 14 °C	0.00	0.30	**<.001**	0.00	0.61	0.599	0.00	0.45	0.998	0.00	0.59	**0.998**	0.00	0.04	**0.998**	0.00	0.23	0.998	0.00	0.20	0.998	0.00	0.19	0.998
	Control 18 °C [a]	2.70	0.64		0.94	0.06		0.22	0.28		-0.17	0.38		-0.25	0.50		-0.07	0.79		-0.27	0.18		0.22	0.28	
	Control 14 °C	0.00	0.30	0.616	0.00	0.61	0.119	0.00	0.45	<.001	0.00	0.59	<.001	0.00	0.04	0.437	0.00	0.23	0.119	0.00	0.20	0.437	0.00	0.19	0.694
	F. columnare 14 °C [b]	0.59	0.38		1.61	0.46		4.53	0.54		4.15	0.78		0.95	0.44		1.57	0.78		1.02	0.35		0.26	0.44	
	Control 18 °C	0.09	0.64	0.320	0.00	0.06	<.001	0.00	0.28	**<.001**	0.15	0.38	**<.001**	0.00	0.50	0.073	0.00	0.79	0.295	0.00	0.18	0.543	0.00	0.28	0.33
	F. columnare 18 °C [b]	1.09	0.14		3.18	0.12		9.12	0.45		4.51	0.84		1.62	0.72		-1.13	0.10		0.39	0.21		0.86	0.13	
	F. columnare 14 °C [b]	0.59	0.38	0.880	1.61	0.46	0.176	4.53	0.54	**<.001**	4.15	0.78	0.880	0.95	0.44	0.880	1.57	0.78	**0.004**	1.02	0.35	0.880	0.26	0.44	0.880
	F. columnare 18 °C [b]	1.09	0.14		3.18	0.12		9.12	0.45		4.51	0.84		1.62	0.72		-1.13	0.10		0.39	0.21		0.86	0.13	
il8	Control 14 °C	0.00	0.21	**0.008**	0.00	0.18	0.320	0.00	0.27	0.995	0.00	0.68	**0.002**	0.00	0.14	**0.997**	0.00	0.12	0.997	0.00	0.14	0.997	0.00	0.03	0.997
	Control 18 °C [a]	1.30	0.10		0.70	0.30		0.16	0.19		-1.51	0.24		-0.02	0.34		0.11	0.15		0.05	0.18		0.07	0.14	
	Control 14 °C	0.00	0.21	0.807	0.00	0.18	0.308	0.00	0.27	<.001	0.00	0.68	<.001	0.00	0.14	0.453	0.00	0.12	0.453	0.00	0.14	0.453	0.00	0.03	0.871
	F. columnare 14 °C [b]	0.30	0.08		1.01	0.49		2.57	0.26		3.09	0.81		0.76	0.57		0.84	0.37		0.79	0.17		-0.08	0.08	
	Control 18 °C	0.00	0.10	0.295	0.00	0.30	0.005	0.00	0.19	**<.001**	0.01	0.24	**0.018**	0.00	0.34	0.391	0.00	0.15	0.078	0.00	0.18	0.639	0.00	0.14	0.39
	F. columnare 18 °C [b]	0.59	0.24		3.68	0.24		8.41	0.23		4.98	0.73		1.54	0.99		-0.69	0.08		0.21	0.38		0.37	0.15	
	F. columnare 14 °C [b]	0.30	0.08	0.761	1.01	0.49	**0.002**	2.57	0.26	**<.001**	3.09	0.81	**0.036**	0.76	0.57	0.664	0.84	0.37	0.113	0.79	0.17	0.761	-0.08	0.08	0.761
	F. columnare 18 °C [b]	0.59	0.24		3.68	0.24		8.41	0.23		4.98	0.73		1.54	0.99		-0.69	0.08		0.21	0.38		0.37	0.15	
il6	Control 14 °C	0.00	0.01	**0.540**	0.00	0.05	0.568	0.00	0.10	0.011	0.00	0.53	**<.001**	0.00	0.13	**0.338**	0.00	0.33	0.24	0.00	0.02	0.54	0.00	0.17	0.681
	Control 18 °C [a]	0.47	0.12		0.35	0.07		-1.26	0.18		-2.29	0.56		-0.66	0.28		-0.76	0.24		0.50	0.33		0.15	0.04	
	Control 14 °C	0.00	0.01	0.995	0.00	0.05	0.995	0.00	0.10	0.995	0.00	0.53	0.995	0.00	0.13	0.995	0.00	0.33	0.872	0.00	0.02	0.98	0.00	0.17	0.995
	F. columnare 14 °C [b]	0.38	0.09		0.33	0.65		-0.02	0.46		0.08	0.62		-0.27	1.42		-0.84	0.30		-0.55	0.16		-0.18	0.35	
	Control 18 °C	-0.05	0.12	0.961	0.00	0.07	0.600	0.00	0.18	**<.001**	-0.05	0.56	**<.001**	0.00	0.28	0.701	0.00	0.24	0.957	0.00	0.33	0.979	0.00	0.04	0.54
	F. columnare 18 °C [b]	-0.25	0.12		1.17	0.49		4.02	0.64		3.72	1.16		0.95	1.65		-0.38	0.11		0.02	0.29		-1.32	0.23	
	F. columnare 14 °C [b]	0.38	0.09	0.901	0.33	0.65	0.879	-0.02	0.46	**0.003**	0.08	0.62	**0.007**	-0.27	1.42	0.801	-0.84	0.30	0.901	-0.55	0.16	0.901	-0.18	0.35	0.801
	F. columnare 18 °C [b]	-0.25	0.12		1.17	0.49		4.02	0.64		3.72	1.16		0.95	1.65		-0.38	0.11		0.02	0.29		-1.32	0.23	
saa	Control 14 °C	0.00	0.60	0.191	0.00	0.43	**0.015**	0.00	0.46	**0.01**	0.00	0.89	**0.017**	0.00	0.55	**0.020**	0.00	0.25	0.977	0.00	0.82	0.977	0.00	0.37	0.298
	Control 18 °C [a]	1.58	0.09		2.61	0.37		2.74	0.56		2.52	0.40		2.42	0.37		-0.15	0.98		0.05	0.55		1.28	0.35	

	Control 14 °C	0.00	0.60	0.849	0.00	0.43	0.849	0.00	0.46	0.849	0.00	0.89	0.808	0.00	0.55	0.168	0.00	0.25	0.048	0.00	0.82	0.22	0.00	0.37	0.849
	F. columnare 14 °C [b]	-0.46	0.68		-1.07	0.50		0.71	0.27		1.31	2.18		2.79	0.64		3.51	0.40		2.55	0.11		0.90	1.60	
	Control 18 °C	-0.01	0.09	0.929	0.00	0.37	0.929	0.00	0.56	**<.001**	0.16	0.40	**0.646**	0.00	0.37	0.372	0.00	0.98	0.929	0.00	0.55	0.393	0.00	0.35	0.65
	F. columnare 18 °C [b]	-0.45	0.28		0.29	0.25		4.92	0.43		1.34	1.41		1.70	0.50		-0.49	0.48		1.61	0.51		1.20	1.07	
	F. columnare 14 °C [b]	-0.46	0.68	>.999	-1.07	0.50	0.875	0.71	0.27	**0.018**	1.31	2.18	>.999	2.79	0.64	0.921	3.51	0.40	**0.024**	2.55	0.11	0.921	0.90	1.60	0.994
	F. columnare 18 °C [b]	-0.45	0.28		0.29	0.25		4.92	0.43		1.34	1.41		1.70	0.50		-0.49	0.48		1.61	0.51		1.20	1.07	
	Control 14 °C	0.00	0.17	**0.052**	0.00	0.08	<.001	0.00	0.13	0.015	0.00	0.30	**0.08**	0.00	0.25	**0.052**	0.00	0.36	0.94	0.00	0.41	0.044	0.00	0.18	0.448
	Control 18 °C [a]	1.14	0.10		2.02	0.49		1.40	0.24		0.97	0.12		1.13	0.47		0.03	0.47		1.20	0.27		-0.49	0.19	
ill10	Control 14 °C	0.00	0.17	0.425	0.00	0.08	0.154	0.00	0.13	0.059	0.00	0.30	<.001	0.00	0.25	0.01	0.00	0.36	0.009	0.00	0.41	<.001	0.00	0.18	0.776
	F. columnare 14 °C [b]	0.55	0.29		0.92	0.51		1.18	0.09		2.41	0.45		1.55	0.57		1.59	0.27		2.51	0.45		-0.13	0.06	
	Control 18 °C	0.03	0.10	0.966	0.00	0.49	0.742	0.00	0.24	**0.003**	0.03	0.12	**0.814**	0.00	0.47	0.966	0.00	0.47	0.814	0.00	0.27	0.814	0.00	0.19	0.08
	F. columnare 18 °C [b]	0.15	0.34		-0.76	0.34		2.29	0.41		0.55	1.04		0.14	0.32		-0.58	0.08		-0.63	0.07		1.55	0.48	
	F. columnare 14 °C [b]	0.55	0.29	0.525	0.92	0.51	**0.047**	1.18	0.09	0.150	2.41	0.45	**0.028**	1.55	0.57	0.080	1.59	0.27	**0.008**	2.51	0.45	**<.001**	-0.13	0.06	**0.047**
	F. columnare 18 °C [b]	0.15	0.34		-0.76	0.34		2.29	0.41		0.55	1.04		0.14	0.32		-0.58	0.08		-0.63	0.07		1.55	0.48	
	Control 14 °C	0.00	0.11	**0.819**	0.00	0.03	0.531	0.00	0.10	0.778	0.00	0.09	**0.868**	0.00	0.07	**0.701**	0.00	0.16	0.868	0.00	0.08	0.778	0.00	0.17	0.868
	Control 18 °C [a]	-0.18	0.17		0.33	0.11		-0.23	0.07		0.12	0.13		0.27	0.12		-0.13	0.04		0.23	0.27		0.11	0.18	
	Control 14 °C	0.00	0.11	0.932	0.00	0.03	0.950	0.00	0.10	0.950	0.00	0.09	0.95	0.00	0.07	0.522	0.00	0.16	0.932	0.00	0.08	0.841	0.00	0.17	0.866
tgfβ	*F. columnare* 14 °C [b]	-0.22	0.13		0.04	0.07		-0.09	0.53		0.14	0.09		0.49	0.15		0.23	0.33		0.34	0.20		-0.30	0.22	
	Control 18 °C	-0.05	0.17	0.572	0.00	0.11	0.984	0.00	0.07	**0.984**	-0.05	0.13	**0.288**	0.00	0.12	0.572	0.00	0.04	0.984	0.00	0.27	0.867	0.00	0.18	0.98
	F. columnare 18 °C [b]	0.32	0.07		-0.11	0.09		0.10	0.07		-0.54	0.42		0.38	0.07		-0.02	0.17		0.23	0.14		0.08	0.06	
	F. columnare 14 °C [b]	-0.22	0.13	0.487	0.04	0.07	0.955	-0.09	0.53	0.955	0.14	0.09	0.256	0.49	0.15	0.955	0.23	0.33	0.934	0.34	0.20	0.955	-0.30	0.22	0.792
	F. columnare 18 °C [b]	0.32	0.07		-0.11	0.09		0.10	0.07		-0.54	0.42		0.38	0.07		-0.02	0.17		0.23	0.14		0.08	0.06	
	Control 14 °C	0.00	0.19	**0.836**	0.00	0.29	0.316	0.00	0.37	0.028	0.00	0.33	**0.651**	0.00	0.39	**0.992**	0.00	0.46	0.384	0.00	0.64	0.384	0.00	0.91	0.858
	Control 18 °C [a]	-0.57	0.36		-1.51	0.60		-2.37	0.33		-0.92	0.07		-0.01	0.20		-1.35	1.36		-1.37	0.17		-0.37	0.15	
	Control 14 °C	0.00	0.19	0.836	0.00	0.29	0.316	0.00	0.37	0.028	0.00	0.33	0.651	0.00	0.39	0.992	0.00	0.46	0.384	0.00	0.64	0.384	0.00	0.91	0.858
igt	*F. columnare* 14 °C [b]	-0.57	0.36		-1.51	0.60		-2.37	0.33		-0.92	0.07		-0.01	0.20		-1.35	1.36		-1.37	0.17		-0.37	0.15	
	Control 18 °C	0.10	0.36	0.665	0.00	0.60	0.980	0.00	0.33	**0.980**	0.00	0.07	**0.093**	0.00	0.20	0.893	0.00	1.36	0.98	0.00	0.17	0.391	0.00	0.15	0.98
	F. columnare 18 °C [b]	-1.52	0.46		-0.44	0.26		-0.20	1.67		3.03	1.31		1.06	1.47		-0.57	0.15		2.15	0.37		-0.25	0.86	
	F. columnare 14 °C [b]	-0.57	0.36	0.959	-1.51	0.60	0.959	-2.37	0.33	0.959	-0.92	0.07	0.480	-0.01	0.20	0.959	-1.35	1.36	0.636	-1.37	0.17	0.439	-0.37	0.15	0.959
	F. columnare 18 °C [b]	-1.52	0.46		-0.44	0.26		-0.20	1.67		3.03	1.31		1.06	1.47		-0.57	0.15		2.15	0.37		-0.25	0.86	
	Control 14 °C	0.00	0.59	**0.963**	0.00	0.20	0.911	0.00	0.23	0.019	0.00	0.88	**0.876**	0.00	0.40	**0.955**	0.00	0.62	0.612	0.00	0.35	0.911	0.00	0.62	0.955
	Control 18 °C [a]	-0.04	0.56		-0.68	0.59		-2.53	0.32		-0.82	0.58		-0.36	0.12		-1.20	0.91		-0.67	0.48		-0.25	0.48	
	Control 14 °C	0.00	0.59	0.888	0.00	0.20	0.994	0.00	0.23	0.657	0.00	0.88	0.888	0.00	0.40	0.994	0.00	0.62	0.98	0.00	0.35	0.994	0.00	0.62	0.079
igm	*F. columnare* 14 °C [b]	-0.68	0.25		-0.13	0.41		-0.98	0.32		-0.65	0.35		0.15	0.26		0.32	0.11		-0.11	0.55		1.77	0.51	
	Control 18 °C	0.11	0.56	0.923	0.00	0.59	0.715	0.00	0.32	**0.597**	0.18	0.58	**0.923**	0.00	0.12	0.923	0.00	0.91	0.899	0.00	0.48	0.017	0.00	0.21	0.92
	F. columnare 18 °C [b]	-0.33	0.53		-1.00	1.14		-1.18	0.60		-0.06	0.24		0.54	0.28		-0.68	0.21		2.47	0.20		-0.48	0.08	

	F. columnare 14 °C [b]	-0.68	0.25	0.907	-0.13	0.41	0.636	-0.98	0.32	0.907	-0.65	0.35	0.833	0.15	0.26	0.907	0.32	0.11	0.560	-0.11	0.55	**0.003**	1.77	0.51	**0.009**
	F. columnare 18 °C [b]	-0.33	0.53		-1.00	1.14		-1.18	0.60		-0.06	0.24		0.54	0.28		-0.68	0.21		2.47	0.20		-0.48	0.08	
	Control 14 °C	0.00	0.19	**<.001**	0.00	0.02	<.001	0.00	0.23	<.001	0.00	0.32	**<.001**	0.00	0.16	**<.001**	0.00	0.12	0.509	0.00	0.12	<.001	0.00	0.29	<.001
	Control 18 °C [a]	1.95	0.21		1.64	0.20		2.86	0.45		1.61	0.18		3.69	0.07		0.21	0.20		1.43	0.27		1.84	0.18	
	Control 14 °C	0.00	0.19	<.001	0.00	0.02	0.045	0.00	0.23	<.001	0.00	0.32	0.062	0.00	0.16	<.001	0.00	0.12	0.062	0.00	0.12	0.003	0.00	0.29	0.077
hsp 47	*F. columnare* 14 °C [b]	1.71	0.04		0.79	0.14		1.51	0.40		0.69	0.18		1.53	0.27		-0.72	0.05		1.12	0.12		-0.54	0.26	
	Control 18 °C	0.08	0.21	0.489	0.00	0.20	0.908	0.00	0.45	**<.001**	-0.07	0.18	**0.672**	0.00	0.07	0.163	0.00	0.20	0.243	0.00	0.27	0.012	0.00	0.18	0.91
	F. columnare 18 °C [b]	0.50	0.13		-0.04	0.07		2.37	0.09		0.23	0.13		0.65	0.21		-0.57	0.15		0.97	0.22		0.11	0.16	
	F. columnare 14 °C [b]	1.71	0.04	**<.001**	0.79	0.14	**0.017**	1.51	0.40	**0.015**	0.69	0.18	0.240	1.53	0.27	**0.015**	-0.72	0.05	0.828	1.12	0.12	0.828	-0.54	0.26	0.073
	F. columnare 18 °C [b]	0.50	0.13		-0.04	0.07		2.37	0.09		0.23	0.13		0.65	0.21		-0.57	0.15		0.97	0.22		0.11	0.16	

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CHAPTER 2: Effect of increased water temperature and *Flavobacterium columnare* infection on swimming behavior and transcriptional responses in juvenile Chinook salmon (*Oncorhynchus tshawytscha*)

A similar version of this chapter is in preparation for submission to
the journal of San Francisco Estuary and Watershed Science

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ABSTRACT

Associated with climate change-driven rising water temperatures, diseases are among the most pressing risk factors that contribute to the imperiled status of salmonids in the Pacific Northwest (PNW). *Flavobacterium columnare* is a significant bacterial pathogen of wild and cultured fish species and one of the etiological agents of columnaris disease. In the Pacific Northwest, columnaris is reported as reemerging, especially during summer and early fall. This study aimed to determine whether, and to what extent, *F. columnare* infection affects Chinook salmon (*Oncorhynchus tshawytscha*) swimming behavior at a commonly occurring elevated temperature, 18 °C. To evaluate this, we determined swimming behavior, infection progression in terms of *F. columnare* abundance in gill, as well as expression of behavior-related genes in brain tissues. Fish were assigned to one of two experimental groups: *F. columnare* challenged or non-challenged, both maintained at 18 °C. Twelve fish per group were sampled at 1-, 22-, and 142-hours post-challenge (hpc). In this study, challenged fish were less receptive to the presence of the novel object, whereas control fish exhibited a range of behavioral responses. *Flavobacterium columnare* abundance, as determined by qPCR was significantly elevated in challenged fish, peaking at 22 hpc. Change in the expression of behavior-related genes was seen in challenged fish. This response, however, did not affect fish behavior in an additive manner. This study provides a foundation for future avenues of research concerning out-migrating Chinook salmon in the Sacramento River.

