

The Effect of Acute Heat Stress on the Chinook Salmon Immune System and on Ability to Combat *Vibrio anguillarum* Infection

by

Emily McKenzie

A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirement for the degree of

Master of Science

in

Biology

Waterloo, Ontario, Canada, 2024

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The frequency, duration, and intensity of heatwaves in western Canada is expected to rise in the coming years. As a result, shallow rivers and streams have also experienced drastic changes, and water temperature may fluctuate by up to 13 °C throughout the duration of a single day. This poses a problem for salmon species, as they travel from marine to freshwater environments to spawn. Additionally, salmon are poikilothermic, meaning their physiological functions are influenced by their surrounding water temperature. Encountering water that is outside of their optimal growth temperature of 11-17 °C may result in behavioural and metabolic changes and, in extreme cases, rapid death. This may also result in increased susceptibility to infectious disease, as warming waters may render them temporarily immunocompromised and increase the virulence factors of some pathogens. Salmon have several cellular mechanisms to survive these stressful events, including heat shock proteins, immune responses, and secretion of glucocorticoids. Interestingly, the concept of thermal preconditioning is emerging as a method to aid salmonids' ability to handle increasing water temperatures – exposure to a controlled, short-term stimulus triggers a physiological response that prepares them for future, more extreme stress exposure. However, thermal preconditioning has never been tested before exposure to a pathogen. Further, the immune effects of heat waves have scarcely been investigated in salmon. In this study, we performed an environmentally applicable mock heat shock on juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and measured their immunological responses up to 14 days afterwards. Chinook salmon also received an injection of live *Vibrio anguillarum* after heat shock to determine if heat deterred their ability to fight a systemic bacterial infection. The transcripts of IL-1 β , IL-8, TNF- α , IL-10, TGF- β , IFN- γ , IL-2, cathelicidin, hepcidin, MHC1 α ,

tapasin, MHC2 α , MHC2 β , HSP47, HSP70, and HSP90 were quantified by qPCR in the spleen, gills, and hindgut, as well as assessment of the stress response by measuring glucose, lactate, cortisol, and HSP47 levels in the plasma. Heat shock did not affect mortality rates due to vibriosis compared to salmon that received *V. anguillarum* alone. Additionally, heat shock mitigated the pro-inflammatory and corresponding anti-inflammatory responses needed to combat infection by initially upregulating *il1b*, *tnfa*, *il8*, and *il10*, then returning to normal levels by three days post-infection. The antimicrobial peptides *camp* and *hamp* played a significant role in combatting infection in both *V. anguillarum* treatment groups. Transcript levels of *hsp47* and *hsp90* were upregulated in response to both heat shock and bacterial infection; however, *hsp70* expression was surprisingly low through the duration of the trial. HSP47 proteins measured by ELISA in the serum did not differ between any treatment groups. Heat shock caused a significant increase in plasma and cortisol lactate concentrations that both returned to basal levels 6-hours post-heat shock. Altogether, these data indicate that the experimental heat shock had a positive preconditioning effect on Chinook salmon and provides new insights on the interactions between the host, environment, and pathogen over a 14-day period. This research also provides a first step in understand how increasing river temperatures affect the salmon immune and stress responses and can help inform decision making in policy and sustainability initiatives to protect Pacific salmon populations in the coming years.

Acknowledgements

Firstly, I would like to thank my supervisor, Dr. Brian Dixon. This project has been a whirlwind of changes and new ideas, and you were always there to support me, with a few laughs along the way. You introduced me to the wonderful and complex world of immunology, which continues to fascinate me the more I study it. I am incredibly grateful that you took a chance on me as a very eager and talkative undergraduate student taking on a BIOL 499 project, because it led me to work in this crazy, wacky lab with a crazy, wacky supervisor that loves fish. To everyone in the Dixon lab, you have made it so much fun to come to work every day, even when our experiments don't work, or we are in a writing slump. Muchas gracias for the Spanish lessons and many, many coffees we shared together. A special thank you to Tania Rodríguez-Ramos for always being there to teach me new (scary) techniques and answering all of my silly questions, and to Xiaoqing (Blandine) Dang for always being there for a bubble tea or croissant date – I am grateful for your friendship.