Keywords: *Flavobacterium columnare*; Columnaris; Temperature**;** Chinook Salmon; swimming behavior; Gene expression

1. INTRODUCTION

There is global concern over fish population declines, particularly for salmonid species (Ruckelshaus et al., 2002). Pacific salmon, *Oncorhynchus spp.*, is a genus of iconic fish species that have significant symbolic, economic, and recreational value to communities in the United States Pacific Northwest (PNW) (Lichatowich and Lichatowich, 2001). Pacific salmon are considered a keystone species for maintaining both terrestrial and aquatic ecosystems (Cederholm et al., 1999; Gende et al., 2002). Chinook salmon (*Oncorhynchus tshawytscha*), the largest in the genus *Oncorhynchus*, are anadromous salmonids native to the North Pacific Ocean. The Sacramento River in Northern California supports four Chinook salmon population runs, named for the time of year adults return to freshwater: fall, late-fall, winter, and spring run, with the last two listed as endangered and threatened, respectively (Moyle et al., 2017). Fall-run has been designated as a "species of concern" according to the National Oceanic and Atmospheric Administration (NOAA) Fisheries (Buchanan et al., 2018). During outmigration, juveniles experience high mortality, primarily due to habitat degradation, climate change, and increased predation risks by non-native species (Miller et al., 2014). Chinook salmon are projected to be further impacted by climate change as water reservoir storage decreases, reducing river flows and increasing water temperatures (Moyle et al., 2017).

Infectious diseases are among the most pressing risk factors linked to declining of Chinook salmon - a risk that may increase with increasing temperatures (Lehman et al., 2020). While disease outbreaks can occasionally have devastating impacts, salmon populations may also be indirectly affected by increased predation, when infections impair physiological performance (Miller et al., 2014). Individuals that exert effort to combat infection may do so at the expense of their swimming performance, diminishing their ability to evade predators (Lehman et al., 2020).

Infected salmon have been found to have decreased critical swimming speed (Kocan et al., 2009; Tierney and Farrell, 2004) and higher predation risks (Mesa et al., 1998; Miller et al., 2014). The extent to which the indirect impacts of infectious diseases affect salmon population dynamics is still poorly understood. This is further complicated by the various and dynamic environmental factors that salmon encounter throughout their migratory life cycle.

Flavobacterium columnare, one of the causative agents of columnaris disease, causes substantial mortality in a wide range of wild and cultured freshwater fish worldwide (reviewed by Declercq et al.,2013; LaFrentz et al., 2022). *Flavobacterium columnare* is ubiquitous in the aquatic environment and outbreaks are often triggered during the spring and summer months (Mohammed and Arias, 2014). *Flavobacterium columnare* can persist in subclinical carrier fish or environmental reservoirs (Kunttu et al., 2012; Suomalainen et al., 2005) posing a threat to out-migrating juvenile salmonids (Becker, 1978; Sebastião et al., 2021). Columnaris disease is notorious for affecting the external mucosal surfaces of fry and fingerlings, causing rapid and widespread destruction of gills, skin, and fins (Lafrentz et al., 2012), and causing nonspecific clinical signs such as lethargy, anorexia, and swimming near the surface. To date, the majority of studies on *F. columnare* have been focused on the mode and dynamics of adhesion (Olivares-Fuster et al., 2011), virulence (Darwish and Ismaiel, 2005; Thomas-Jinu and Goodwin, 2004), and the host's adaptive immunity (Grabowski et al., 2004; Shoemaker et al., 2011). *Flavobacterium columnare* as a cause of mortality may be significantly greater in *toto* than just the mortality directly attributable to the bacterium. There is very little information on how this bacterium affects salmon behavior and how changes in behavior could affect the capacity of salmon to avoid predation or forage for food.

Organismal behavior integrates all physiological and genetic networks' activities, thus there is a potential to infer pathophysiological changes from altered behavior of infected fish, including swimming performance (Castro et al., 2013). Behavioral tests have recently garnered interest in understanding how co-occurring changes in host behavior and pathogen burden would aid in elucidating useful host-pathogen causation linkages to declines in performance (Estes et al., 2021; Gopko et al., 2019; Lee et al., 2015; Yi et al., 2019). These types of tests have recently been adapted for use with aquatic species to examine a multitude of cognitive/emotional processes including memory capacity, aggression, locomotion, boldness, and anxiety-like behavior (Hamilton et al., 2022). Open field and novel object approach tests have been used extensively in behavioral neuroscience research and are well-validated measures of anxiety-like behavior (Hamilton et al., 2021a; Johnson et al., 2022; Maximino et al., 2010; Stewart et al., 2012; Szaszkiewicz et al., 2021). The open field test evaluates fish locomotion in response to simultaneous opposing incentives, such as exploration for foraging or mating or minimization of movement to avoid potential predators (Champagne et al., 2010). Movement serves as the foundation for many behavioral traits that are assessed through behavioral tests (Ramos, 2008), hence quantifying different aspects of locomotion is common in behavioral research. The open field test can therefore provide insight into anxiety-related behaviors including thigmotaxic (wall-hugging) behavior (Basnet et al., 2019). In our study, we utilized the open field test to examine fish locomotion, in particular, total distance moved, mean velocity, and meandering. The novel object approach test involves placing an object that the fish has not previously experienced in the center of an open field arena (Johnson and Hamilton, 2017). The novel object approach test measures anxiety as the proportion of time spent inspecting a novel object (Johnson and Hamilton, 2017; Hamilton *et al.*, 2021a, 2021b).

Neuropeptides are diverse neuron-secreted peptides that have neuromodulatory, neurotransmitter, or hormonal functions as well as various pluripotent functions. These functions include stimulating the endocrine system to balance immunity to pathogen and immunological tolerance, regulating the circadian rhythm, and regulating appetite, as summarized by (Li et al., 2022b). Neuropeptides are derived from neuropeptide precursor genes that have undergone multiple duplications and losses in fish (Hu et al., 2016). Characterizing the molecular basis of the interaction between pathogen and host in the context of behavioral change is essential. Changes in the expression of genes can demonstrate their role in behavioral variation and/or phenotypic plasticity, which would allow for the development of biomarkers of behavior in the lab and in the field (Fitzpatrick et al., 2005). Few studies have particularly shown an association between gene expression and other physiological parameters or fitness-related traits crucial for fish conservation (Connon et al., 2018; Houde et al., 2019; Oomen and Hutchings, 2017).

Sub-lethal behavioral effects caused by bacterial infection can have long-lasting repercussions on fish ecological fitness through effects on predator avoidance and foraging success. Therefore, the focus of our study was to investigate how *F. columnare* challenge affects a range of behavioral indices in Chinook salmon. Specifically, we conducted the current study to determine whether *F. columnare* infection can alter the behavior of juvenile fall-run, as a sentinel for other runs of Chinook salmon. We quantified locomotion and anxiety-like behavior. We used the open field test to assess locomotion followed by a novel object approach test to quantify anxiety. Additionally, we assessed *F. columnare* abundance in gills, and examined changes in expression of genes associated with behavior, in the brain tissue. These measures could help to gain insight into alterations in physiological pathways that may contribute to changes in swimming behavior.

2. MATERIALS & METHODS

2.1 Experimental fish and acclimation

All fish care and use protocols were reviewed and approved by the University of California Davis (UC Davis) Institutional Animal Care and Use Committee (IACUC Protocol # 19499). Juvenile fall-run Chinook salmon fry (~3 months of age) were transported from the Mokelumne River Hatchery (California Department of Fish and Wildlife, Clements, CA, USA) to the Center for Aquatic Biology and Aquaculture (University of California, Davis, CA, USA). Fish were mainrained in a flow-through ultraviolet-filtered well water 980-L tank with continuous aeration at 11 °C. Fish were fed with a pelleted salmon diet (Salmon Sink; Skretting, Toole, UT, USA) at 2% body weight per day. Prior to the experiment, fish were acclimated to 18 °C, with temperature adjusted at a rate of 1 °C day^{-1}. A temperature of 18 °C was chosen since this is an elevated temperature, typically recorded in PNW watersheds (Billman et al., 2014); when conditions (high temperature, low flow) are favorable to *F. columnare* epizootics (Austin and Austin, 2016; Wakabayashi, 1991). After reaching the target temperature, fish were acclimated for a further 14 days prior to *F. columnare* challenge. Ten fish were euthanized with tricaine methanesulfonate (MS-222; 500 mg L^{-1}, buffered with sodium bicarbonate) at the end of the acclimation period, and sampled for necropsy and posterior kidney bacterial culture, to confirm that fish were *F. oolumnare*-free prior to the bacterial challenge.

2.2 Bacterial isolate and growth conditions

Flavobacterium columnare (117/MK981921) used in this study was isolated from a Chinook salmon's kidney from a hatchery in California during a 2017 outbreak (Sebastião et al., 2021). The bacterium was sourced from frozen stock, streaked onto modified-Shieh agar (MSA;

LaFrentz and Klesius, 2009) supplemented with tobramycin (1 μg mL^{-1}; Decostere *et al.*, 1997), and incubated at 28 °C for 48 h. Two flat, rhizoid, yellow colonies with irregular margins tightly adherent to the agar were selected and inoculated into 5 mL of modified-Shieh broth (MSB) supplemented with tobramycin and incubated at 28 °C for 17–18 h with shaking at 180 rpm (Declercq et al., 2013). This initial culture was then introduced into 110 mL of MSB in a 500 mL Erlenmeyer flask and incubated at 28 °C for ~17–18 h with shaking at 180 rpm. Cultures were then adjusted using MSB to an optical density of 0.9 read on a UV/Vis photometer (BioPhotometer Plus, Eppendorf AG, Hamburg, Germany) at 600 nm [OD_{600}] to produce ~10^9 colony-forming units [CFU] mL^{-1}.

2.3 *Flavobacterium columnare* dose determination challenge

To identify a suitable *F. columnare* dosage that could be used for the challenge experiment, an acute challenge was conducted using triplicate groups of ten fish per tank (mean fork length ± SD, 8.4 ± 0.6 cm; mean mass ± SD, 8.2 ± 1.7 g) receiving air-equilibrated well-water flow-through at a rate of 700 mL $minute^{-1}$, with dissolved oxygen (DO) levels kept at ~9 mg O_2 L^{-1}.. *Flavobacterium columnare* challenge was carried out as described by Soto *et al.* (2008) with slight modifications. Briefly, water levels were reduced, water flow was suspended, and constant aeration was maintained. *Flavobacterium columnare* suspension was inoculated into 10 L tank water to achieve a challenge concentration of 1.33×10^6, 2.67×10^6, 1.33×10^7, 2.67×10^7, and 2.67×10^8 CFU mL^{-1} for 2 h in static conditions. A sixth group was mock-challenged by immersion for 2 h in 10 L of water containing comparable volumes of sterile MSB. Dead fish were removed, and mortality was recorded, twice daily for 96 h. For the challenge experiment, therefore, we chose an

infection dose of ~ 1.8×10^7 CFU mL^{-1} to ensure infection and sufficient survivors for assessing behavior following *F. columnare* challenge over a study period of 6 days.

2.4 *Flavobacterium columnare* laboratory-controlled challenge

Fish (n = 120) were haphazardly assigned to aerated 6, 130-L fiberglass tanks containing ~100-L (20 fish $tank^{-1}$) receiving air-equilibrated well-water flow-through at a rate of three liters $minute^{-1}$. Fish were assigned to one of two experimental groups in triplicate tanks per treatment: *F. columnare* challenged or non-challenged. On the day of the challenge, water levels were reduced from 100-L to 50-L, water flow was suspended, and constant aeration was maintained. To achieve a final dose of ~ 1.8×10^7 CFU mL^{-1}, 300 mL of cultivated MSB was diluted in 1-L of water before adding this solution directly to the tank. As natural infection route of *F. columnare*, fish were exposed *via* bath immersion for 2 h before water flow was restored. Comparable volumes of MSB were used in mock immersion challenges for fish in the control group. A subset of four fish from each replicate tank (12 fish $group^{-1}$) was arbitrarily sampled at 1, 22, and 142 hours post-challenge (hpc). For statistical analysis, challenges were staggered and repeated three times (n = 3) to account for batch effects across a total of two weeks.

2.5 Behavioral testing

Fish were observed in standardized behavioral tests intended to detect swimming behavioral responses following *F. columnare* challenge (Fig. 2.1).

2.5.1 Open field test (Fig. 2.1A)

Individual fish were gently transferred from their respective challenge or control tanks and carefully placed into the center of 55-gal (66.04 cm diameter) opaque, white plastic bucket (henceforth “arenas”), filled to a height of 18 cm with oxygenated well water. A camera (Dragon

Touch, Frederick, MD, USA) was placed within the lids of each bucket. Fish were allowed to adjust for ~5 min before the initiation of behavioral testing. Individual fish movement was tracked for 15-min, and their swimming activity was filmed using a video camera set ~1.0 m above the arena. A line was placed on the interior of the bucket to indicate a water fill line, to ensure constant filming conditions across trials. A second line was placed on the outside of the lid and bucket to indicate the placement of the lid, ensuring that the camera was always in the same location (Leal et al., 2021).

2.5.2 Novel object approach test (Fig. 2.1B)

The novel object approach test is designed to assess boldness or exploration (Toms et al., 2010). Following the open field test described above, a multicolored object used to rule out any intrinsic color preference (Hamilton et al., 2021a), was placed in the middle of the testing arena to quantify potential approach and time spent near the novel object. The object was slowly fastened to the arena with Velcro, after which a further fifteen-minute test commenced, during which the fish were free to swim in the arena and explore the novel object. Water in the arena was replaced after each fish to keep the temperature at 18 °C ± 1 °C, to prevent potential cross-infection and the build-up of waste products.

2.5.3 Behavior Quantification

For both the open field and novel object approach tests, two sections were marked out at the bottom of each arena by concentric circles, to delineate inner (0-23 cm diameter), and outer (23-66 cm diameter) zones. Movement was tracked for each fish and analyzed using EthoVision XT tracking software (version 15, Noldus, Leesburg, VA, USA), which determined multiple outputs for individual fish (Table 2.1). Total distance moved and mean velocity were measured for control and challenged fish. Time spent in the different zones of the arena was used to determine

thigmotaxis and anxiety. Other behavioral endpoints, such as absolute turn angle, absolute angular velocity, and meandering were measured to categorize locomotion. Heatmaps were generated in EthoVision, displaying a color range based on the mean for each group.

2.6 Tissue sampling and *F. columnare* re-isolation

After the behavioral trials, fish were removed from the arenas and immediately euthanized with buffered MS-222. Gills and posterior kidney were streaked on MSA plates and incubated at 28 °C for 48 h. Presumptive identification of isolates was based on colony color and morphology. Right gill arches and brain were excised, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent analyses: *F. columnare* copy number ng^{-1} genomic DNA, and gene expression, respectively.

2.7 Genomic DNA extraction, total RNA extraction, and cDNA synthesis

A QIAcube system 230 V (Qiagen, Redwood City, CA, USA) was used to extract genomic DNA (gDNA) and total RNA from gills using the DNeasy Blood and Tissue Kit (Qiagen) and RNeasy Plus Mini Kit (Qiagen) respectively, according to the manufacturer's protocol. Extracted gDNA and RNA were assessed for quality (by measuring the A260:280 and A260:230 ratios) and concentration (ng μL^{-1}) using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The integrity of the RNA was assessed by electrophoresis on a 1% w/v agarose gel. QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA from 1 μg of total RNA in 20 μL following the manufacturer's protocol. Generated cDNA template was diluted 1:10 with DEPC-treated water and then stored at −20 °C until analysis.

2.8 *Flavobacterium columnare* detection and quantification of in gills

Probe-based quantitative PCR (qPCR) was used for the detection and quantitation of *F. columnare* in gills targeting a 113 bp nucleotide region of the chondroitin AC lyase gene of *F. columnare* (GenBank accession number AY912281; (Panangala et al., 2007; Xie et al., 2004). *Flavobacterium columnare*-specific primers (forward 5′-CCTGTACCTAATTGGGGAAAAG AGG-3′ and reverse 5′-GCGGTTATGCCTTGTTTATCATAGA-3′) and a dual-labeled probe (5′-ACAACAATGATTTTGCAGGAGGAGTATCTGATGGG-3′) were used for specific detection of *F. columnare* (Panangala et al., 2007). Quantitative PCR reactions consisted of 6 μL of TaqMan™ Environmental Master Mix 2.0 (ThermoFisher Scientific), 0.4 μM of each primer, 0.08 μM of the probe, 5 μL of template gDNA, and nuclease-free water to a total volume of 12 μL. Plasmid DNA with specific sequences corresponding to *F. columnare* (Eurofins Genomics LLC, Louisville, KY, USA) was used to generate a standard curve of known copy numbers, as well as for assay quality control and quality assurance. All DNA samples along with 10-fold serially diluted plasmid standards were amplified in 384-well plates in a QuantStudio7 Flex Real-Time PCR System (Real-time PCR Research and Diagnostics Core Facility, UC Davis). All reactions were tested in triplicate under standard amplification conditions: 50 °C for 2 min; 95 °C for 10 min; followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Fluorescent signals were analyzed using QuantStudio™ Design and Analysis Software v2 (Thermo Fisher Scientific). *Flavobacterium columnare* presence in gills was calculated as copy number ng-1 of gDNA, hereafter referred to as abundance, and presented as log-transformed.

2.9 Candidate gene expression analysis in the brain

Quantitative PCR was used to examine the expression profile for six target genes that represent specific functions associated with behavior in the brain of fishes, namely:

- Serotonin (5-hydroxytryptamine, *5ht*): regulates emotion, behavior, and cognition (Herr et al., 2017; Roumier et al., 2019). It is well-recognized that the *5ht* system in the brain is essential for systems that control different stress-coping mechanisms, such as the suppression of aggressive and impulsive behavior (Koolhaas et al., 1999; Mahar et al., 2014). The expression of two paralogue receptors; *5ht1aα* and *5ht1aβ* were evaluated.
- Corticotropin-releasing factor receptor 1(*crfr1*): mediates hypothalamic-pituitary-interrenal (HPI) axis activation in teleost fish (Flik et al., 2006).
- Brain-derived neurotrophic factor (*bdnf*) is a protein of the neurotrophin family, which comprises neurotrophin (NT), nerve growth factor (NGF), and has important physiological roles including synaptic plasticity, neurogenesis, and neuronal maturation (Cacialli et al., 2016; Lu et al., 2013)
- Arginine vasotocin (*avt*): provides integrative regulation of many aspects of fish physiology and behavior, including circadian and seasonal biology, responses to stress, metabolism, reproduction, cardiovascular function, and osmoregulation (reviewed by Balment et al. (2006).
- Gamma-aminobutyric acid receptor-associated protein (*gabarap*): is a ubiquitin-like modifier, initially identified as a ligand of the gamma subunit of the $GABA_A$ receptor (Wang et al., 1999; Chen et al., 2000). The latter is a ligand-gated ion channel that mediates rapid inhibitory synaptic transmission in the central nervous system (CNS) of vertebrates and invertebrates (Chen and Olsen, 2007; Mohrlüder et al., 2009), and is

activated by gamma-aminobutyric acid (GABA), an important inhibitory neurotransmitter (Chen and Olsen, 2007).

Primer pairs (Table 2.2) for each of the above genes were either designed using Primer3 software from NCBI sequences, or retrieved from published literature, and were synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). Primer efficiency was tested using 10-fold serial dilutions of cDNA from pooled RNA samples. Each qPCR reaction was carried out in a final volume of 12 µL; 6 µL of 2x PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 µM of the respective forward and reverse primers, and 5 µL of cDNA, and RNase free water to volume. All measurements were performed in triplicate on 384-well plates using the QuantStudio7 Flex Real-Time PCR Systems together with QuantStudio Design and Analysis Software v2. The cycling conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min. A dissociation curve was performed at 95 °C for 15s, 60 °C for 1 min, and 95 °C for 15 s with each run to confirm specificity. Transcript abundance for investigated genes was normalized to three reference genes: 60S ribosomal protein L7, β-Actin, and glyceraldehyde-3-phosphate dehydrogenase. Consequently, normalized values were calibrated to control group, at the corresponding sampling time. Relative expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and is presented as $\log_2$-transformed fold-change.

2.10 Statistical analyses

All statistical tests were performed in GraphPad Prism v9.4.1(GraphPad Software, USA). Survival curves for the preliminary challenge were compared against controls using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. Data from all measured behavioral variables

were analyzed using two-way ANOVA with Fisher's LSD test. Differences in *F. columnare* abundance and gene expression between respective control and *F. columnare* challenged groups were tested for significant differences using a *t*-test and Holm-Šídák multiple comparisons. Challenged groups across time; at 1, 22, and 142 hpc, were tested for significance using two-way ANOVA and Tukey's multiple comparisons tests. A *p*-value of < 0.05 was considered statistically significant.

3. RESULTS

An acute exposure was conducted to determine the suitable dose (CFU mL^{-1}) for assessing juvenile fall-run Chinook salmon behavior in response to *F. columnare* infection. Higher mortality rates were seen starting at 48 hpc. Infection doses of 2.67×10^7 CFU mL^{-1} and 2.67×10^8 CFU mL^{-1} resulted in 46.7% and 90% mortality respectively, at the 96 h time point (Fig. 2.2 A). Therefore, for the challenge experiment assessing behavior, we chose an infection dose of $\sim 1.8 \times 10^7$ CFU mL^{-1}. Significantly lower survival rate was observed in *F. columnare*-challenged fish when compared to the control ($p < 0.001$; Fig. 2.2 B). Mortalities began at 22 hpc and reached 46.2 % mortality on 142 hpc. No mortality was observed in control fish receiving MSB.

3.1 Clinical signs following challenge with *Flavobacterium columnare*

Flavobacterium columnare-challenged fish exhibited clinical signs and lesions consistent with those summarized by Declercq et al. (2013), including lethargy, anorexia, and swimming near the surface. The main gross findings observed in challenged fish were pale gills with yellow-white patches (Fig. 2.3A, B), discolorations of the skin, mostly initiated at the head and base of the dorsal fin (Fig. 2.3C), and frayed fins and erythematous skin (Fig. 2.3D).

3.2 Behavior testing

Behavioral changes between open field and object approach tests were greater in control fish compared to challenged fish, which showed lower behavioral responses to novel object in the latter group (Fig. 2.4). Total distance moved and mean velocity were significantly lower in control fish during the novel object approach test ($p = 0.046$ and $p = 0.037$, respectively) at 142 hpc (Fig, 2.4A and B). During the open field test, challenged fish spent more time in the inner zone compared to control fish at 1 hpc ($p = 0.024$; Fig. 2.4C); however, once the novel object was introduced, the control fish spent more time in the inner zone than they did during the open field test ($p = 0.004$, Fig. 2.4C). Overall, control fish spent more time exploring the novel object than challenged fish. These were qualitatively evident in the heat maps shown in Supplementary Fig. S2.1; presented as bright red spots resulting from fish halted for long periods in the inner zone, exploring the novel object. Correspondingly, time spent in the outer zone was significantly lower in control fish during the novel object approach compared to the open field test at 22 hpc ($p = 0.01$; Fig. 2.4D). Challenged fish had lower absolute turn angle (Fig. 2.4E), absolute angular velocity (Fig. 2.4F), and absolute meander (except at 1 hpc, Fig. 2.4G) than control fish, though non-significant. None of the measured behavioral endpoints were significantly different between challenged and control groups in either the open field or the novel object approach tests, likely due to high variability within groups. We frequently observed, while analyzing behavioral videos, that challenged fish swam erratically during the open field, and the novel object approach tests.

3.3 *Flavobacterium columnare* infection prevalence and abundance

Pure cultures consistent with *F. columnare* were recovered from the gills of challenged fish as early as 1 hpc at a prevalence of 75 ± 9.64%, and 69.43 ± 7.35% at 22 hpc (Fig. 2.5). No significant difference was determined between 1 hpc and 22 hpc ($p = 0.76$). At 142 hpc, *F. columnare* cultures were recovered from one fish, at a prevalence of 2.77%, which was significant from 1 hpc and 22 hpc ($p = 0.034$ and $p = 0.037$, respectively, Fig. 2.5). Cultures were morphologically recognized as golden-yellow, flat, rhizoid colonies with irregular margins, tightly adherent to the agar. In kidney, *F. columnare* was re-isolated later at 22 hpc at a prevalence of 8.33% (Fig. 2.5). *Flavobacterium columnare* was not re-isolated from any control fish at any sampling points.

Changes in *F. columnare* abundance between challenged and control fish were evident as early as 1 hpc (Fig. 2.6). At 1 and 22 hpc challenged fish had significantly higher *F. columnare* abundance compared to controls ($p = 0.003$ and $p < 0.001$, respectively; Fig. 2.6). *Flavobacterium columnare* abundance peaked at 22 hpc and was significantly higher than 1 hpc and 142 hpc ($p < 0.0001$ and $p < 0.0001$, respectively; Fig. 2.6). At 142 hpc, there was a significant decrease in abundance in challenged fish compared to challenged fish at 1 hpc ($p = 0.009$), with no significant difference compared to control fish (Fig. 2.6).

3.4 Candidate gene expression in the brain

Behavior-related genes were differentially expressed in challenged fish compared to control fish (Fig. 2.7). At 1 hpc, expression of *5ht1aβ* (Fig. 2.7A) but not *5ht1aα* (Fig. 2.7B), *crfr1* (Fig. 2.7C), and *bdnf* (Fig. 2.7D) were significantly lower in challenged fish compared to control fish ($p = 0.001$, $p = 0.003$, and $p = 0.001$, respectively). At 22 hpc, significantly greater expression of *5ht1aβ* ($p = 0.032$; Fig. 2.7B), *bdnf* ($p = 0.018$; Fig. 2.7D), and *avt* ($p = 0.013$; Fig. 2.7E) was

detected in challenged fish when compared to challenged fish at 1 hpc. At 22 hpc, challenged fish had significantly greater expression of *gabarap* ($p = 0.022$; Fig. 2.7F) when compared to control fish. Furthermore, in challenged fish, expression of *gabarap* was significantly higher at 22 hpc compared to 1 hpc and 142 hpc ($p = 0.007$ and $p = 0.006$, respectively). All other comparisons showed no significant differences.

4. DISCUSSION

Elucidating the consequences of climate warming and infectious diseases is a critical challenge for the conservation of "at-risk" aquatic species, such as Chinook salmon. The main objective of this study was to ascertain whether, and to what extent, the behavior of juvenile Chinook salmon would be affected by exposure to *F. columnare* at suboptimal, elevated temperatures. Three major findings were made: First, whereas challenged fish were less receptive to the presence of the novel object, control fish exhibited a greater range of behavioral responses compared to challenged fish. Second, there were significant differences in *F. columnare* abundance between challenged fish over time, with pathogen abundance rapidly increasing, but showing potential for recovery over time. Lastly, the *F. columnare* challenge elicited differential expression of behavior-related genes as the *F. columnare* infection progressed.

Considering *F. columnare* pathophysiology, infection is associated with high morbidity and mortality and manifests as necrosis of the gills, and erosion and ulceration of the skin. Skin lesions typically originate at the base of the dorsal fin and extend ventrally on either side of the body forming a circumferential pale band, creating the classic "saddleback" lesion that is often associated with columnaris disease (Declercq et al., 2013). Therefore, corresponding impacts on

behavior would be expected. Recent studies have linked pathogen presence and/ or prevalence (Connon et al., 2012; Jeffries et al., 2014) and external signs of disease (Hostetter et al., 2011) to successful salmon migration. Others have found infection to increase predation risk of migrants (Hostetter et al., 2012; Mesa et al., 1998; Schreck et al., 2006). *Renibacterium salmoninarum*-infected Chinook salmon, for example, were shown to be predated on at considerably higher numbers compared to non-infected fish when exposed to smallmouth bass (*Micropterus dolomieu*) predators (Mesa et al., 1998).

Our main prediction was that *F. columnare* infection would impact fish behavior. Fish behavior is a crucial indication of fish well-being and offers the experimental benefits of direct observation, sensitivity, and non-invasiveness to understand performance in laboratory studies (Martins et al., 2012). The potential for a bacterial infection to affect fish swimming behavior has been reported. Movement of zebrafish challenged with *Edwardsiella tarda* substantially decreased in response to pathological development as time progressed (i.e., 1 to 3 days post-challenge) (Lee et al., 2015), and the critical swimming speed of Atlantic salmon (*Salmo salar L.*) challenged with *Aeromonas salmonicida* was significantly impaired when compared to control at day 6 post-injection (Yi et al., 2016). In our study, we observed decreased locomotor activity (e.g., total distance moved, and mean velocity), and decreased anxiety (e.g., time spent in the inner zone) in control fish during the novel object approach test compared to the open field test. As control fish were exploring, they spent more time in the inner zone where the novel object was located, this exploration behavior was confirmed by the heat maps. Exploration is a recognized personality trait that influences how animals move around and interact with objects and other individuals in their environment (Maiti et al., 2019). In our study, challenged fish did not show any significant locomotion differences in the open field test compared to the novel

object approach test at any time point; i.e., challenged fish appeared to be less perceptive of the change in their environment, being non-responsive to the introduced novel object. However, it is possible that sub-clinical infections (i.e., sustained exposure to *F. columnare* for a longer period of time), could result in different outcomes Lehman et al. (2020) interpret that individuals who exert energy to fight infection may do so at the expense of their swimming prowess, making it harder to evade predators, forage, and navigate migration routes. This could explain unresponsive behavior of challenged fish to the novel object during the novel object approach test.

As early as 1 hpc, viable *F. columnare* was recovered from gills of challenged fish. The highest abundance in the gills was detected at 22 hpc, which was also confirmed by qPCR. These results suggest that *F. columnare* survived and multiplied in challenged fish gills for at least 22 hpc. These results are in accordance with the prior observations of Abdelrazek et al., Submitted. Yet mere exposure to *F. columnare* and the degree of infection does not always result in disease development. However, disease ensues when the host sustains sufficient damage to perturb homeostasis (Casadevall and Pirofski, 1999). Therefore, there are two general, and not mutually exclusive consequences for increased abundance. One: *F. columnare* pathogenesis would trigger a physiological response at the molecular level (i.e., gene expression); or two, infections can impact the physiological performance at the individuals level, including their mobility, visual acuity, behavior and tolerance and ability to effectively respond to additional stressors (Miller et al., 2014).

Two physiological responses evolved that allow animals to survive pathogen infections and overcome distress: the immune response and stress response systems (Azeredo et al., 2017). *Flavobacterium columnare* challenge elicited differential expression of genes involved in the

regulation of behavior. Specifically, expression of serotonin (5-hydroxytryptamine, *5ht1aβ*), corticotropin-releasing factor receptor1 (*crfr1*), brain-derived neurotrophic factor (*bdnf*), and gamma-aminobutyric acid receptor-associated protein (*gabarap*). Interestingly, expression of investigated genes followed the same trend, albeit with varying magnitudes. Lower gene expression was observed at 1 hpc, whilst greater expression levels were typically observed at 22 hpc.

The brain serotonergic system plays a key role in coordinating autonomic, behavioral, and neuroendocrine stress responses (Backström and Winberg, 2017). Nonetheless, association between pathogenic infection-induced behavioral alterations in animals and the *5ht* system is still unclear. According to our findings, *F. columnare*-challenged fish exhibited lower transcript abundance of both serotonin paralogues (*5ht1aα* and *5ht1aβ*) at 1 hpc, before the transcripts returned to the level of the control fish; at 22 hpc and 142 hpc, implying that this acute response was not sustained. Similarly, the serotonergic marker expression was suppressed in the brain of Nile tilapia (*Oreochromis niloticus*) following *Streptococcus agalactiae* challenge, implying a potential impact on behavior during bacterial infection (Li et al., 2022a). Yi et al. (2019) indicated that *S. agalactiae* infection affected the swimming behavior of Nile tilapia, and the serotonin level in the brain, intestine, and stomach was significantly reduced post-challenge. Rainbow trout (*Oncorhynchus mykiss*) infected with *Flavobacterium psychrophilum* (Muñoz et al., 2019) and *Eleginops maclovinus* infected with *Francisella noatunensis* (Quilapi et al., 2022) both showed an early increase in brain serotonin content; however, this response was not at the transcription level. Levels of 5-HT within the brain have also been correlated with changes in locomotor activity in goldfish (*Carassius auratus*) (Fenwick, 1970), and the Texas killifish (*Fundulus grandis*) (Fingerman, 1976). Winberg et al. (1993) demonstrated that inhibition of

brain serotonergic activity caused a significant increase in the activity levels of arctic charr (*Salvelinus alpinus*). These studies demonstrate that serotonin and locomotor activity can change concurrently. 5HT-related effects, however, are further coordinated by additional neuromodulators; particularly, two neuropeptides: corticotropin-releasing factor (*crf*) and arginine vasotocin (*avt*), of interest based on their role in the stress axis (Backström and Winberg, 2017).

The biological function of corticotropin-releasing factor (*crf*) is mediated through its receptors, namely *crfr1* and *crfr*2 (Arai et al., 2001). *Crfr1* mediates hypothalamic-pituitary-interrenal (HPI) axis activation in teleost fish (Flik et al., 2006). We found that expression of *crfr1* in the brain of *F. columnare*-challenged fish was significantly lower than in controls at 1 hpc, followed by the subsequent increase of the expression levels. Similarly, *Trypanoplasma borrelia* infection and constraint stress caused by netting resulted in downregulation of *crfr1* expression in the gills and skin of common carp (*Cyprinus carpio*) (Mazon et al., 2006), and previously in response to restraint stress in common carp (Huising et al., 2004). Differences in the expression level of *crfr1* could be a consequence of challenge with *F. columnare*; however, we are unable to conclude whether changes in *crfr1* observed in challenged fish are solely due to infection.

In fish, brain-derived neurotrophic factor (*bdnf*) is primarily synthesized in the brain, specifically in primary sensory neurons, with relatively small amounts of this peptide also found in the retina, ear, lateral line, gut, and gonads (Blanco et al., 2020). It has established roles in neuronal survival and differentiation, synaptic plasticity, and neurotransmitter release summarized by Lucon-Xiccato et al. (2023). Because of *bdnf*'s extensive cellular and molecular effects on the brain, it has been hypothesized that it may play a significant role in determining

individuals' behavior and, more critically, its dysfunctions (Lucon-Xiccato et al., 2023). In our study, *bdnf* followed the same trend of regulation as other genes, with *F. columnare*-challenged fish exhibiting lower expression at 1 hpc. This down-regulation of *bdnf* expression coincided with the significant down-regulation of expression observed in *crfr1*, and *5ht1aβ* in challenged fish. The subsequent increase of *bdnf* expression observed at 22 hpc, could be a sign of brain injury in challenged fish. Adult zebrafish exhibited significant increases in *bdnf* mRNA levels one-day following traumatic brain injury (TBI), thereafter, gradually reducing over time (Cacialli et al., 2018). Given the full restoration of *bdnf*, authors concluded that *bdnf* might contribute to generating a permissive environment that promotes the development of a new neuronal population in injured brain. In an experimental *Streptococcus pneumoniae*-meningitis, Bifrare et al. (2005) demonstrated that *bdnf* injection protects the rat brain from different types of brain damage. Hence, the authors emphasized the importance of *bdnf* in bacterial infections. In particular, the receptor for *bdnf* is expressed in a wide variety of neurons across the brain, which is s a prerequisite for a beneficial consequence in diseases impacting many brain regions, such as bacterial meningitis. It thus could be plausible that *bdnf* is a component of an endogenous protective system during bacterial infection. Additionally, Lucon-Xiccato et al. (2023) suggested a role of *bdnf* in controlling levels of behavioral activity, sociability, and anxiety-like behavior in zebrafish. The differences in *bdnf* transcription levels could be indicative of the presumed lack of perception of the introduced object in *F. columnare*-challenged fish, in our study.

The neuropeptide arginine vasotocin (*avt*), is a teleost homolog of mammalian arginine vasopressin (*avp*). In the central nervous system of fish, *avt* functions as neurotransmitters and/or neuromodulators, and it is recognized to play a role in modulating reproductive processes and a variety of related social behaviors (Godwin et al., 2000). Additionally, it is known that

avt regulates several behavioral reactions, including those that are connected to sexual and aggressive behavior and the establishment of social hierarchies (Gesto et al., 2014). While our findings displayed similar expression of *avt* with other investigated genes; downregulation followed by upregulation in *F. columnare* challenged fish, no difference in *avt* expression was measured in comparison to control fish. Our understanding of the physiological role of *avt* in fishes and especially in salmon is limited and needs elucidation. In teleost, the *avt* system seems to be involved in the coordination of physiological responses to various stressors, such as handling, social confrontation, or osmotic disruption, which have been found to elevate pituitary and plasma *avt* levels (Backström et al., 2011; Gilchriest et al., 2000; Kulczykowska, 2001; Mancera et al., 2008). According to *in vitro* research utilizing rainbow trout pituitaries, *avt* may function as an adrenocorticotrophic hormone (ACTH) secretagogue during the stress response, synergizing with CRF (Baker et al., 1996). Additionally, it has been proposed that the *avt* system may interact with brain monoaminergic systems to control aggressive behavior in fish during social stress (Backström and Winberg, 2009; Semsar et al., 2004). However, studies on the specific role of *avt* response in fish following bacterial infection are lacking.