I would also like to thank both of my committee members for their support and expertise with this project. Dr. Paul Craig, I would be absolutely lost during fieldwork without you. Your ideas have made this project a complete, well-rounded story. Dr. Narveen Jandu, we have come a long way from your seminar class I took in 2019. Without your advice all of those years ago, I would never be the biologist I am today. Thank you also to Drs. John and Ann Heath, who were courteous enough to allow me to use their hatchery, equipment, and salmon to make this project possible. You are two of the most talented and generous scientists I have ever met.

Thank you to my family for ensuing in me a diligent work ethic with a reminder to have fun along the way. You have always been there through the highs and lows of grad school, rooting me on along the way. I am so lucky to have such a strong support system. By the way, if you're reading this, hi Mom! Finally, I would like to thank my cat Churro, who put in his best efforts to help me write this thesis by walking across my laptop several times (sorry, but those parts got cut. He tried his best, though).

Dedication

To my father, John. My time with you was cut too short. I hope I have made you proud.

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List of Abbreviations

<i>16s</i>	16S ribosomal subunit
<i>18s</i>	18s ribosomal subunit
<i>actb</i>	Beta actin
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
<i>camp</i>	Cathelicidin
cDNA	Complementary DNA
CFU	Colony forming units
CHSE-214	Chinook salmon embryonic cell line
CT_{MAX}	Critical thermal maximum
<i>cx6cl</i>	Cytochrome oxidase subunit 6C-1
DO	Dissolved oxygen
DPI	Days post-infection
DPT	Days post-treatment
<i>ef1a</i>	Elongation factor 1 α gene
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
<i>gadph</i>	Glyceraldehyde-3-phosphohate dehydrogenase
GALT	Gut associated lymphoid tissue
gDNA	Genomic DNA
GIALT	Gill associated lymphoid tissue
<i>hamp</i>	Hepcidin
HS	Heat shock
HSP	Heat shock protein
HSP47	Heat shock protein 47
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
I.P.	Intraperitoneal
IFN-γ	Interferon gamma
IL-10	Interleukin 10
IL1B	Interleukin 1 beta
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
LPS	Lipopolysaccharide
MALT	Mucosal associated lymphoid tissue
MHC	Major histocompatibility complex

MWCO	Molecular weight cutoff
OD	Optical density
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PMA	Phorbol-myristate-acetate
Poly I:C	Polyinosinic:polycytidylic acid
PST	Pacific standard time
qPCR	Quantitative polymerase chain reaction
<i>RecA</i>	Recombinase A
SEM	Standard error of the mean
TBS	Tris buffered saline
TGFB	Transforming growth factor beta
TNFA	Tumor necrosis factor alpha
T_C	Cytotoxic T cell
T_H	Helper T cell
T_{REG}	Regulatory T cell
TSA	Tryptic soy agar
TSB	Tryptic soy broth

Chapter 1

Introduction

1.1 Salmonids in British Columbia

Pacific salmon (*Oncorhynchus* spp.) represent a large and dominant component of Pacific Northwest bodies of water. They are distributed along the coast in California, towards the Canadian and Alaskan coasts, and near the Arctic Ocean, as well as along the coasts of Russia, Japan, and Korea (Irvine & Fukuwaka, 2011). Along with their role in the aquatic ecosystem, salmon are an important food source and gained a crucial economic role as a commercial commodity (Criddle & Shimizu, 2014). Chinook salmon (*Oncorhynchus tshawytscha*) are the largest of the Pacific salmon in North America. Due to their relatively low abundance compared to other salmon species (such as pink (*O. gorbuscha*), chum (*O. keta*), and sockeye (*O. nerka*) salmon) and their large size, their meat is considered some of the highest quality and is commercially valuable (Ohlberger et al., 2018). However, since the 1970's, salmon populations have been decreasing to dangerously low wild levels, and Fisheries and Oceans Canada reports that Chinook salmon populations are reaching critically low levels in Southern British Columbia (DFO, 2019). There are several factors that affect populations, such as disease, fishing, and alterations in river discharge (Irvine & Fukuwaka, 2011). Notably, climate change effects on Pacific salmon populations have been an increasing research area of interest. Along the American and Canadian coasts in 1990, there was a sharp decline in Chinook salmon catch levels, notably occurring simultaneously with significant climate and aquatic environment shifts (Beamish et al., 1999; Noakes et al., 2000). It is now well understood that climate change has the

potential to severely affect the viability of Pacific salmon populations (Muñoz et al., 2015) and that more research into the extent of this impact and how salmon respond to their environmental changes is crucial (Honea et al., 2016).