Gamma-aminobutyric acid receptor-associated protein (*gabarap*) is crucial for synaptic plasticity, autophagy, and apoptosis (Mohrlüder et al., 2009). As a result of gabarap and its crucial role in GABAA receptor, it poses the question of whether *gabarap* expression changes in response to *F. columnare* infection, which has not been discussed to date. In our study, *F. columnare* challenged fish exhibited higher expression of *gabarap* at 22 hpc compared to control fish, concomitantly with the highest bacterial abundance. Kim et al. (2018) demonstrated that GABAergic activation promoted antimicrobial responses against *Mycobacteria*, *Salmonella*, and *Listeria* infections *in vitro* and *in vivo*. Expression of *gabarap* was up-regulated and linked to

anxiety-like behaviors, including an increase in shoal average speed, a preference for dark settings (scototaxis), and rapid exploration, in crabs exposed to seawater with elevated CO_2 levels (Ren et al., 2018).

Individuals perceive and react differently to their environment, and this affects their robustness to challenges such as stress and diseases (Vindas et al., 2017). Behavioral monitoring may bridge the gap between micro-scale (i.e., molecular level) and macro-scale (i.e., population/community level) monitoring (Lee et al., 2015), thus supporting ongoing efforts aimed at determining the effect of pathogens on fish populations. Furthermore, changes in gene expression of infected fish compared to non-infected conspecific might be functionally associated with the behavioral change but could also merely be the consequence of being exposed to infectious agents (Grecias et al., 2020). While our study evaluated behavioral and transcriptional impacts of *F. columnare* infection on Chinook salmon under laboratory-controlled conditions, we were unable to establish a causal linkage. Our findings come with some limitations; high biological heterogeneity within a group, low fold-change in expression of investigated genes in brain tissue, and small sample size all may have contributed to the nonsignificant difference in swimming behavior and significant, but low level, difference in gene expression. Acknowledging these limitations will enhance future experimental design and, in turn, advance our knowledge of consequences of infectious diseases that are likely to remain a conservation issue as climate change continues to reshape host-environment-agent dynamics.

In our study, we emphasize how a single modification to a behavioral test, such as the introduction of a novel object, can change the inferred outcome. Given the increased interest in the elucidating consequences of exposure to pathogens, it will be important to continue to

investigate how different selective pressures (e.g., infectious diseases and climate change) may influence swimming behavior and their effects on the overall animal's performance.

Funding

This research was supported by the Delta Water Quality and Ecosystem Restoration Grant Program from the California Department of Fish and Wildlife (CDFW; Proposition 1 grants # P1696002, P1896051, and Q1996036 to REC, ES, and collaborators).

Acknowledgments

We thank Dr. Michaiah J. Leal for assistance with setting up the swimming behavior tests and Dr. Anne E. Todgham for providing all materials and support necessary for the swimming behavior tests.

5. FIGURES & TABLES

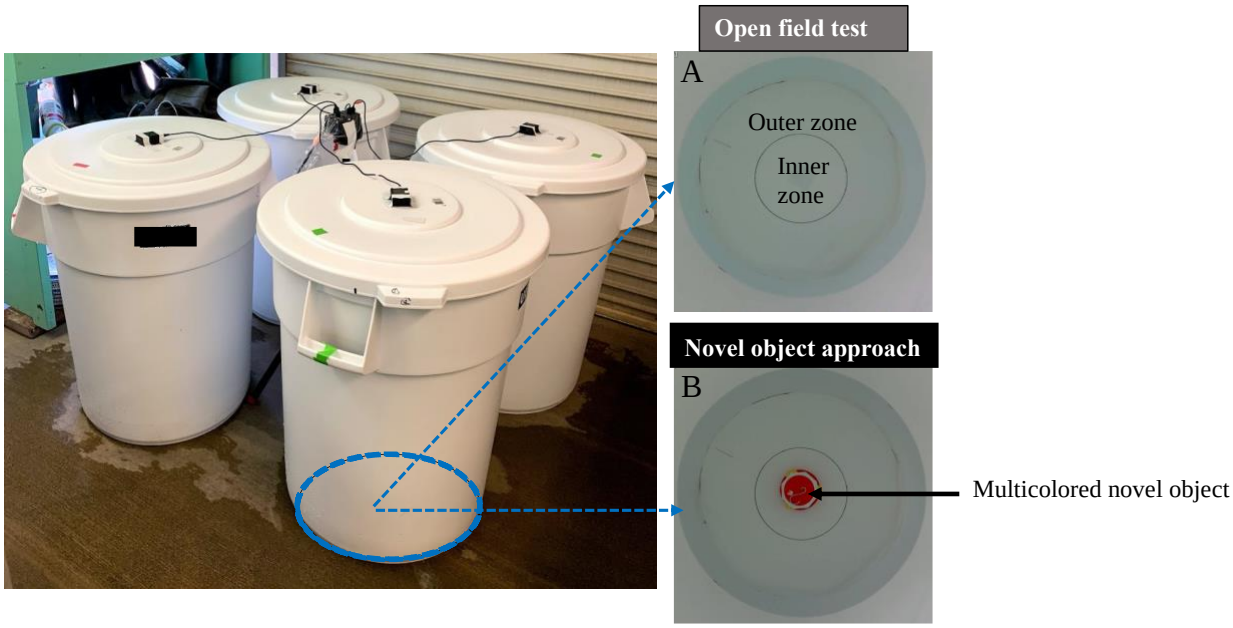


Figure 2.1: Experimental arena: A) open field, and B) novel object approach tests used for assessing swimming behavior of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) challenged by immersion with modified-Shieh broth (MSB) or *Flavobacterium columnare* at 18 °C.

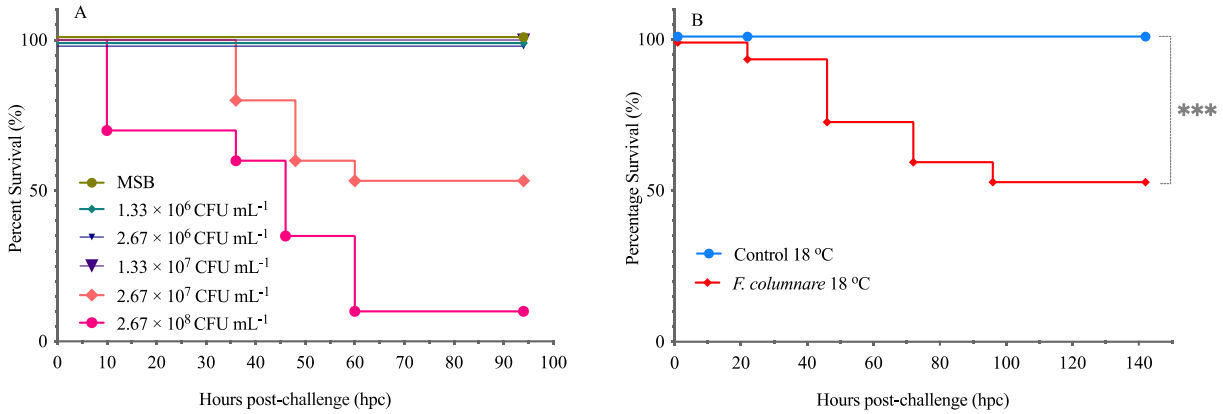


Figure 2.2: Percentage survival (%) of juvenile Chinook salmon (*Oncorhynchus tshawytscha*. A) triplicate groups of ten fish were challenged by immersion with modified Shieh broth (MSB) or different CFU mL^{-1} of *F. columnare* to determine a median lethal dose (LD_{50}), B) challenged with modified-Shieh broth (MSB; control) or 1.8 x 10^7 CFU mL^{-1} *Flavobacterium columnare,* for 2 h in static conditions *via* bath immersion at 18 °C and monitored for 142 h. Asterisks indicate statistical significance (*** $p < 0.001$), as determined by log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests.

Table 2.1: Measured behavioral endpoints in Chinook salmon (*Oncorhynchus tshawytscha*) for the open field and novel object approach tests using EthoVision XT tracking software (version 15).

Endpoint	Body point	Measurement	Unit
Total distance moved	Center point	Total	cm
Velocity	Center point	Mean	cm/s
Time in inner zone	Center point	Cumulative duration	s
Time in thigmotaxis zone	Center point	Cumulative duration	s
Absolute turn angle	Center point	Mean	◦
Absolute angular velocity	Center point	Mean	◦/s
Absolute meander	Center point	Mean	◦/cm

Table 2.2: Primer sequences for genes used in quantitative polymerase chain reaction (qPCR) analyses in juvenile Chinook salmon (*Oncorhynchus tshawytscha*).

Gene		Forward primer sequence (5′–3′) Reverse primer sequence (5′–3′)	Primer efficiency	Reference
5-hydroxytryptamine receptor 1A-alpha	F R	GCA AGG CAT CGT TGT ATT CCC A CGG AAA CCT TCG TGT CTC GT	106.6	This study
5-hydroxytryptamine receptor 1A-beta	F R	TTG ATC ATG CGT TCC CAG CCG A AAA GGA ATG TAG AAC GCG CCG A	102.22	Thörnqvist et al. (2015)
Corticotropin-releasing factor receptor 1	F R	ACTG GGG CAT GTG CCA AAT AG ATG ATG CAG CGC CAG AAA GC	92.53	This study
Brain-derived neurotrophic factor	F R	GGC GTT GGG AAG CAG TAG AG AGC GAG GGT AAC TAA GGG GG	101.38	*Abdelrazek et al.
Arginine Vasotocin	F R	TGC GTC ATC GGA CTC CTA GC TGT CTG ATG CCG GTG TCC TG	104.65	This study
Gamma-aminobutyric acid-receptor-associated protein	F R	AGC ATT AGC AAC AAG GCG ATCA GCT AGC CTA TGC CTG CCC TT	106.67	This study
60S Ribosomal gene 7l	F R	GCA AGG TCG GGA ACT TCT ACG CCC CTG ATC CTG ATG ACG AA	98.93	Mauduit et al. (2022)
β-actin	F R	GGA CTT TGA GCA GGA GAT GG ATG ATG GAG TTG TAG GTG GTC T	100.87	Bjork et al. (2014)
Glyceraldehyde-3-phosphate	F R	CGC GTC GCT GAC CTG TT TGT GAC ACT GGA GGG TGT GAG T	99.35	*Abdelrazek et al.

*Submitted

Figure 2.3: Gross findings in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) challenged with *Flavobacterium columnare* at 18 °C. A, B) pale gills with yellow-white patches (arrow), C) discolorations of the skin (mostly initiated at the base of the dorsal fin, arrows), D) frayed fins and erythematous skin (arrows).

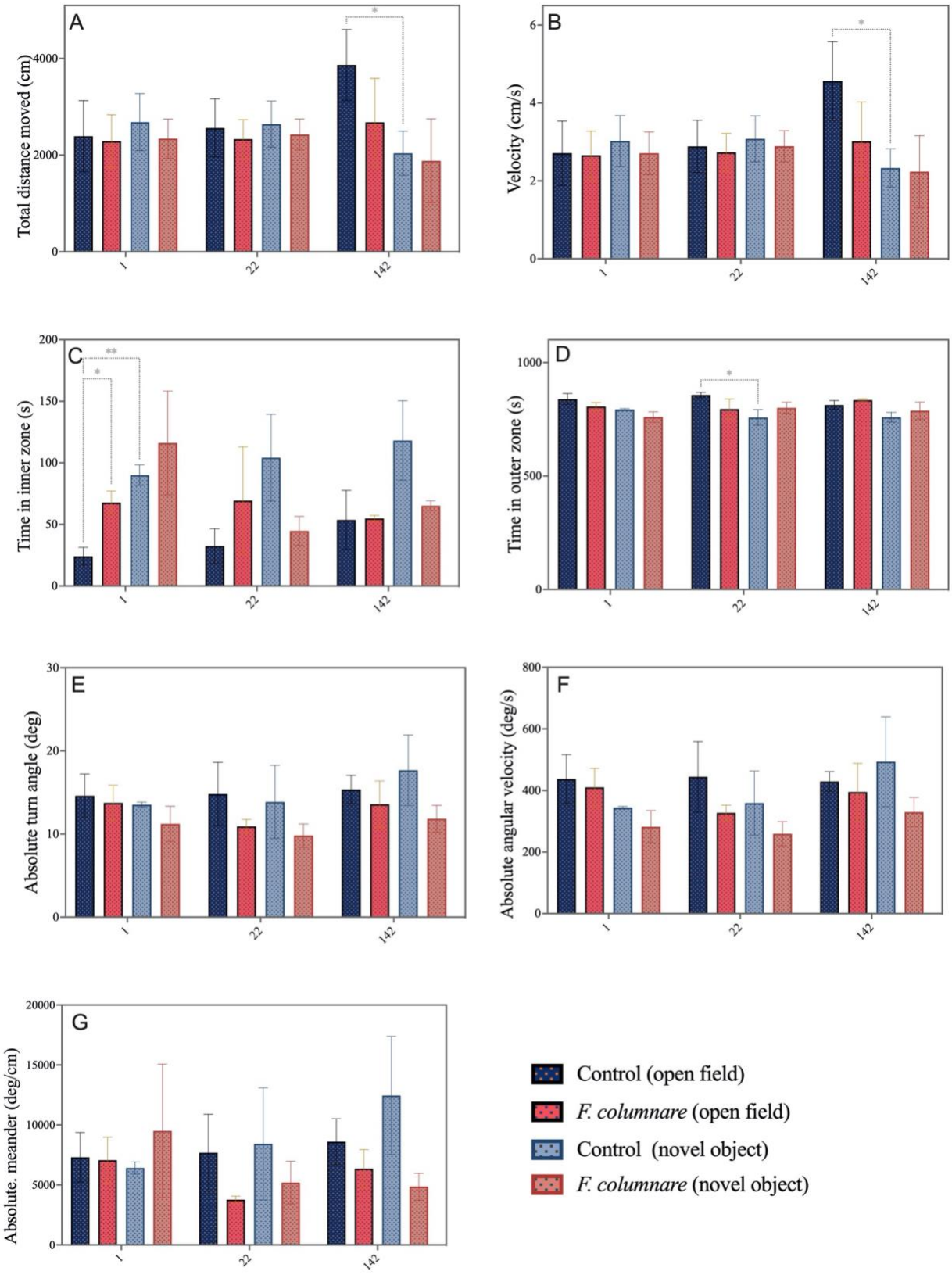

A
Total distance moved (cm)
0
2000
4000
B
Velocity (cm/s)
0
2
4
6
C
Time in inner zone (s)
0
50
100
150
200
D
Time in outer zone (s)
0
500
1000
E
Absolute turn angle (deg)
0
10
20
30
F
Absolute angular velocity (deg/s)
0
200
400
600
800
G
Absolute. meander (deg/cm)
0
5000
10000
15000
20000
1
22
142
Control (open field)
F. columnare (open field)
Control (novel object)
F. columnare (novel object)
Hour post-challenge (hpc)

Figure 2.4: Behavioral endpoints measured in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) following a challenge by immersion with modified Shieh broth (MSB; control) or *Flavobacterium columnare* at 18 °C. A) total distance moved (cm), B) mean velocity (cm/s), C) time in inner zone (s), D) time in the outer zone (s), E) absolute turn angle (◦), F) absolute angular velocity (◦/s), G) absolute meander (◦/cm) L). Asterisks indicate statistical significance (***** $p < 0.05$, and ****** $p < 0.01$) as measured using two-way ANOVA with Fisher's LSD test (n=3).

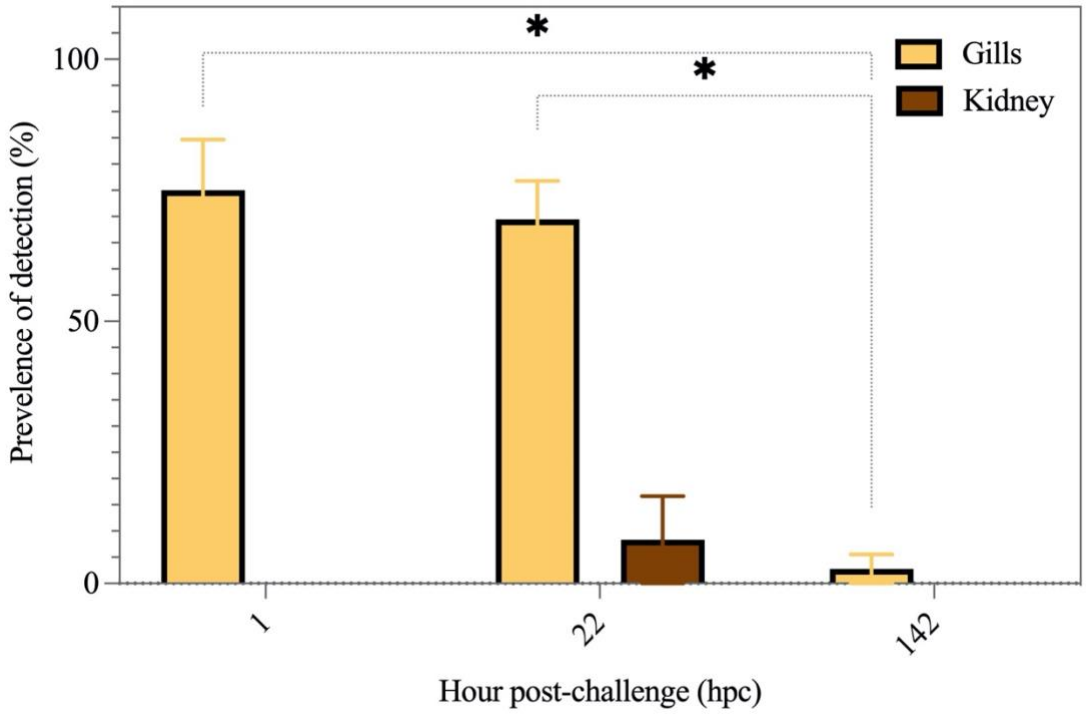


Figure 2.5: Re-isolation of viable *Flavobacterium columnare* from gills and kidney of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) at select time points; 1-, 22-, and 142-hours post-challenge. Asterisks indicate statistical significance (* $p < 0.05$) as measured using two-way ANOVA and Tukey's multiple comparisons tests ($n = 3$).