1.2 Heat waves and river temperatures

There is a strong link between the increasing global temperature and the prevalence of heat waves. The frequency, duration, and intensity of heatwaves have been increasing in recent years and is expected to continue increasing globally in the coming decades (NOAA, 2021; Piccolroaz et al., 2018; Tassone et al., 2022). Heat waves are characterized by a disproportionate change in temperature for three consecutive days or longer (Smoyer-Tomic et al., 2003). Further, as the annual number of days of extreme heat have doubled between 1996 and 2021 (Tassone et al., 2022), heat waves are a growing area of concern to the government and to the public (Zuo et al., 2015). Heat waves result in several downstream consequences, such as increased water temperatures in shallow streams and rivers (Zuo et al., 2015). There is a strong positive correlation between daily water and air temperature, and shallow rivers are particularly susceptible to temperature fluctuations, as thermal energy transfer occurs mostly on the air-water level (Graf, 2019; Piccolroaz et al., 2018). Thus, for every 1 °C increase in air temperature, stream temperature correspondingly rises approximately 0.4-0.6 °C (Morrill et al., 2005). This results in a large change in water temperature throughout a 24-hour period, typically greater than 8 °C per day (Gallant et al., 2017). In British Columbia, river temperatures fluctuated from 10 °C to 23 °C over the course of seven days in July of 2003 & 2004 (Parkinson et al., 2016), leading

to increased organism mortality, shifts in the river ecosystem, and a decrease in water quality (Graf, 2019; Tassone et al., 2022).

1.3 Temperature impact on salmon

Increasing water temperatures have several consequences for teleosts. As poikilothermic animals, salmon's body temperatures fluctuate with their surrounding environment, meaning that their physiological functions are influenced by the water temperature (Bonachea, 2019; Morvan et al., 1998). As a result, they have preferential temperatures, at which their ability to survive, feed, and grow is at its maximum, as well as thermal tolerance limits – the upper and lower temperature limits at which salmon can survive (Ern et al., 2023). Salmonids have an optimal growth temperature of between 11-17 °C, a critical thermal maximum (CT_{MAX}) that starts around 29-31 °C (Recsetar et al., 2012), and display behavioural and metabolic changes when exposed to acute changes in temperature (Sullivan et al., 2000; see Figure 1.1).

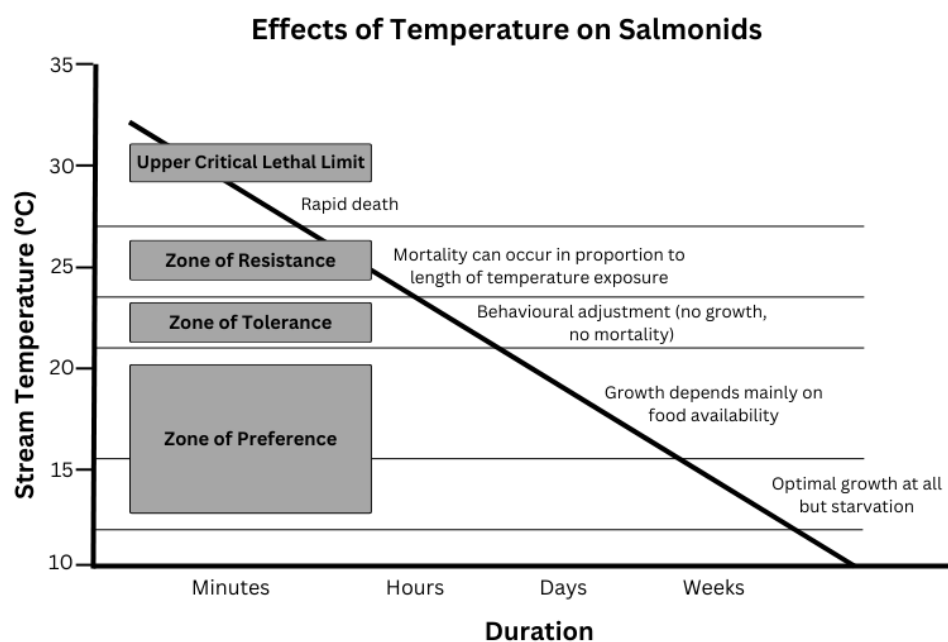


Figure 1.1 Biological effects of temperature on salmonids

The physiological and behavioural characteristics of salmonids vary with temperature. There is a relationship between the temperature, duration, and fish species that determine the potential effects when fish are exposed to temperatures outside their zone of preference. Upper critical temperature limits can cause death within minutes to hours of exposure. Figure adapted from (Sullivan et al., 2000).