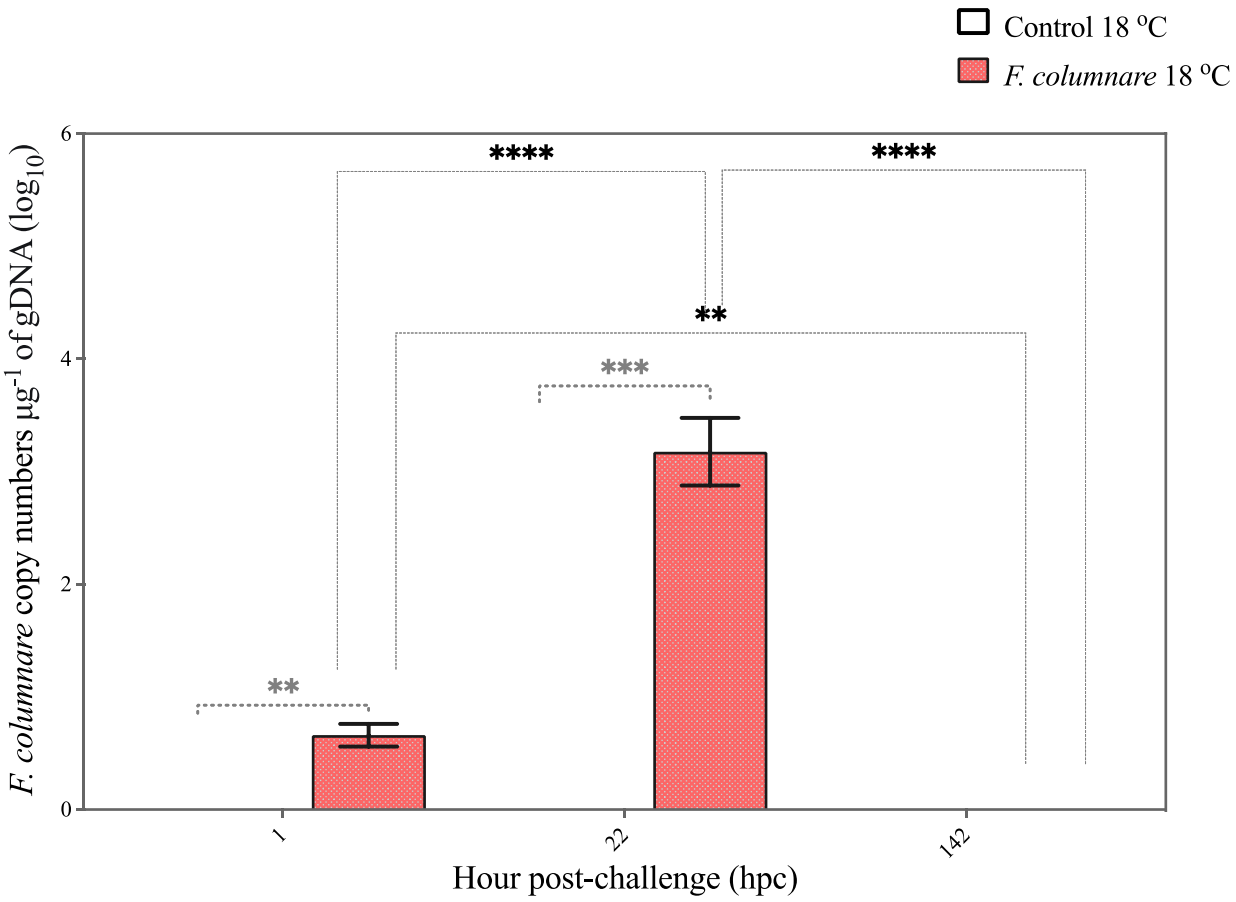


Figure 2.6: *Flavobacterium columnare* detection in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) at select time points; 1-, 22-, and 142-hours post-challenge, presented as $\log_{10}$-transformed copy number μg^{-1} of gDNA in gills of control or challenged fish and (n = 3) exposed at 18 °C. Asterisks indicate statistical significance (** $p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). No *F. columnare* was recovered from controls at any time point, nor challenged fish at 142 hpc.

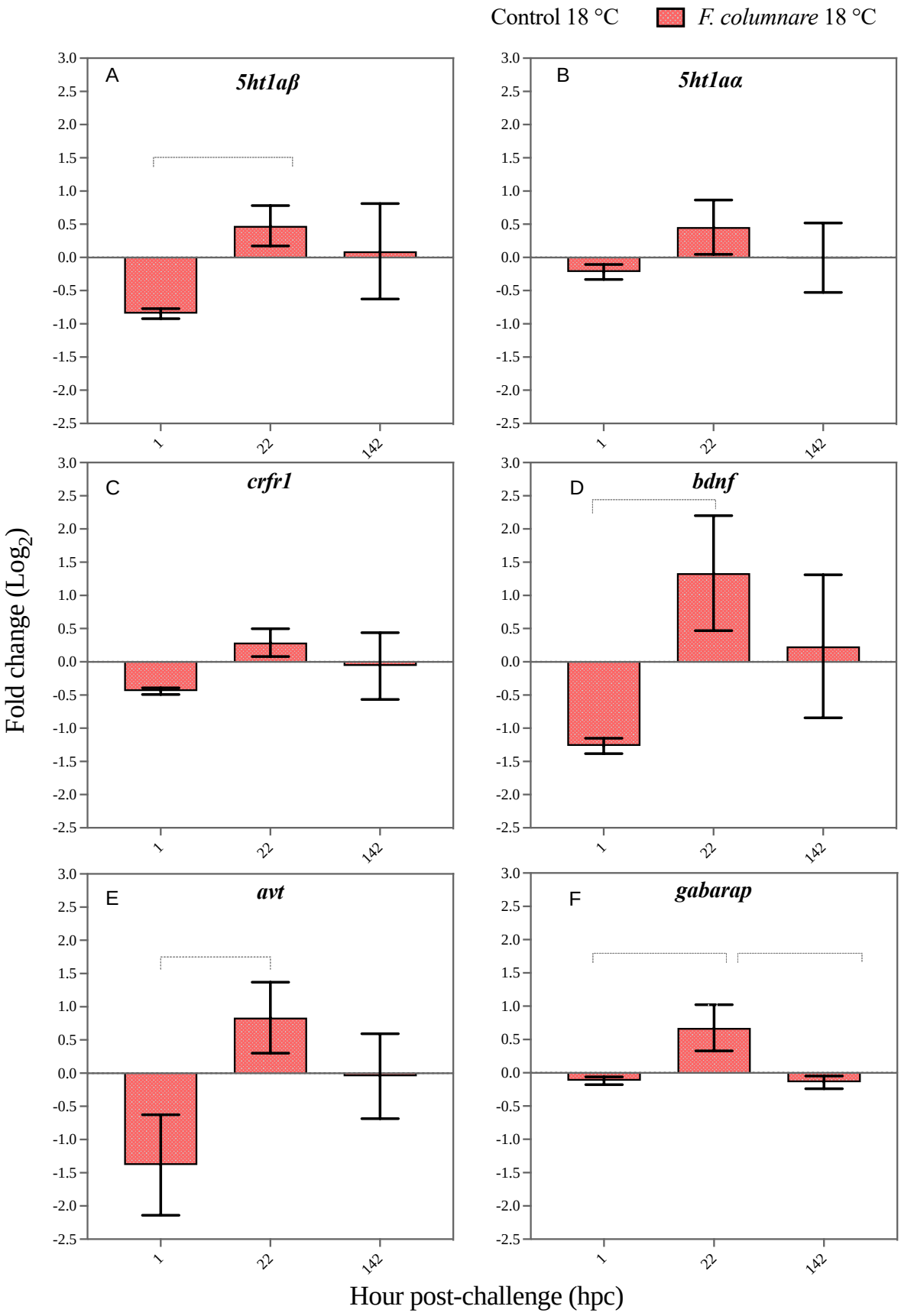
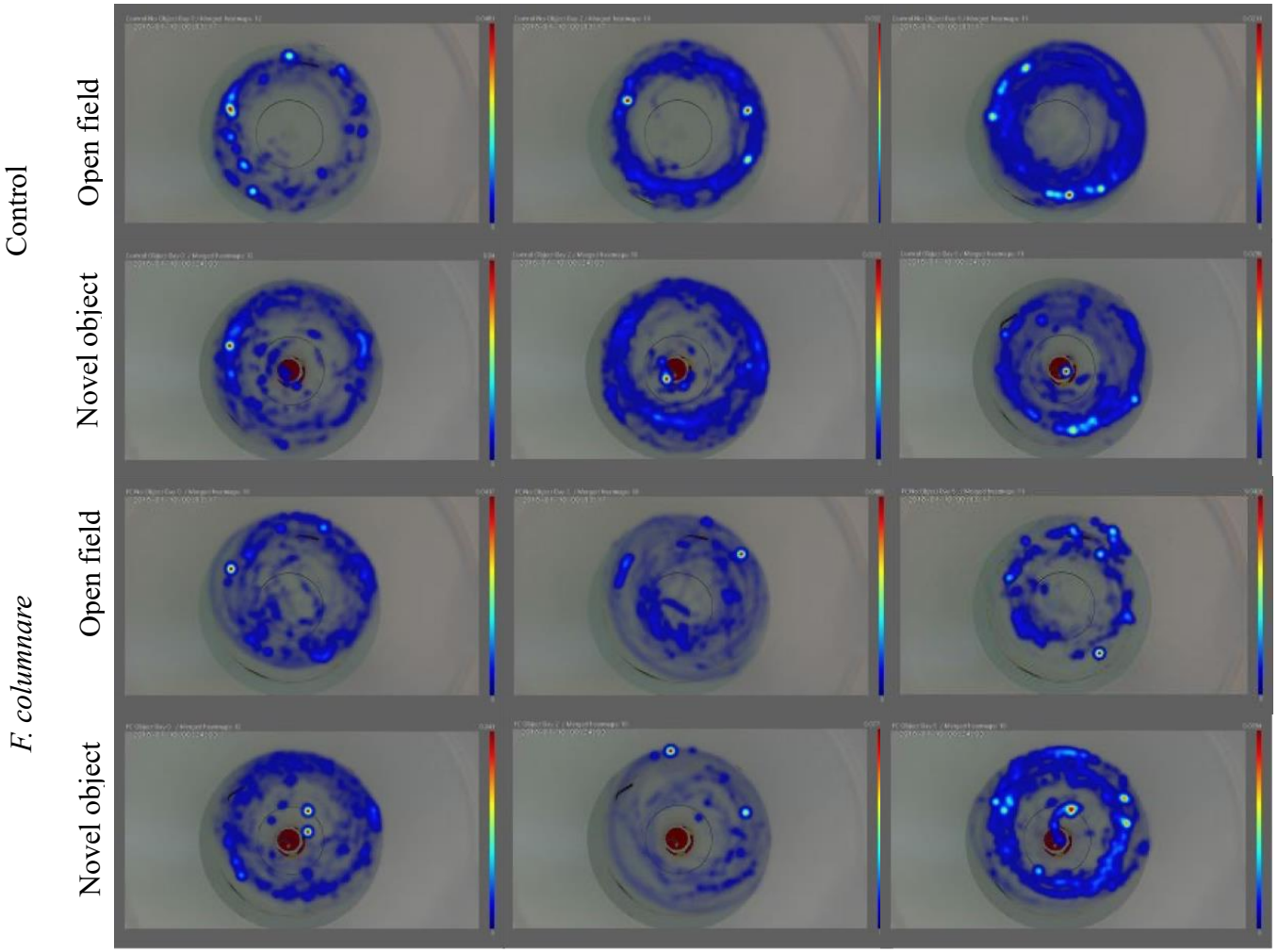

Control 18 °C
F. columnare 18 °C
A
5ht1aβ
B
5ht1aα
C
crfr1
D
bdnf
E
avt
F
gabarap
Fold change (Log2)
Hour post-challenge (hpc)
1
22
142

Figure 2.7: Gene expression in brain tissue of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) challenged by immersion with modified Shieh broth (MSB; control) or *Flavobacterium columnare* at 18 °C. Gene expression of A) 5-hydroxytryptamine receptor 1A-beta (*5ht1aβ*), B) 5-hydroxytryptamine receptor 1A-alpha (*5ht1aα*), C) corticotropin-releasing factor receptor 1(*crfr1*), D) brain-derived neurotrophic factor (*bdnf*), E) arginine vasotocin (*avt*), and F) gamma-aminobutyric acid receptor-associated protein (*gabarap*). Gene expression was normalized against the housekeeping genes *rpl7*, act*β*, and *gapdh* and subsequently expressed as fold change ($\log_2$) relative to expression of respective control fish ($n = 3$). Asterisks indicate statistical significance (***** $p < 0.05$, and ****** $p < 0.01$). Light grey asterisks indicate statistical significance between control and challenged fish.



Supplementary Figure S2.1: Example of swimming behavior of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) challenged by immersion with modified-Shieh broth (MSB; control) or *Flavobacterium columnare* at 18 °C assessed by open field and novel object

approach tests. Group heatmaps show a colored representation of the combined mean location of each group of fish during a 6-days study period. Heat maps depict the averaged movement patterns of all fish in a given group. Fish immobility resulted in glaring red blotches, which showed that control fish had halted to explore the novel object, whereas continuous movement along the arena's edge appeared as a dazzling halo in warm colors.

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CHAPTER 3: Responses to pathogen exposure in sentinel juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) in the Sacramento River, California

A similar version of this chapter was submitted for publication by the journal of Conservation Physiology

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ABSTRACT

Infectious diseases are one of many factors affecting different salmon populations worldwide. This study investigated how the prevalence and abundance of pathogens (microparasites and bacteria) in fish tissues could affect expression of genes involved in immune system functioning, general stress, and host development of juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Juveniles were deployed in sentinel cages for 21-days, at the Red Bluff Diversion Dam, an area where fish have been previously found to be impacted by *Ceratonova shasta* and *Parvicapsula minibicornis*, in the Sacramento River, California, USA. Gill, kidney, and intestinal tissue were sampled at day 0, 7-, 14-, and 21-days post-deployment (dpd). Pathogen detection and host response were assessed by a combination of conventional PCR and quantitative PCR (qPCR), along with supporting histopathological evaluation. Our findings showed that deployed fish were infected with parasites: *Ceratonova shasta*, *Parvicapsula minibicornis*, and *Ichthyophthirius multifiliis* and the bacteria, *Flavobacterium columnare* and *Rickettsia*-like organism. Co-infection was common among sentinel fish, with *C. shasta* and *P. minibicornis* being the two dominant pathogens. Upregulation of immune and stress-related genes was detected in gills (7-dpd) before they were detected in kidneys (14-dpd) indicating that gill biopsy may be useful as early-warning and non-lethal target tissue. We found that expression of investigated genes was altered following deployment, and may represent irreversible impacts on salmon, thus posing a threat to the population. This study provides a foundation for future avenues of research investigating prevalent pathogens that affect out-migrating Chinook salmon in the Sacramento River and offers crucial knowledge for future conservation efforts.

Keywords: Fish diseases, Sacramento River, Pathogens, Gene expression, Chinook salmon

1. INTRODUCTION

Exposure to pathogens (disease-causing organisms) can play an important role in salmon ecology and population health (Chapman et al., 2021; Johnson et al., 2015; Sofonea et al., 2017; Teffer et al., 2022). Additionally, this role may be dynamic as rapid environmental changes can disrupt the host-pathogen relationships for salmon, affecting pathogen species composition, host immune responses, and overall animal performance (Altizer et al., 2011; Evans et al., 2011; Lehman et al., 2020). For example, while many opportunistic pathogens may be endemic in a watershed, diseases may only manifest when other factors, such as poor environmental conditions, first compromise the salmon host (Lehman et al., 2020).

The epidemiological triad of host-environment-agent is the core concept used to describe how pathogen exposure levels, host susceptibility, and the environment in which host and pathogen interact, determine disease dynamics (Dicker et al., 2006). Traditionally, research on these interactions has focused on single pathogen studies. In many systems, however, salmon are exposed to a variety of pathogens, and these multiple exposures may synergize and not result in simple additive effects on the host. While collective pathogen community composition is recognized as having greater ecological relevance (Sofonea et al., 2017, 2015), predicting outcomes of multiple exposures remains challenging.

Multiple host-related factors influence the outcome of pathogen infections for salmon, including tissue damage, the host immune response to prevent, control, and eliminate pathogens, and detrimental consequences caused by inflammation. Gene expression measured from salmon gill biopsies can indicate whether a fish is responding physiologically to pathogen infections, as well as the magnitude of that response (Connon et al., 2012; Jeffries et al., 2014; Teffer et al., 2017; Mauduit et al., 2022). However, sampling gills has not been confirmed as a reliable indicator of systemic infections. In fish, the kidney is a major lympho-hematopoietic organ (Zapata et al., 2006). It is routinely sampled in piscine

pathological procedures with the intent to determine the extent of systemic infection (Bjørgen and Koppang, 2021).

Pacific salmon, *Oncorhynchus* spp., are iconic fishes with significant cultural, economic, and recreational value (National Research Council, 1996; Lichatowich and Lichatowich, 2001), and are keystone species for aquatic ecosystems (Cederholm et al., 1999; Gende et al., 2002). Pacific salmon populations have declined across their ranges due to overexploitation, habitat loss, reduced prey availability, and pathogens (Rand, 2002; Ruckelshaus et al., 2002; Kent, 2011**;** Miller et al., 2014; Deeg et al., 2022). The Central Valley fall-run Chinook salmon *(Oncorhynchus tshawytscha),* the focus of this study, has been designated as a "species of concern" by NOAA Fisheries, necessitating extensive research to quantify and understand factors threatening its survival (Buchanan et al., 2018).

During outmigration, juvenile Chinook salmon may be exposed to a range of pathogens that can affect growth, development, distribution, behavior, predation risk, and long-term survival (Myrick and Cech, 2001**;** Myrick and Cech, 2004). Many known salmonid pathogens are endemic, particularly the myxozoan microparasites *Ceratonova shasta* (an intestinal parasite of salmonids that causes severe enteronecrosis and mortality) and *Parvicapsula minibicornis* (Bartholomew et al., 2007; Hallett et al., 2012), which share the same invertebrate annelid host: *Manayunkia occidentalis* (Atkinson et al., 2020). Of particular concern in the Sacramento River and the focus of this study, is a site described by Foott et al. (2017) as an "infectious zone," a stretch of river that extends approximately 64 kilometers, between Anderson River Park and Red Bluff Diversion Dam (RBDD), in which higher *C. shasta* and *P. minibicornis* infection rates have been observed. Other pathogens of concern including *Ichthyophthirius multifilis, Candidatus Branchiomonas cisticola*, *Flavobacterium psychrophilum*, and *Rickettsia*-like organism have been linked to

histopathology and immune system function in out-migrating winter-run Chinook Salmon caught in screw traps at Red Bluff Diversion Dam (Hasenbein et al., Unpubl. data).

The goals of this study were to determine the risk of pathogen infection and to evaluate the impact of infection on sentinel juvenile Chinook salmon within this infectious zone. The specific objectives were to 1) determine the prevalence and abundance of pathogens in fish tissue deployed to river conditions at the RBDD site of the Sacramento River (CA, USA) for a 21-day study period; and 2) determine consequences of pathogen exposure on salmon histological and physiological responses; expression of immune, stress, and development-related genes. We hypothesized that sentinel salmon would be co-infected with multiple pathogens, resulting in complex host-pathogen dynamics, with detrimental effects on fish health.