With the rise in global water temperatures, it is becoming increasingly likely that salmon will encounter water at their upper tolerance limit while they travel from saltwater to freshwater to spawn (Chiaramonte et al., 2016). Notably for Chinook salmon that spawn in the summer, increased water temperature is considered an important variable in spawning success (Honea et al., 2016). Further, as increased water temperatures last longer throughout the day, it leaves little time for acclimation to or recovery from high temperatures (Gallant et al., 2017). Increased heat may also reduce the amount of dissolved oxygen (DO) available for respiration (Smoyer-Tomic et al., 2003). If stream temperatures continue to rise, the DO may reach critically low levels, making the journey even more difficult for salmonids to complete (Morrill et al., 2005). Therefore, it is important that we study the effects of increased river temperatures to determine any sublethal effects they may impose on salmonids.

1.4 Heatwaves and infectious diseases

The susceptibility of an organism to a disease is influenced by the interaction between the host themselves, their environment, and the pathogen (Marcogliese, 2008). Water temperature can influence both host susceptibility and pathogenicity of the infectious agent (Chiaramonte et al., 2016). Thermal stress of salmon may result in temporary immunocompetence, increasing

their susceptibility to disease (Marcogliese, 2008). Further, increased water temperatures improve the virulence factors of some pathogens (Lages et al., 2019; Marcogliese, 2008). It is predicted that, in general, increased temperature will cause the organism to have a greater susceptibility to disease and will increase the pathogenicity of infectious agents (Chiaramonte et al., 2016).

1.4.1 *Vibrio anguillarum*

Vibrio anguillarum (*V. anguillarum*) is a Gram-negative, comma-shaped bacterium that grows readily in freshwater and saltwater (Frans et al., 2011). It is the causative agent of vibriosis, a highly fatal disease found in over 50 species of fish, crustaceans, and bivalves (Frans et al., 2011). Signs of disease include hemorrhagic skin lesions, gill necrosis, respiratory distress, lethargy and exophthalmia (Ina-Salwany et al., 2019). These infections can also quickly become systemic and cause mortality as soon as three days post-infection (Hickey & Lee, 2017).

V. anguillarum grows readily at its optimal temperature of 25 °C, but can survive at temperatures ranging from 5 °C to 30 °C (Frans et al., 2011; Larsen et al., 2004). In warmer water temperatures (25 °C as opposed to 15 °C), *V. anguillarum* shows increased growth, chemotaxis, and motility gene expression (Lages et al., 2019), and the number of vibriosis outbreaks are reported to increase with an increase in water temperature (Guijarro et al., 2015). Further, in a study assessing the effects of water temperature on mortality rates of juvenile Coho salmon infected with *V. anguillarum*, it was discovered that the time from exposure to death and were proportional to increasing temperatures, with the average days to death being 2.7 at 21 °C compared to 11.8 at 9 °C (Groberg Jr. et al., 1983).

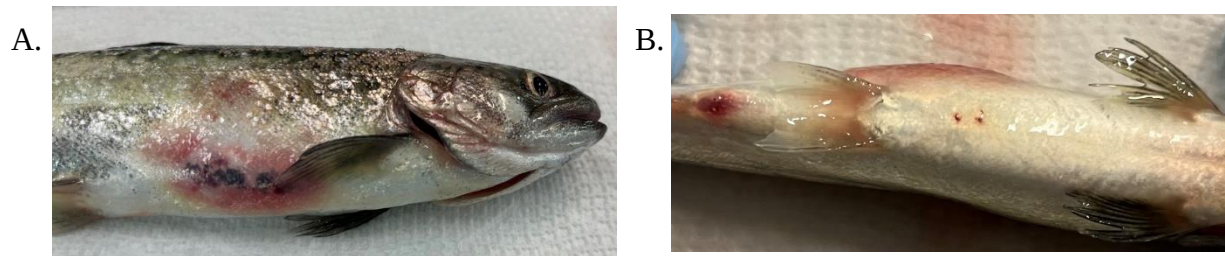


Figure 1.2 Symptoms of vibriosis in juvenile Chinook salmon

Symptoms of vibriosis, caused by the pathogen *V. anguillarum*, observed in this study include red and swollen lesions in the skin (A, B), reddened gills, and bleeding from the anus resulting from internal hemorrhaging (B).

1.5 The salmonid immune system

Teleosts have developed several innate and adaptive immune mechanisms in order to protect themselves from pathogens. Innate immunity is a non-specific first line of defense that teleosts rely on heavily, due to the limitations of the adaptive system (for example, slow proliferation of lymphocytes and limited antibody repertoire) and the large amount of pathogens that fish encounter in an aquatic environment (Aoki et al., 2008; Semple & Dixon, 2020; Uribe et al., 2011). However, the adaptive immune system provides a stronger, more specific response towards pathogens, is necessary for developing immunological memory, and both are needed for successful clearance and future protection against infectious diseases (Semple & Dixon, 2020).

1.5.1 Immune organs

Primary and secondary lymphoid organs

Similarly to mammals, teleosts have immune organs that are crucial to the development and maturation of leukocytes, including bone marrow, lymph nodes, and the spleen. However, fish lack many of these organs and instead rely solely on the anterior kidney (also referred to as

the head kidney) and spleen (Soulliere & Dixon, 2017). The head kidney is a primary lymphoid organ that acts equivalently to bone marrow in mammals, responsible for the production of B- and T-cell progenitors (Soulliere & Dixon, 2017). The head kidney also contains macrophages and monocytes and is involved in the secretion of cytokines as a secondary lymphoid organ (Soulliere & Dixon, 2017; Uribe et al., 2011). The spleen is another secondary lymphoid organ which induces and intensifies immune responses (Press & Evensen, 1999). Macrophages line the walls of the spleen and phagocytose antigens, and B and T cells are activated to produce a large adaptive response (Castro et al., 2013; Soulliere & Dixon, 2017).

Mucosal associated lymphoid tissue

The mucosal-associated lymphoid tissue (MALT) is present in the gut, skin, and gills, of teleosts. These tissues are in constant contact with potential pathogens in the aquatic environment and thus require several immune barriers in order for teleosts to combat potential pathogens (Salinas, 2015). MALT also serves to mediate mucosal homeostasis by generating both innate and adaptive immune responses in the face of a threat to the organism's health (Yu et al., 2020a). Contrary to mammals, teleosts lack organized lymphoid structures such as Peyer's patches, however, MALT contain T and B cells and resident immune cells to defend the mucosal surface (Peterson, 2015; Salinas, 2015; Yu et al., 2020a).

The gut associated lymphoid tissue (GALT) of fish is less organized than in mammals, but teleosts have a more diffusely organized immune system within the gut itself (Rombout et al., 2011). Mucosal T cells are the major leucocyte population in the gut, and neutrophils, macrophages, and eosinophils are also present (Peterson, 2015; Rombout et al., 2011). Notably,

the hindgut is the preferential site of bacterial antigen uptake and has the largest role in mucosal immunity (Peterson, 2015). Within another MALT, the gill-associated lymphoid tissue (GIALT), the mucus layer acts as a physical barrier to microorganisms and contains antimicrobial peptides and lysozymes for a non-specific immune capability. Beyond the mucus layer, B cells, macrophages, and granular cells are found within the epithelium (Peterson, 2015).

1.5.2 Cytokines

One crucial component of the immune system are cytokines – small signalling proteins that, when bound to their associated receptors, cause a signalling cascade that further coordinates an immune response (Aoki et al., 2008; Mulder et al., 2007). These molecules are grouped into several broad categories based on their function, including pro-inflammatory effects, anti-inflammatory effects, and activation of other immune cells. The upregulation of cytokine transcripts during infection makes them ideal biomarkers for identifying stressful conditions, such as heat stress or pathogenic stimulation (Aoki et al., 2008).

1.5.3 Pro-inflammatory response

The inflammatory immune response is a highly regulated system responsible for the activation of immune cells and initial pathogen clearance (Grayfer & Belosevic, 2012). They are produced in response to several pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) (Dinarello, 2009), phorbol-myristate-acetate (PMA) (Engelsma et al., 2001) and polyinosinic:polycytidylic acid (poly(I:C)) (Li et al., 2018). These cytokines are often the first step in response to infection or stress, as their actions on the cell result in a downstream, larger immune response (Hou et al., 2021).