2. MATERIALS & METHODS

2.1 Sentinel fish exposure

All fish care and protocols were reviewed and approved by the University of California, Santa Cruz Institutional Animal Care and Use Committee (UCSC IACUC), protocol no. Danim1911. Fall-run Chinook salmon eggs were obtained from the in-basin Coleman National Fish Hatchery (CNFH; near Anderson, California, USA) and raised at the National Oceanic and Atmospheric Administration Southwest Fisheries Science Center (SWFSC) in Santa Cruz, California, USA. Juvenile fall-run Chinook salmon (mean fork length ± SD, 7.5 ± 0.5 cm; mean mass ± SD, 4.6 ± 0.9 g) were transported to the deployment location in rotomolded coolers and monitored regularly to maintain ~13.5 °C and dissolved oxygen > 90% during transport.

Sentinel fish were deployed for 21 days from May 19th to June 9th, 2020. Fish were held in cylindrical cages (diameter 50 cm; height 80 cm; n = 3 cages; 54 fish per cage) in the

Sacramento River at Red Bluff Diversion Dam (Fig. 3.1). Cages were constructed with a welded steel rod skeleton, with 4 mm plastic drainage mesh covering the bottom and sides, and a 6 mm mesh polyester netting covering the tops of cages, to allow food to enter and exit. All cages were suspended from the water surface using flotation devices and were fastened to the RBDD. River temperature and flow were monitored at the RDB station gauge, adjacent to the RBDD (cdec.water.ca.gov; Fig. 3.2).

2.2 Tissue collection

Seventy-five juvenile Chinook salmon were sampled in total: Fish were sampled prior to deployment at the SWFSC facility and used as reference samples on day 0 (n = 15), and at 7-, 14-, and 21-day post-deployment (dpd) at the RBDD site (n = 20; 6 to 7 fish per cage). Prior to tissue sampling, fish were euthanized using an overdose of buffered tricaine methanesulfonate (MS-222; 500 mg L^{-1}; Western Chemical, Ferndale, WA), weighed (g), and fork length measured (L_F; mm). Gill, kidney, and intestinal tissue were collected and preserved in RNAlater (Life Technologies Carlsbad, CA, USA) before being transported at ambient temperature to the corresponding laboratory. Gill and kidney samples were stored at -80 °C at the University of California, in Davis, on arrival, while the intestine samples were shipped to Oregon State University. On day 21 (the endpoint of the study), gill, kidney, and intestine from an additional 20 fish were collected for histopathological assessments.

2.3 Genomic DNA extraction, total RNA extraction, and cDNA synthesis

Prior to extraction, gill and kidney tissue were independently homogenized using a Qiagen TissueLyser LT (Qiagen, Valencia, CA, USA). A QIAcube system 230 V (Qiagen) was used to extract genomic DNA (gDNA), and total RNA from gill and kidney tissue using the DNeasy Blood and Tissue Kit (Qiagen), and RNeasy Plus Mini Kit (Qiagen) respectively,

according to the manufacturer's recommendations. Extracted gDNA and RNA were assessed for quality (A260:280 and A260:230 ratios) and concentration (ng μL^{-1}) using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Integrity of RNA was assessed by electrophoresis on a 1% w/v agarose gel. A QuantiTect Reverse Transcription Kit (Qiagen) was used to synthesize cDNA from 1 µg of total RNA following the manufacturer's protocol, with the exception that reaction volumes were scaled to a final volume of 40 µL. Fish intestine samples were digested using a modified 'boiled-crude' method of Palenzuela et al. (1999): incubation at 56 °C for 1–2 h with 180 µL buffer ATL (Qiagen) and 20 µL proteinase K to digest tissue, followed by heat denaturation at 85 °C for 15 min, then dilution 1:100 prior to amplification in PCR.

2.4 Pathogen detection in gill, kidney, and intestinal tissue

Probe-based quantitative PCR (qPCR) was used for the detection and quantitation of eight pathogens known or suspected to cause disease in salmon in gill and kidney tissue. This included four parasites and four bacteria (Table 3.1). Custom TaqMan X20 Gene Expression Assays, FAM (ThermoFisher Scientific, Waltham, MA, USA) were used. All primers and TaqMan probes utilized in this study (Table 3.1) were sourced from (Teffer et al., 2017) and validated by (Miller et al., 2016), except for *F. columnare*; which was sourced from (Panangala et al., 2007). qPCR consisted of 6 µL TaqMan Environmental Master Mix 2.0 (ThermoFisher Scientific), 0.9 µM each primer, 0.25 µM probe, 5 µL template gDNA, and nuclease-free water to a total volume of 12 µL. Plasmids with specific sequences corresponding to each pathogen (Eurofins Genomics LLC, Louisville, KY, USA) were used to create standard curves of known copy numbers, and for assay quality control and quality assurance. All DNA samples were amplified in 384-well plates in a QuantStudio7 Flex Real-Time PCR System (Real-time PCR Research and Diagnostics Core Facility, UC Davis), with

10-fold, serially diluted plasmid standards. All reactions were tested in triplicate under standard amplification conditions: 2 min at 50 °C; 10 min at 95 °C; followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C**.** Fluorescent signals were analyzed using QuantStudio Design and Analysis Software v2 (Thermo Fisher Scientific). While copy number ng^{-1} of gDNA is not directly comparable to pathogen abundance (except in the case of single-copy genes assessed in a bacterium or virus infection; reviewed by Chapman et al. (2021), the results for each pathogen in gill and kidney were represented by copy number ng^{-1} of gDNA, hereafter referred to as pathogen abundance. Extracted intestine gDNA was assayed solely for *C. shasta* ITS-1 rDNA region using conventional PCR (described in Atkinson et al. (2018)). Amplicons were visualized by gel electrophoresis, and PCR result was given as negative ("-" no band) and positive.

2.5 Gene expression of Chinook salmon following deployment

Quantitative reverse transcription PCR (RT-qPCR) was used to examine the expression profile for 11 target genes associated with immune function, general stress, and development responses in gill and kidney tissue (Table 3.2), but not in intestinal tissue. Primer pairs for each gene were either designed using Primer3 software from NCBI sequences, or retrieved from published literature, and synthesized by Integrated DNA Technologies (IDT; Coralville, IA, USA). Standard curves were generated for each primer set using cDNA synthesized from pooled RNA. Each qPCR was in a final volume of 12 µL: 6 µL of 2X PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 µM of the respective forward and reverse primers, and 5 µL of cDNA, and RNase free water to volume. All measurements were performed in triplicate on 384-well plates using the QuantStudio7 Flex Real-Time PCR Systems together with QuantStudio Design and Analysis Software v2. The cycling conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for

15s, 55 °C for 15s, and 72 °C for 1 min. A dissociation curve analysis was performed at 95 °C for 15s, 60 °C for 1 min, and 95 °C for 15 s with each run to confirm specificity. Gene expression data were normalized to the mean of three reference genes: 60S ribosomal protein L7, β-Actin, and Glyceraldehyde-3-phosphate dehydrogenase, and expression of all genes was then calibrated to the reference fish sampled at day 0. Relative expression of target genes was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and is presented as $\log_2$-transformed fold-change.

2.6 Histopathology

At 21-dpd, one-gill filament, posterior kidney tissue, and 3-6 mm of the distal small intestine were fixed in Davidson's fixative for 48 h, then stored in 70% ethanol for 24 h before being processed into 5 μm paraffin sections and stained with hematoxylin and eosin (Humason, 1979). Each resulting slide was examined blindly, at both low (40X) and high magnification (400X), and each fish received a score based on an initial tissue examination. Histological ratings for *C. shasta (Cs)* in intestinal tissue and *P. minibicornis* (*Pm*) in kidney tissue, the parasites' primary infection sites, were graded on a scale of 0 to 2, with 0 = no parasites, 1 = parasite present in the respective tissue but with minimal inflammatory changes, and 2 = multifocal lesions associated with the parasite infection. *Cs2* rating of intestine refers to lamina propria hyperplasia, necrotic epithelium/sloughing, and necrotic muscularis. *Pm2* rating of kidney refers to interstitial hyperplasia, necrotic interstitium or tubule, interstitial granuloma, glomerulonephritis, and protein casts within the glomeruli or tubules.

2.7 Statistical analyses

We used a non-parametric Kruskal-Wallis to test whether pathogen abundance and gene expression in tissues changed in sentinel fish over the course of the study (i.e., from 0-, 7-, 14-, and 21-dpd) due to changing exposure conditions (Kruskal and Wallis, 1952). We applied a significance threshold of *p-value* = 0.05 and followed up any statistically significant result with a multi-comparison test with a Bonferroni error correction (Abdi, 2007). We assumed that gill and kidney tissue would respond differently and therefore analyzed them as separate outcomes. Pathogen abundance data were analyzed as $\log_{10}$ transformed values to detect changes that occurred over an order of magnitude, while differences in gene expression were analyzed as $\log_2$-transformed fold-change to evaluate changes in gene expression that doubled. All analyses were performed in R (version 2.15.2; R-CoreTeam 2021).

We used Principal Component Analysis (PCA: Jolliffe, 2002) to identify correlations between the expression of certain genes and pathogen exposure, as well as to determine which pathogens and genes explained most of the variation in the data. Specifically, we ran PCAs that included the three pathogens detected most frequently (i.e., *C. shasta* and *P. minibicornis*, and *I. multifiliis*) with the 11 target genes. We focused on these three pathogens as they were likely responsible for the majority of immune responses as determined by prevalence of detection and level of abundance. Separate PCAs were performed using gill and kidney data as we hypothesized these tissues would respond differently, and we ran PCA after scaling (and centering) all data since pathogen abundance and gene expression units differed. To formally test which principal components were statistically significant and which variables were significantly correlated with those principal components, we used a permutation-based approach as outlined by Vieira (2012).

3. RESULTS

During the 21-day study period, RBDD temperatures were 12.7 - 16.8 °C, with daily fluctuations of 0.6 to 1.7 °C (Fig. 3.2A). Upstream inputs from tributaries and reservoir releases caused daily river discharge fluctuations of up to 200 m^3 sec^{-1} (Fig. 3.2B).

3.1 Pathogen prevalence and abundance

Prevalence and pathogen abundance (measured as log_{10} copy numbers per ng of gDNA) varied for each pathogen and across sampling points, but both tended to increase over time. Of the eight pathogens screened for, three parasites: *C. shasta, P. minibicornis,* and *I. multifiliis,* and the bacteria *F. columnare* and RLO were detected in fish deployed at RBDD (Fig. 3.3). Gill and kidney were negative for all assayed pathogens in reference fish sampled at day 0, confirming that all fish were free of pathogens prior to deployment.

Ceratonova shasta and *P. minibicornis* were the pathogens detected most frequently, with 100% prevalence (20/20) at 7-, 14-, and 21-dpd for gill, and 14-, and 21-dpd for kidney. Abundance of *C. shasta* and *P. minibicornis* also increased significantly by 14- and 21-dpd (Supplementary Data S3.1, $p < 0.05$) when compared to 7-dpd in both gill and kidney (Fig. 3.3A, 3.3B). *C. shasta* detection in the intestine as measured by conventional PCR was relatively consistent with the detection in the kidney. In the intestine, positive samples were detected by 7-dpd in 55% of samples (11/20), followed by an increase in prevalence to 100% by 21-dpd (Table 3.3).

The additional pathogens detected (*I. multifiliis, F. columnare,* and RLO) were often near or below 50% prevalence over the duration of the study, with pathogen abundance staying relatively stable at different sampling points. *I. multifiliis* prevalence increased above 50% by 14- and 21-dpd in gill tissue and abundance was also significantly higher during this time period, while both prevalence and abundance in kidney tissue remained relatively low during the duration of the study (Fig. 3.3C). *F. columnare* and RLO (Fig. 3.3D and 3.3E,

respectively) were detected in gill and kidney by 7-, 14-, and 21-dpd. However, there were no differences in pathogen abundance, nor prevalence in gill and kidney between reference fish at day 0 and RBDD-deployed fish at 7-,14-, and 21-dpd.

3.2 Gene Expression

Immune, general stress, and development-related genes were influenced by river deployment and were differentially expressed between fish sampled from the reference fish at day 0 and RBDD-deployed fish (Fig. 3.4). In gill tissues, expression of proinflammatory cytokines was significantly upregulated over time (Supplementary Data S3.2); tumor necrosis factor α (*tnfα*, Fig. 3.4A), interleukin1β (*il1β*, Fig. 3.4B), acute-phase protein serum amyloid A (*saa*, Fig. 3.4C), and interleukin 8 (*il8*, Fig. 3.3D) at 7-, 14-, and 21-dpd, respectively, when compared to the reference fish at day 0. The genes *tnfα* (Fig. 3.4A), and interleukin-6 (*il6*, Fig. 3.4E), were the only pro-inflammatory genes to be significantly upregulated in the kidney by 7-, and 14-dpd, respectively. The regulatory gene, interleukin 10 (*il10*) was significantly upregulated in the gill tissue of RBDD-deployed fish at 7-, 14, and 21-dpd (Fig. 3.4F), and transforming growth factor β (*tgfβ*) was significantly upregulated at 14-dpd (Fig. 3.4G). Similarly, in the kidney IL-10 was significantly upregulated at 7-, 14- and 21-dpd (Fig. 3.4F), and *tgfβ* expression was significantly upregulated at 7-, and 21-dpd (Fig. 3.4G). Expression of adaptive immune genes; immunoglobulin T (*igt*) was significantly upregulated in kidney at 14- and 21-dpd (Fig. 3.4H), and immunoglobulin M (*igm*) was significantly upregulated in gills at 7-, 14-, and 21-dpd, (Fig. 3.4I). In kidney, a significant upregulation in IgM gene expression was found by 21-dpd (Fig. 3.4I).

Heat shock protein 47 (*hsp47*), which is associated with the general stress response, was significantly upregulated in gills by 7-dpd, followed by a significant down-regulation at 14- and 21-dpd (Fig. 3.4J) compared to reference fish at day 0. *Hsp47* expression in kidney was

similar to that of gill tissue with significant downregulation at 14-, and 21-dpd (Fig. 3.4J) compared to reference fish at day 0, although not significant at 7-dpd. The development gene, brain-derived neurotrophic factor (*bdnf*) was significantly upregulated in the kidney at 21-dpd (Fig. 3.4K).

3.3 Association between pathogen abundance and transcriptional responses

Principal component analysis was applied to the $\log_{10}$ copy number of pathogens, and the fold change of differentially regulated genes in the fish sampled at 0-, 7-, 14-, and 21-dpd (Fig. 3.6). The two dimensions (PC1 and PC2) explained 53% of the total variation in gill tissue. PC1 separated reference fish at day 0 from RBDD-deployed fish at 7-, 14-, and 21-dpd and explained 39% of the variation in the data (Fig. 3.6A). Genes significantly correlated ($p < 0.05$) with PC1 included five pro-inflammatory (*il1β*, *il8*, *il6*, *ssa*, and *tnfα*), one anti-inflammatory (*il10*) gene, one adaptive gene (*igm*), and one general stress gene (*hsp47*). Those significantly correlated with PC2 included one pro-inflammatory (*il6*) gene. The pathogens *C. shasta* and *P. minibicornis* were significantly correlated with PC1 and with an anti-inflammatory (*il10*) gene, with *I. multifiliis* also correlated with PC1 and 4 pro-inflammatory genes (*il1β*, *il8*, *saa*, and *tnfα*).

In kidney samples, the two dimensions (PC1 and PC2) explained 63% of the total variation. This PCA revealed a different clustering pattern than in gills. Particularly, PC1 distinguished reference fish at day 0 and RBDD fish at 7-dpd in one cluster, from RBDD-deployed fish at 14- and 21-dpd and explained 43% of the variation in the data (Fig. 3.6B). Genes significantly correlated ($p < 0.05$) with PC1 included the pro-inflammatory (*il6*, *il8*, and *tnfα*), one anti-inflammatory (*bdnf*) gene, and two adaptive genes (*igm* and *igt*). Those significantly correlated with PC2 were all pro-inflammatory (*ssa*, *il-1β*, and *il-8*) genes. The

pathogens *C. shasta* and *P. minibicornis* were significantly correlated with PC1 and correlated with an anti-inflammatory (*il10*) gene.

3.4 Histopathology

By 21-dpd, the prevalence of *P. minibicornis* in kidney samples and *C. shasta* in intestine samples were 100% and 95% respectively (Table 3.4). The majority of these infections were deemed light and were accompanied by minimum to no histological signs of inflammation (rating 1). *P. minibicornis* trophozoites were detected in low numbers in kidney glomeruli, associated with mild glomerulonephritis (Fig. 3.6A). Trophozoites of *C. shasta* were observed within the lamina propria and kidney granulomas in four fish, but there was no inflammation or enteronecrosis in the intestinal tissue (Fig. 3.6B).

4. DISCUSSION

This study used both molecular and histopathological approaches to determine the presence of pathogens of concern, and their impacts on the physiological and pathological responses of sentinel juvenile fall-run Chinook salmon. Our findings, indicate that 1) fall-run Chinook salmon are exposed to multiple potential pathogens when out-migrating from the Sacramento River, 2) gill biopsies can be used to assess salmon pathogen exposure and health, and 3) a targeted panel of gene expression assays can potentially elucidate how salmon respond to pathogen exposure. Overall, this study adds weight of evidence in support of the utility of molecular tools to investigate the physiological status of wild fish within the context of conservation.

4.1 Pathogens

Our findings indicate that Chinook salmon in the Sacramento River are exposed to multiple pathogens, posing a risk to out-migration survival. Specifically, sentinel fish at RBDD were exposed to three microparasites: the myxozoans *C. shasta and P. minibicornis,* the ciliate *I. multifiliis*; and two bacteria: *F. columnare* and RLO. Previous studies elsewhere in the Pacific Northwest have also identified that salmon are likely to encounter a wide range of pathogens and microparasites (Jeffries *et al.*, 2014; Miller *et al.*, 2014). These exposure events may not only result in direct mortality but also have sublethal effects that consume the host's energy that would otherwise be used for growth, survival, and reproduction (Bakke and Harris, 1998). Incorporating the effects of multiple pathogen exposures into population models of salmon survival will improve understanding of population dynamics in systems such as the Sacramento River and should be a goal for future research.

The most prevalent pathogens among sentinel fish sampled at 21-dpd using histologypathological examination were *C. shasta* and *P. minibicornis*. Observing that the prevalence and abundance of these pathogens followed similar trends (i.e., increasing over time) was not unexpected, as both share the same invertebrate annelid host: *Manayunkia occidentalis* (Atkinson et al., 2020), which sheds the infectious life stage of these pathogens. Co-infection by *C. shasta* and *P. minibicornis* has been previously reported in salmon in California rivers, especially when water temperatures are high (Stocking *et al.*, 2006; Foott *et al.*, 2007; Foott *et al*., 2017; Voss *et al*., 2019; Lehman *et al.*, 2020; Mauduit *et al.*, 2022). *C. shasta* is the etiological agent of enteronecrosis also known as ceratomyxosis (Hallett and Bartholomew, 2012), and is regarded to be a major cause of mortality in juvenile salmonids throughout systems in North America's Pacific Northwest (Fujiwara *et al.*, 2011; Foott *et al*., 2019). *P. minibicornis* infects the kidney of hosts and has been detected in streams along the Pacific Northwest (Foott et al., 2007).