Interleukin 1 beta (IL-1 β) is one of the first cytokines expressed in an immune response (Frenette et al., 2023). The secretion of the mature protein from resident macrophages, dendritic cells, and some non-immune cells (such as epithelial cells) results in the migration of phagocytes to the site of infection (Zou & Secombes, 2016). IL-1 β has also been shown to increase the expression of other pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF- α) and interleukins 6 and 8 (IL-6 & IL-8), (Zou & Secombes, 2016). Secondly, TNF- α is an important activator of the immune response, as it is responsible for the chemotaxis and phagocytic enhancement of neutrophils and monocytes (Grayfer & Belosevic, 2012). It also plays an important role in the activation and proliferation of macrophages and triggers the expression of itself, IL-1 β , and IL-8 (Hong et al., 2013). Lastly, IL-8 is a potent chemokine which recruits neutrophils to the site of inflammation (Omaima Harun et al., 2008). IL-8 is produced as a pro-peptide and in healthy tissue, basal levels are low (Santana et al., 2016). However, active IL-8 is produced locally at the site of tissue inflammation is upregulated in response to cellular stress, pathogens, or other cytokines (Sáenz-Martínez et al., 2021; Santana et al., 2016).

1.5.4 Anti-inflammatory response

Anti-inflammatory cytokines are important modulators of an immune response and often act correspondingly to inflammation during bacterial infection (Semple & Dixon, 2020). The balance between pro-inflammatory and anti-inflammatory cytokines is a dynamic system that changes in order to control the immune response and prevent damage to host tissues as much as possible (Opal & DePalo, 2000; Semple & Dixon, 2020). These cytokines, including interleukin

10 (IL-10) and transforming growth factor beta (TGF- β), activate tissue repair mechanisms and inhibit the actions of pro-inflammatory responses in tissue to limit excessive inflammation (Opal & DePalo, 2000; Wei et al., 2015).

IL-10 is produced by almost all leukocytes but is mainly produced by, and functions in conjunction with, regulatory T cells (T_{REG} cells) (Belkaid, 2007; Semple & Dixon, 2020). It is well established that IL-10 has anti-inflammatory properties through dampening the effects of pro-inflammatory cytokines, as well as inhibiting the production of antiviral cytokines in Type I-helper T (T_H1) cells (Semple & Dixon, 2020; Wang et al., 2010; Wei et al., 2015). TGF- β plays a dual role, possessing both anti-inflammatory and tissue repair properties. Immunologically, TGF- β prevents the activation of T cells to self-antigens and protects against excessive inflammation by inhibiting activities of macrophages, neutrophils, and Type 17-helper T (T_H17) cells (Sanjabi et al., 2009; Zhang et al., 2022). Once pro-inflammatory signals begin to diminish, TGF- β and other growth factors can begin tissue repair as the source of tissue damage is cleared (Wahl et al., 1989).

1.5.5 Communication with the adaptive immune system

In order to mount a full immune response towards the first exposure of a pathogen, after the non-specific innate immune response there must be activation of an adaptive immune response, which is more flexible, more specific, and can provide the organism with protection against future exposures to the infectious agent. Interleukin-2 (IL-2) and interferon-gamma (IFN- γ) are two examples of cytokines whose actions stimulate the activation or differentiation of

other aspects of the immune system, which allows for a larger and more robust immune response.

IL-2 is a main promoter of T cell growth and differentiation into T helper 1 (T_H1) and T helper 2 (T_H2) cells (Wang et al., 2018). In salmonids, its expression is highly controlled, is secreted mainly by activated CD4⁺ T cells, and is stimulated by T cell activation and bacterial infection (Díaz-Rosales et al., 2009; Wang et al., 2018; Zhang et al., 2024). IL-2 increases the expression of the apoptotic factors perforin and granzyme, while decreasing IL-17, insinuating that it has an inhibitory effect on the production of T_{REG} cells (Zhang et al., 2024). Secondly, IFN- γ is a cytokine produced exclusively by cells of the immune system, notably by T_H1 cells, CD8⁺ cytotoxic T cells (T_C cells), and antigen presenting cells (monocytes and macrophages) (Bhat et al., 2018; Robertsen, 2006). Cells infected with a pathogen will secrete IFN- γ , which upregulates hundreds of inducible genes, notably, MHC II, several chemokines, and tapasin (Bhat et al., 2018; Grimholt et al., 2020; Robertsen, 2006). IFN- γ is regulated by other cytokines; for example, its production is upregulated by IL-2 and downregulated by TGF- β (Schoenborn & Wilson, 2007). It also promotes its own expression by T cells, acting as a positive feedback loop (Schoenborn & Wilson, 2007).

1.5.6 Antimicrobial peptides

Antimicrobial peptides (AMPs) are an ancient form of immunity and their small peptides sequences of approximately 18-46 amino acids are highly conserved amongst vertebrates (Chang et al., 2006; Katzenback, 2015). They have broad and non-specific antimicrobial activity and are induced during bacterial infection (Santana et al., 2016). mRNA levels of AMPs also increase in

response to IL-8 (Santana et al., 2016). AMPs work by disrupting the cell membranes of bacteria or bypassing the pathogen's membrane, working directly on intracellular targets and are recognized as essential components of the innate immune system (Chang et al., 2006; Semple & Dixon, 2020).

Cathelicidins are found largely on mucosal surfaces and in neutrophils of teleosts (Santana et al., 2016) and are induced in response to bacteria as early as 24 hours post-infection (Katzenback, 2015). Transcript levels also increase in response to the pro-inflammatory cytokines IL-1 β and IL-6 and several Gram-positive and Gram-negative bacteria (Katzenback, 2015; Furlan et al., 2018). Hepcidin is another family of AMPs that, in addition to its antimicrobial functions, also regulates iron transport and maintains iron homeostasis for the host (Valenzuela-Munoz et al., 2017). This also becomes important in *V. anguillarum* infection, as this pathogen uses iron as a cofactor to synthesize many of its key enzymes (Guijarro et al., 2015). In addition, hepcidin can regulate inflammatory responses and is upregulated in response to bacterial, viral, and fungal pathogens (Alvarez et al., 2014; Katzenback, 2015).

1.5.7 Antigen presentation

The antigen presentation pathways are the main connectors between the innate and adaptive immune responses. At their core, the major histocompatibility complex (MHC) receptors and their accessory molecules are responsible for the processing, loading, and in some cases presenting of antigen peptides (Johnstone & Chaves-Pozo, 2022; Wong-Benito et al., 2023). Intracellular or endogenous antigens (such as viruses) and extracellular or exogenous antigens (such as bacteria and including *V. anguillarum*) are processed using the MHC I and II

pathways, respectively (Johnstone & Chaves-Pozo, 2022). MHC I molecules are found on all nucleated cells and will be loaded with the antigen peptide in the endoplasmic reticulum (ER) (Neefjes et al., 2011; Semple & Dixon, 2020). MHC I is assisted by several chaperones, including tapasin, which is involved in the loading of high-affinity peptides to the MHC I molecules (Neefjes et al., 2011; Wong-Benito et al., 2023). Once the peptide is loaded, MHC I leaves the ER for the cell surface, where it will present the peptide to CD8⁺ T_C cells and results in the death of the infected cell (Wong-Benito et al., 2023). Opposingly, MHC II molecules are only found in the ER of antigen presenting cells (APCs) and are composed of α and β dimers (Neefjes et al., 2011; Semple & Dixon, 2020). Their peptides are loaded in the endosomal-lysosomal compartment before departing for the cell surface (Semple & Dixon, 2020; Wong-Benito et al., 2023). Their presentation to CD4⁺ T_H cells results in B cell differentiation, antibody production, and cytokine release (Wilson, 2017).

1.5.8 Heat shock proteins

Though not often categorized as part of the immune system, heat shock proteins (HSPs) play a major role in protein regulation under stressful conditions. HSPs are involved in the maintenance of protein folding and functionality and make up 5-10% of total proteins in healthy cells (Mohanty et al., 2018; Stewart & Young, 2004). When teleosts experience stress, such as heat shock or bacterial infection, HSPs transcripts undergo rapid upregulation as the risk of misfolded proteins increases (Mohanty et al., 2018). There are also several connections between HSPs and the immune system. For example, HSPs can induce the production of pro-inflammatory cytokines (Mohanty et al., 2018), are involved in the collection of peptides for

antigen presentation (Stewart & Young, 2004), and act as intracellular signaling molecules in T cell-mediated immune responses (Roberts et al., 2010).