For *C. shasta and P. minibicornis*, pathogen detection in gill samples collected at 21-dpd aligned with histopathology at 21-dpd in that both had a prevalence of detections at or near 100%. These findings further support the use of molecular disease diagnostics using gill biopsy samples to assess infection status in species of concern as has been done elsewhere (Jeffries *et al.*, 2014; Miller *et al.*, 2017) and imply these methods may provide utility as a non-lethal method to infer disease impacts. It is important to note, however, that the presence of specific pathogens in fish tissue does not always imply disease establishment and progression, especially for pathogens that are ubiquitous in the aquatic environment. To determine the pathological significance of pathogens in fish, a combination of molecular and histopathological approaches is required.

In the present study, *I. multifiliis*, a protozoan that has been detected in watersheds worldwide, was the third most frequently detected pathogen. The largest reported outbreak occurred in the Klamath Basin in 2002 when *I. multifiliis*, along with *F. columnare* infections, caused the largest salmon die-off in the American West's history (Belchik *et al.*, 2004). Prevalence and abundance estimates of *I. multifiliis* based on gill sampling used in this study, however, may be an underestimate of true prevalence because this pathogen primarily infects the skin tissue of fish (Buchmann, 2020). Therefore, our sample protocol may be underestimating exposure to *I. multifiliis* in the Sacramento River and future studies should evaluate the utility of collecting swabs over the skin of salmon for pathogen detection.

Flavobacterium columnare, the etiological agent of the highly contagious columnaris disease, can occur as both primary and secondary infections (Shoemaker and LaFrentz, 2015). The low prevalence and pathogen abundance of *F. columnare* observed in this study is interesting considering that this pathogen is transmitted from fish-fish, that young fish are often more susceptible to infection, and that crowding is a known risk factor. These characteristics of columnaris would seem to imply that sentinel cage studies would be a high-

risk environment for fish. The risk of columnaris is often considered to be greater when temperatures exceed 20 °C (reviewed by Declercq *et al.* (2013)). In our study, peak temperatures did not exceed 17 °C and therefore the timing of the study may have mitigated some of the columnaris risks.

4.2 Physiological responses to deployment at the RBDD

4.2.1 Immune response

Sentinel salmon at RBDD exhibited a pronounced immune response potentially due to pathogen exposure and infection; these were sentinel-deployed fish that are likely responding to multiple environmental parameters. Specifically, higher levels of pro-inflammatory cytokines and chemokines (*tnfα*, *il1β*, *saa*, *il8*, and *il6*) were often seen in gill samples by 7-dpd and typically seen later in kidney samples by 14-dpd. This earlier response observed in gill samples could be due to cytokines that are produced at the pathogen entry site, controlling the ability of phagocytes (i.e., cells able to engulf some pathogens) to eliminate invading pathogens by driving inflammatory signals (Bose and Farnia, 1995). *Tnfα* is frequently the first cytokine released during the pro-inflammatory signaling cascade in mammals and fish, triggering the release of *il1β* and other chemokines such as *il8* (Holopainen et al., 2012). Our data support this timeline, with *tnfα* being the only measured transcript that was significantly upregulated from day 0 in both the gill and kidney. Up-regulation of *il1β* in the early stages of infection is crucial for initiating the inflammatory response (Long et al., 2019), which might explain why *il1β* in gill tissues was upregulated by 7-dpd. *saa* levels declined steadily during the study period in gill samples, presumably indicating time-dependent inflammatory responses. *saa* is an acute-phase protein that increases in response to inflammation (Jensen et al., 1997; Long et al., 2019; Rebl et al., 2009), and is induced in fish in response to viral, bacterial, and parasitic infections (Braden et

al., 2015; Chettri et al., 2014; Sutherland et al., 2014; Villarroel et al., 2008). *Il8* was significantly upregulated in gill tissues, but not in kidney tissues, which might indicate an immune response to pathogen infections only at the entry site. Similar increases in *il8* have been found in other fish species exposed to bacteria, viral products, and cellular stress (Abdelkhalek et al., 2009; Pérez-Cordón et al., 2014). In contrast, *il6* was upregulated in the kidney, but not in gill tissues. This upregulation of *il6* could make it particularly important as a marker of systemic activation of proinflammatory cytokines (Barton, 1997). Overall, this dataset implies a prolonged elevation in the inflammatory response of salmon deployed in the Sacramento River over 21 days and a potentially detrimental effect on salmon due to inflammation.

Upregulation of regulatory cytokines (e.g., *il10* and *tgfβ*) allows for modulation of the pro-inflammatory response during infection (Munoz et al., 1991) limiting the potentially detrimental effects of an excessive inflammatory response (Opal and DePalo, 2000). Excessive *il10* is regarded as a critical biomarker following pathogen infections in fish, where moderately controlled *il10* mRNA expression levels indicate recovery and overproduction is associated with severe infection (Bailey et al., 2017; Gorgoglione et al., 2013). Furthermore, *tgfβ* has been demonstrated to be upregulated in multiple fish species in response to bacterial infections; *F. columnare* (Ravindra et al., 2019), *F. columnare* and *Edwardsiella ictaluri* (Wang *et al*., 2018), and *Aeromonas hydrophila* (Dang et al., 2016), and parasite infection; *Tetracapsuloides bryosalmonae* (Holland et al., 2003) and *Gyrodactylus derjavini* (Lindenstrøm et al., 2004). While we cannot conclude this from our research, early expression of *il10*, and *tgfβ* at 7-dpd in gill and kidney tissues, before the proinflammatory responses at 14-dpd, in response to pathogen infections could be a pathogen-driven strategy to counteract the host's inflammatory response causing immune suppression and, as a result, impairing the host's ability to control infections (Barrett and Bartholomew, 2021; Bjork et al.,

2014; Korytář et al., 2019). *l10* and *tgfβ* can suppress pro-inflammatory cytokine genes (such as *il1* and *tnf*) while also directing adaptive immune pathways involving B lymphocytes (Abbas et al., 1996; D'Amico et al., 2000; Dinarello, 2000; Syahputra et al., 2019; Zou and Secombes, 2016).

Pathogen infections elicited an adaptive immune response in sentinel salmon. Specifically, expression of *igt*, which plays a specific role in adaptive immune response (Castro et al., 2013) was significantly upregulated at 14- and 21-dpd in kidney tissues. Up-regulation of *igm* in gill tissues over the study period, and in kidney at 21-dpd, could be indicative of chronic or prolonged infections. Although our findings suggest that the immune system mounted an adaptive response to pathogen infections, molecular and histopathological screening continued to detect pathogens by 21-dpd. Hence the host immune response was either 1) insufficient to clear the parasites, 2) emerged too late after the tissue was already damaged by the initial inflammatory response, or 3) negatively affected the host and contributed to the pathology observed (Taggart-Murphy, 2018). Further work is needed to develop a better understanding of the effects of pathogen infections on the kinetics of antibody-mediated immunity.

4.2.2 Stress response

Temperatures at RBDD were within the tolerance range of Chinook salmon (Poletto et al., 2017), but fish experienced higher mean temperature at 7-dpd than at 0-, 14-, and 21-dpd (up to 16.8 °C; Fig 3.2) and higher temperature fluctuations (up to 1.7 °C) potentially causing thermal stress (Gallant et al., 2017). This was associated with the differential expression of *hsp47*, a gene that codes for a chaperone protein that promotes protein stabilization against heat stress and is regarded as a reliable biomarker of thermal stress (Akbarzadeh et al., 2018).

4.2.3 Development response

Brain-derived neurotrophic factor was significantly upregulated in the kidney at 21-dpd, which coincided with the upregulation pattern of pro-inflammatory genes (*tnfα*, and *il6*). Expression of *bdnf* in kidney tissue has recently been reported to be associated with nephropathy (Tao et al., 2018), and it has been further proposed as a biomarker for glomerular kidney injury (Endlich et al., 2018), having determined its role in glomerular development, morphology, and function in both zebrafish and rodents. The significant upregulation of *bdnf* in the kidney of deployed juvenile salmon could thus be a host-driven strategy for renal tissue repair, given observation of kidney tissue damage associated with *P. minibicornis*.

4.3 Overall health status of deployed fish

Principal component analysis of pathogen abundance and gene expression in both gill and kidney tissues differentiated responses across deployment duration, as well as across immune system functioning status of deployed juvenile Chinook salmon. While multiple genes explained the variation across sample time points, expression of one gene, in particular, the regulatory cytokine *il10* in gill samples, was strongly associated with the presence of *C. shasta* and *P. minibicornis*. Early infection was demonstrated in gill tissues, along with an indication of potential systemic infection and disease progression throughout the deployment period of these two pathogens.

Kidney samples further described the systemic infection and determined disease progression in the sentinel fish, with pro-inflammatory and regulatory genes clustering separately, seemingly in response to pathogen infections. Specifically, in kidney, expression of *bdnf* was associated with *P. minibicornis* abundance, further supporting the use of *bdnf* expression as a biomarker for kidney injury and potential tissue repair. Such molecular diagnostic methods could be highly sensitive in detecting early-stage impacts to pathogens,

even before cellular damage or external lesions appear (Miller et al., 2017). For infectious diseases, molecular tools can potentially distinguish between early and latent infections, in which the agent is detected but there is minimal host response due to early pathogen activity. This could be especially beneficial if such methods would function in instances where many infectious pathogens are present, particularly for conservation-based approaches that would benefit from non-lethal sampling.

In conclusion, infectious diseases are likely to remain a conservation issue as climate change continues to reshape host-environment-agent dynamics in the Sacramento River and elsewhere. To be able to predict the trajectory of these dynamics, will require the use of tools outlined in this study as well as others. For example, while we were able to observe immune responses of salmon to pathogen exposure and tie this to histologically observed infection states, we were not able to observe the survival outcome of the exposed fish, due to the lethal sampling methods used. Future work should merge non-lethal sampling methods employed in this study with observational-based approaches used to assess outmigration survival, to more clearly characterize the impact of pathogen exposure on salmon population health.

While our study focused on the most common pathogens in Chinook salmon, it did not cover all possible pathogens. Therefore, changes in genes expression could have been linked to other pathogens and/or anthropogenic factors (e.g., contaminants) necessitating cautious interpretation of the current findings. Shortcomings of the current study include the fact that the temporal trends of pathogen prevalence represent a group-level effect and may not be accurate for individual fish because we did not follow the same fish over time. Furthermore, gene expression data was calibrated against reference fish sampled at day 0 instead of using corresponding time point controls. Additional studies that improve on these shortcomings will allow for a more holistic picture of salmon conservation by improving our understanding of one of the many stressors, pathogens, affecting salmon populations.

Funding

This research was supported by the Delta Water Quality and Ecosystem Restoration Grant Program from the California Department of Fish and Wildlife (CDFW; Proposition 1 grant # Q1996036 to MED, SLH, and REC, and #P1896051 to REC and collaborators).

Acknowledgments

We thank Celeste Valdivia for assistance with DNA extraction and Bill Poytress from the United States Fish and Wildlife Service Red Bluff office for assisting with sentinel cage placement.

5. FIGURES & TABLES

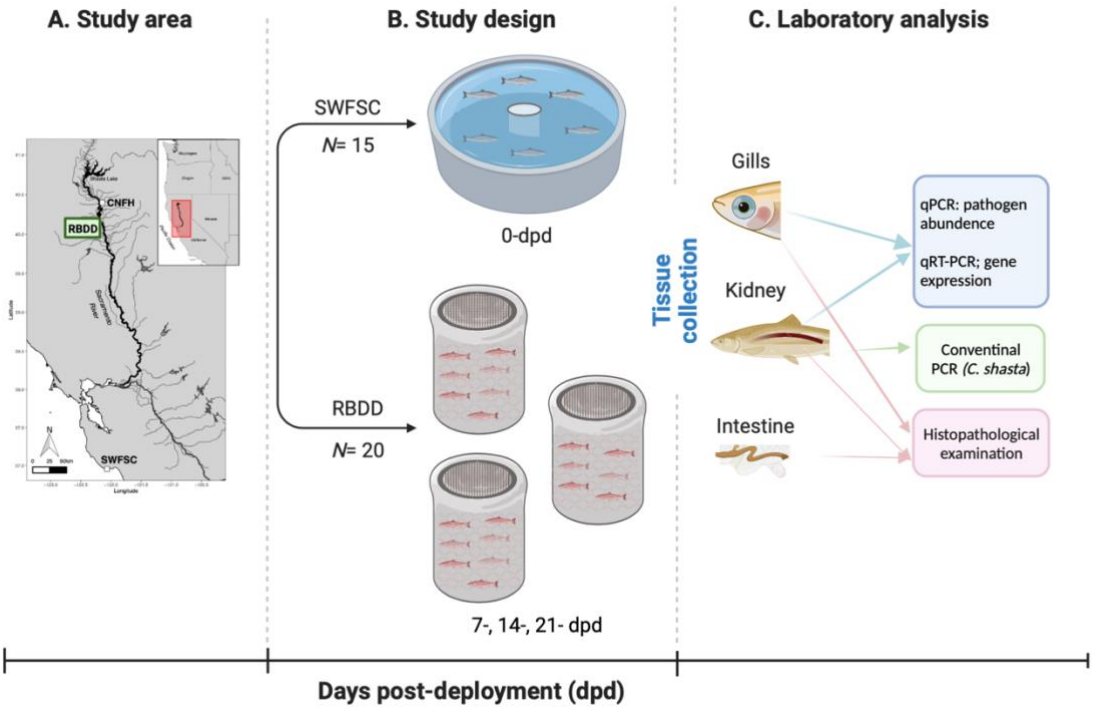

Figure 3.1: A) Map of the study area and fish facilities - Coleman National Fish Hatchery (CNFH), Red Bluff Diversion Dam (RBDD), and the Southwest Fisheries Science Center (SWFSC); **B)** Fish (*N*=15) were sampled at SWFSC on day 0, and (*N*=20) at each of 7-, 14-, and 21-days post-deployment (dpd) at the RBDD. **C)** Pathogen presence and host response were assessed by a combination of qPCR, conventional PCR, and histopathological evaluation.

Table 3.1: Pathogens screened for in quantitative polymerase chain reaction analyses in juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*).

Pathogen Species	Agent type	Reference
Ceratonova shasta	Parasite	Teffer et al. (2017)
Parvicapsula minibicornis	Parasite	Teffer et al. (2017)
Myxobolus cerebralis	Parasite	Teffer et al. (2017)
Ichthyophthirius multifiliis	Parasite	Teffer et al. (2017)
Candidatus Branchiomonas cysticola	Bacterium	Teffer et al. (2017)
Flavobacterium columnare	Bacterium	Panangala et al. (2007)
Flavobacterium psychrophilum	Bacterium	Teffer et al. (2017)
Rickettsia-like organism	Bacterium	Teffer et al. (2017)

Table 3.2: Biomarkers of host immunity, stress, and reference genes that were evaluated using quantitative polymerase chain reaction analyses in juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*).

Gene	Abbreviation	Function	Reference
Tumor necrosis factor alpha	*tnfα*	Proinflammatory	Mauduit et al. (2022)
Chemokine Interleukin 1β	*il1β*	Proinflammatory	Mauduit et al. (2022)
Serum amyloid protein A	*saa*	Proinflammatory	Mauduit et al. (2022)
Chemokine Interleukin 8	*il8*	Proinflammatory	Mauduit et al. (2022)
Chemokine Interleukin 6	*il6*	Proinflammatory	Bjork et al. (2014)
Interleukin 10	*il10*	Anti-inflammatory	Bjork et al. (2014)
Transforming growth factor β	*tgfβ*	Anti-inflammatory	Bjork et al. (2014)
Immunoglobulin T	*igt*	Adaptive	*This study
Classical Immunoglobulin	*igm*	Adaptive	Mauduit et al. (2022)
Heat Shock protein serpin H1	*hsp47*	General stress	*This study
Brain derived neurotropic factor	*bdnf*	Development	*This study
60S Ribosomal gene 7l	*rpl7*	Reference gene	Mauduit et al. (2022)
β-actin	*actβ*	Reference gene	Bjork et al. (2014)
Glyceraldehyde-3-Phosphate Dehydrogenase	*gapdh*	Reference gene	*This study

* See supplementary Table S1for primer sequence

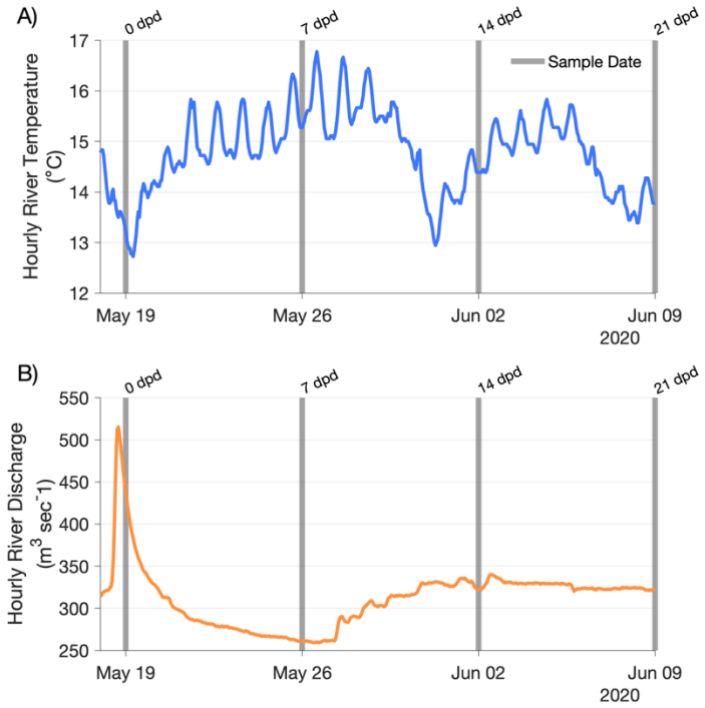

Figure 3.2: **A)** Water temperature (°C) and **B)** river discharge ($m^3 sec^{-1}$), measured during the 21-day study at the Red Bluff Diversion Dam. Data were collected every 15 minutes at the RBD monitoring station (cdec.water.ca.gov). Vertical grey lines represent the sample dates.

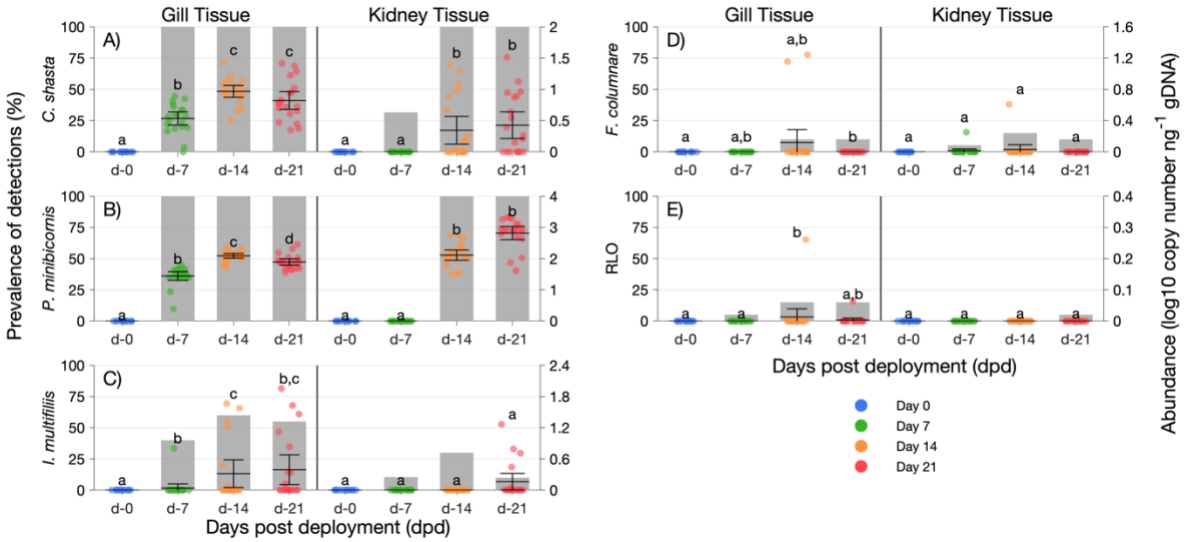

Figure 3.3: Bar plots of pathogen prevalence (grey bars, primary y-axis) overlaid with jitter plots of pathogen abundance (colored dots, secondary y-axis) in juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) at four sampling events (0-,7-, 14-, and 21-days post-deployment (dpd)) at Red Bluff Diversion Dam in gill and kidney tissue. **A)** *Ceratonova shasta* (*C. shasta*), **B)** *Parvicapsula minibicornis* (*P. minibicornis*), **C)** *Ichthyophthirius multifiliis* (*I. multifiliis*), **D)** *Flavobacterium columnare* (*F. columnare*), **E)** *Rickettsia*-like organism (RLO). No pathogens were detected in reference fish sampled at day 0. Lowercase letters denote significantly different ($p < 0.05$) ranks (2±SEM).

Table 3.3: *Ceratonova shasta* genomic DNA was assayed using conventional PCR in the intestine of juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) at four sampling events (day 0, 7-, 14-, and 21-days post-deployment (dpd)) at Red Bluff Diversion Dam. Amplicons were visualized by gel electrophoresis, and PCR result was given as negative ("-" no band) and positive.

Sample event	Prevalence
0 dpd	(0%) 0/15
7 dpd	(55%) 11/20
14 dpd	(95%) 19/20
21 dpd	(100%) 20/20

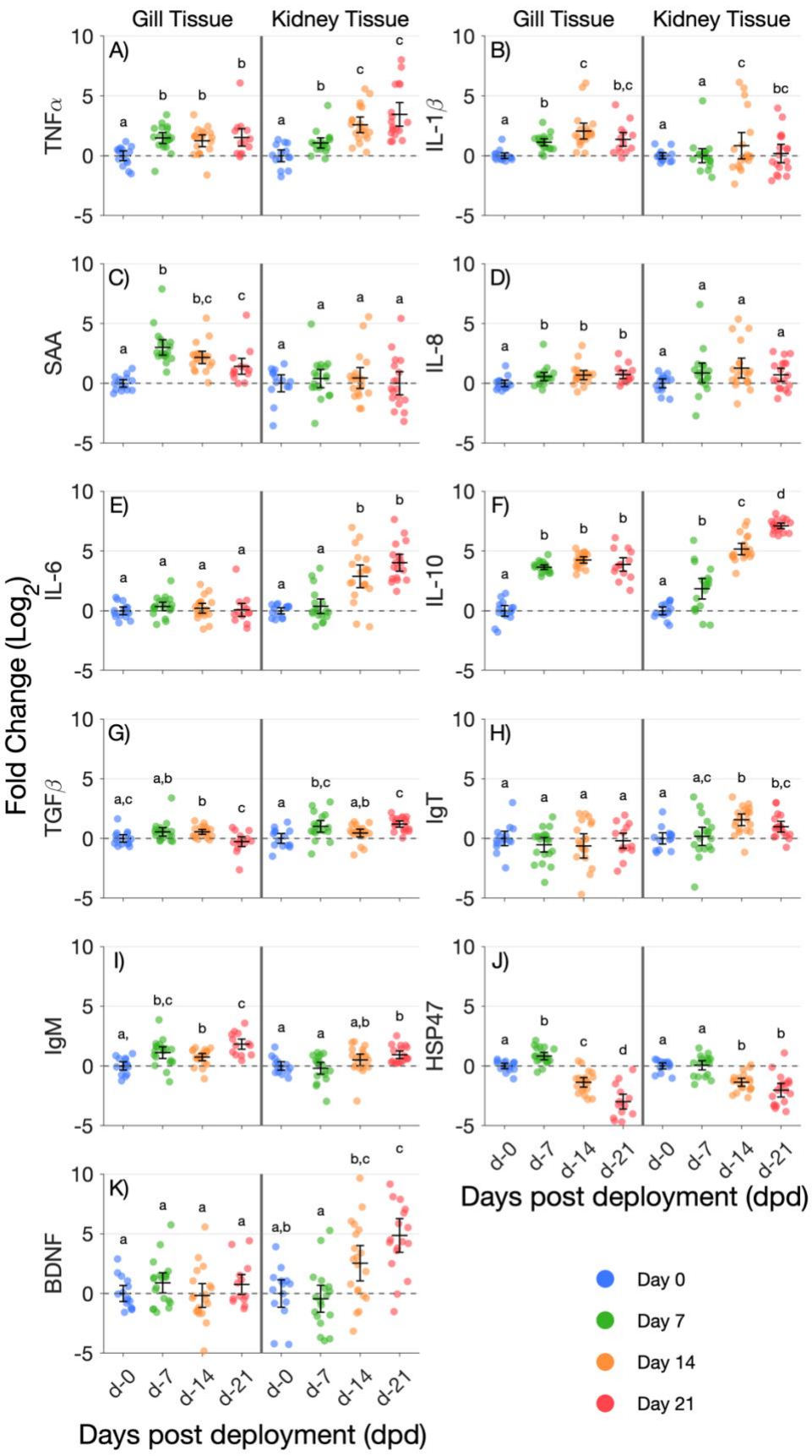
Gill Tissue
Kidney Tissue
A)
B)
C)
D)
E)
F)
G)
H)
I)
J)
K)
TNFα
IL-1β
SAA
IL-8
IL-6
IL-10
TGFβ
IgT
IgM
HSP47
BDNF
Fold Change (Log2)
d-0
d-7
d-14
d-21
Days post deployment (dpd)
Day 0
Day 7
Day 14
Day 21

Figure 3.4: Gene expression in juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) at four sampling events (0-,7-, 14-, and 21-days post-deployment (dpd)) at Red Bluff Diversion Dam (RBDD) in gill and kidney tissue presented in $\log_2$ fold changes of selected genes: Pro-inflammatory genes: **A)** tumor necrosis factor α (*tnfα*), **B)** interleukin 1β (*il1β*), **C)** acute phase protein serum amyloid A (*saa*), **D)** interleukin 8 (*il8*), **E)** interleukin-6 (*il6*), Regulatory genes: **F)** interleukin 10 (*il10*), **G)** transforming growth factor β (*tgfβ*), Adaptive immune genes: **H)** immunoglobulin T *(igt*), **I)** immunoglobulin M (*igm*), General stress gene **J)** heat shock protein 47 (*hsp47*), and Development; **K)** brain-derived neurotrophic factor (*bdnf*). Lowercase letters denote significantly different ($p < 0.05$) ranks (2±SEM). Gene expression was normalized against the reference genes: 60S ribosomal protein L7, β-Actin, and glyceraldehyde-3-phosphate dehydrogenase and calibrated against reference fish sampled at day 0.

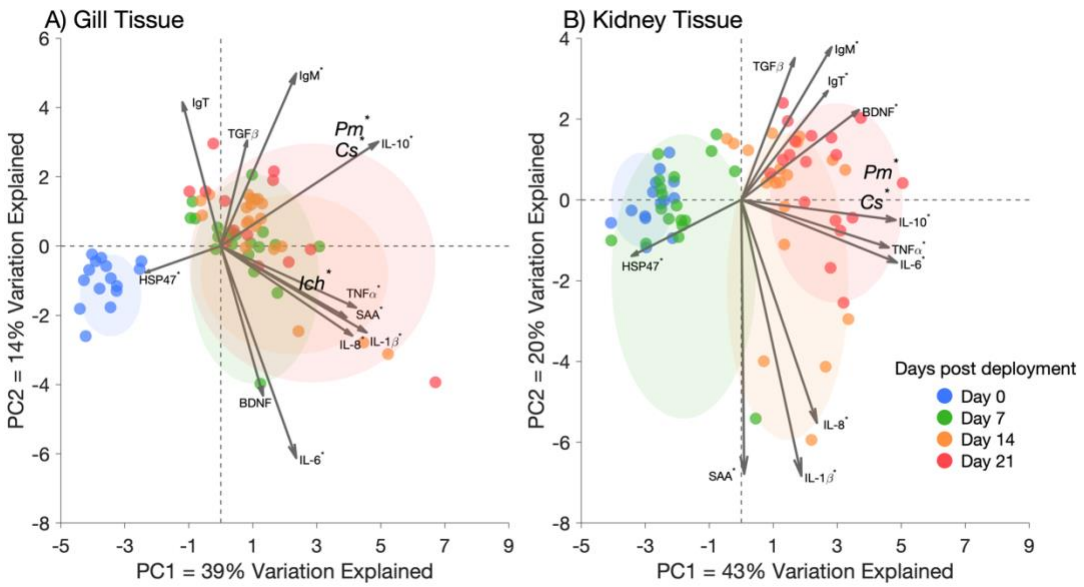

Figure 3.5: Principal component analysis plot for the gene expression profile in juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) at four sampling events (day 0, 7-, 14-, and 21-days post-deployment (dpd)) at Red Bluff Diversion Dam. **A)** gill and **B)** kidney, using $\log_2$ fold-changes for genes, and $\log_{10}$ pathogen abundance; *Ceratonova shasta (Cs), Parvicapsula minibicornis (Pm),* and *Ichthyophthirius multifiliis (*Ich*)*. Genes and pathogens significantly correlated with PC1 or PC2 are indicated with an asterisk.

Table 3.4: Prevalence of infection (number positive/total sample) for *Ceratonova shasta* (*Cs*) in the intestine, *Parvicapsula minibicornis* (*Pm*) in the kidney, and gill parasites in histological specimens (1= infection with little disease, 2= disease state) in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) at 21 days post-deployment.

Pathogen	Prevalence
*Cs*1	(89%) 17/19*
*Cs*2	(5%) 1/19
*Pm*1	(80%) 16/20
*Pm*2	(20%) 4/20

* Presumptive *Cs* trophozoites are visible in one-gill lamellae

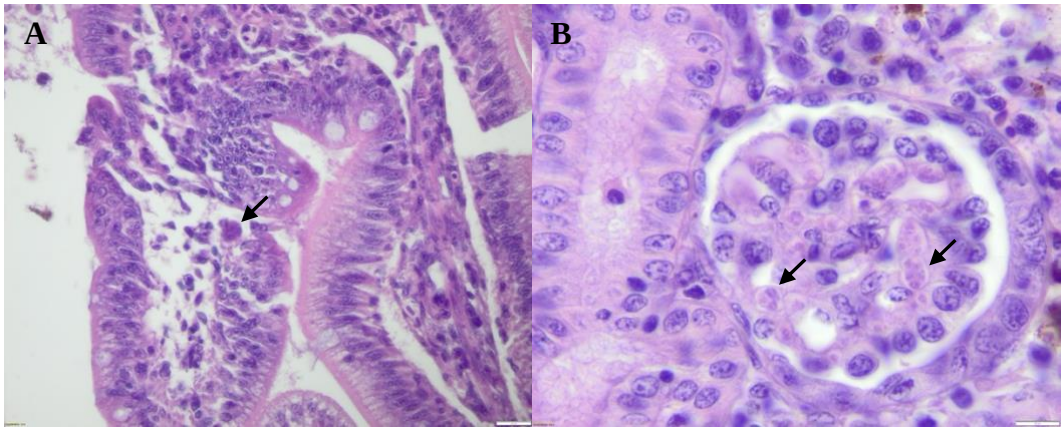

Figure 3.5. Hematoxylin and eosin (H&E) stained histological sections of juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) at 21-day post-deployment, arrows **A)** *Ceratonova shasta* trophozoite within lamina propria (Space is an artifact of processing), **B)** *Parvicapsula minibicornis* trophozoites within glomerulus with a moderate degree of glomerulonephritis. Scale bars = 10 µm.

Supplementary Table S3.1: Primer sequences developed in-house that were evaluated using quantitative polymerase chain reaction analyses in juvenile fall-run Chinook salmon.

Gene	Function		Forward primer sequence (5′–3′) Reverse primer sequence (5′–3′)
Immunoglobulin T	Adaptive	F	AGC TCC GCG TGG CTA AGA AT
		R	CAG GTA CTG GGT GGT GCC AA
Heat Shock protein serpin H1	General stress	F	TCC CTT CAT CTT CCT GGT GAA
		R	CCT TTG GGT CGC ACC ATT C
Brain derived neurotropic factor	Anti-inflammatory/ Development	F	GGC GTT GGG AAG CAG TAG AG
		R	AGC GAG GGT AAC TAA GGG GG
Glyceraldehyde-3-Phosphate Dehydrogenase	Reference gene	F	CGC GTC GCT GAC CTG TT
		R	TGT GAC ACT GGA GGG TGT GAG T

Supplementary data S 3.1: *P*-values for pathogen abundance in gill and kidney tissue of juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) at four sampling events (day 0, 7-, 14-, and 21-days post-deployment (dpd)) at Red Bluff Diversion Dam. A non-parametric Kruskal-Wallis test was used to analyze: *Ceratonova shasta* (*C. shasta*), *Parvicapsula minibicornis* (*P. minibicornis*), *Ichthyophthirius multifiliis* (*I. multifiliis*), *Flavobacterium columnare* (*F. columnare*), and *Rickettsia*-like organism (RLO). Any statistically significant results were then followed by a multi-comparison test with a Bonferroni error correction.

Gill tissue					Kidney tissue		
	dpd	7	14	21	7	14	21
C. shasta	0	0.000	0.000	0.000	0.077	0.000	0.000
	7	-	0.000	0.028	-	0.000	0.000
	14	-	-	0.295	-	-	1.000
P. minibicornis	0	0.000	0.000	0.000	0.022	0.000	0.000
	7	-	0.000	0.000	-	0.000	0.000
	14	-	-	0.014	-	-	0.000
I. multifiliis	0	0.010	0.000	0.001	1.000	1.000	0.150
	7	-	0.029	0.609	-	1.000	0.340
	14	-	-	1.000	-	-	0.730
F. columnare	0	1.000	0.260	0.040	1.000	1.000	1.000
	7	-	1.000	0.430	-	1.000	1.000
	14	-	-	1.000	-	-	1.000
RLO	0	1.000	0.022	1.000	-	0.660	1.000
	7	-	0.027	1.000	-	0.400	1.000
	14	-	-	0.765	-	-	0.640

Supplementary data S 3.2: *P*-values for gene expression in gill and kidney tissue of juvenile fall-run Chinook salmon (*Oncorhynchus tshawytscha*) at four sampling events (day 0, 7-, 14-, and 21-days post-deployment (dpd)) at Red Bluff Diversion Dam. A non-parametric Kruskal-Wallis test was used to analyze: tumor necrosis factor α (*tnfα*), interleukin 1β (*il1β*), acute phase protein serum amyloid A (*saa*), interleukin 8 (*il8*), interleukin 6 (*i6*), interleukin 10 (*il10*), transforming growth factor β (*tgfβ*), immunoglobulin T (*igt*), immunoglobulin M (*igm*), heat shock protein 47 (*hsp47*), and brain-derived neurotrophic factor *(bdnf*). Any statistically significant results were then followed by a multi-comparison test with a Bonferroni error correction.

Gill tissue					Kidney tissue		
tnfα	dpd	7	14	21	7	14	21
	0	0.000	0.006	0.034	0.040	0.000	0.000
	7	-	1.000	1.000	-	0.002	0.000
	14	-	-	1.000	-	-	1.000
il1β	dpd	7	14	21	7	14	21
	0	0.000	0.000	0.001	1.000	1.000	1.000
	7	-	0.024	1.000	-	1.000	1.000
	14	-	-	0.843	-	-	1.000
saa	dpd	7	14	21	7	14	21
	0	0.000	0.000	0.002	1.000	1.000	1.000
	7	-	0.124	0.005	-	1.000	1.000
	14	-	-	0.116	-	-	1.000
il8	dpd	7	14	21	7	14	21
	0	0.043	0.016	0.024	0.280	0.210	0.990
	7	-	1.000	1.000	-	1.000	1.000
	14	-	-	1.000	-	-	1.000
il6	dpd	7	14	21	7	14	21
	0	0.700	1.000	1.000	1.000	0.000	0.000
	7	-	1.000	0.710	-	0.004	0.000
	14	-	-	1.000	-	-	0.311
il10	dpd	7	14	21	7	14	21
	0	0.000	0.000	0.000	0.022	0.000	0.000
	7	-	0.009	1.000	-	0.000	0.000
	14	-	-	1.000	-	-	0.000
tgfβ	dpd	7	14	21	7	14	21
	0	0.053	0.018	1.000	0.047	0.716	0.002
	7	-	1.000	0.048	-	0.492	1.000
	14	-	-	0.013	-	-	0.010
igt	dpd	7	14	21	7	14	21
	0	1.000	1.000	1.000	1.000	0.000	0.040
	7	-	1.000	1.000	-	0.030	0.413
	14	-	-	1.000	-	-	0.356
igm	dpd	7	14	21	7	14	21
	0	0.007	0.015	0.000	1.000	0.234	0.005
	7	-	0.577	0.377	-	0.333	0.021
	14	-	-	0.023	-	-	1.000
hsp47	dpd	7	14	21	7	14	21
	0	0.00	0.00	0.00	1.000	0.000	0.000
	7	-	0.00	0.00	-	0.000	0.000
	14	-	-	0.01	-	-	0.230
bdnf	dpd	7	14	21	7	14	21
	0	1.000	1.000	1.000	1.000	0.282	0.001

	7	-	0.490	1.000	-	0.029	0.000
	14	-	-	1.000	-	-	0.183

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