HSPs are classified, and accordingly named, based on their protein's molecular weight, such as HSP47, HSP70, and HSP90 (Xie et al., 2015). HSP47, the smallest protein of these three, is localized in the endoplasmic reticulum and is involved in the processing of procollagen (Deane & Woo, 2010). It also plays an important protective role during heat stress (Wang et al., 2015). HSP47 was shown to upregulate macrophage and ROS activity after heat stress in rainbow trout (Wang et al., 2015). HSP70 functions mainly as a chaperone protein involved in protein assembly, movement, and secretion (Roberts et al., 2010). It is also the most highly conserved HSP molecule in aquatic animals and is widely used as a biomarker for stress (Baharloeï et al., 2021; Deane & Woo, 2010). Immunologically, HSP70 has anti-apoptotic activity (Deane & Woo, 2010) and its expression levels have been shown to increase for up to three days after bacterial infection (Baharloeï et al., 2021). Lastly, HSP90 also functions as a molecular chaperone in the cytosol, endoplasmic reticulum, and mitochondria, but is also involved in protein degradation, cell signaling, and intracellular transport (Xie et al., 2015). HSP90 is considered essential for survival of the cell and is upregulated in response to several stressors (Deane & Woo, 2005; Roberts et al., 2010); however, its response (or lack thereof) during infection is variable (Deane & Woo, 2005; Wong & Do, 2017).

1.6 The teleost molecular stress response

It is well known that increased temperature leads to a physiological stress response in salmonids (Anttila et al., 2014; Logan & Somero, 2011). More specifically, heat stress can lead

to activation of the neuro-endocrine system and the release of cortisol, a well-known biomarker for stress in an organism (Lemos et al., 2023; Vijayan & Moon, 1994; Pickering & Pottinger, 1989). Cortisol is the main glucocorticoid released during stress in teleosts. It is also fast-acting and can be released just 3-5 minutes after exposure to a stressor (Sopinka et al., 2016). Cortisol is heavily involved in transcriptional regulation; for example, it stimulates the expression of HSPs and ubiquitin, both of which are involved in protein regulation and misfolded protein degradation (Kassahn et al., 2009; Sopinka et al., 2016).

Further, changes in the endocrine system also result in a secondary response, such as inducing metabolic changes in the fish to increase the amount of glucose available to use as energy. For example, during physical stress, there is an accumulation of lactate due to the anaerobic metabolism of glycogen, the fish's energy stores. This lactate will undergo conversion to glucose to both provide energy to the fish and restore glycogen (Pagnotta & Milligan, 1991). Therefore, levels of glucose, lactate or both may increase in the plasma during a stressful event, such as heat shock (Vaage et al., 2023). Stress responses are necessary to provide sufficient energy and oxygen for the fish to survive the stressor (Vaage et al., 2023). However, both primary and secondary stress responses can lead to behavioural changes, disease resistance, and impaired growth in salmon (Lemos et al., 2023), and thus it is important to measure these factors during and after heat shock and *V. anguillarum* infection.

1.7 Thermal preconditioning

Thermal preconditioning is a technique that can potentially help salmon acclimatize to long-term stressors. Thermal preconditioning involves exposing fish to a controlled, short-term

stimulus that will trigger a physiological response that will better prepare them for future stressors (Guo et al., 2023; Sapozhnikova et al., 2023). This technique relies on the idea that fish have plasticity, thus, the organism can respond to a change in their environment with a change in their molecular and cellular responses (Johnston, 2006; Sessions et al., 2021). In this sense, salmon may have the ability to benefit from preconditioning – the first stress exposure provides a “cellular buffer” that allows salmon to withstand a second stress exposure (Guo et al., 2023). So far this has proven true, as recent studies in salmonids have shown positive effects of thermal preconditioning on phagocyte cell levels (Sapozhnikova et al., 2023), cardioprotective functioning (de Preux Charles et al., 2016), and more flexible heat shock responses (Sessions et al., 2021). Preconditioning of rainbow trout has also shown to decrease cortisol levels and IL-1 β transcripts when exposed to a secondary thermal test compared to trout that were not preconditioned (Guo et al., 2023).

Many studies on preconditioning in teleosts focus on the biological effects of a short and subsequent long, more extreme heat stress, hypoxia, or a combination of these two factors (Guo et al., 2023; Williams et al., 2019). However, research exploring preconditioning as a protective measure against a pathogen is scarce. In one study, repeated, short-term hypoxic challenges during disease progression were beneficial against the virus *Piscine orthoreovirus* in Atlantic salmon (Lund et al., 2017). However, there have been no studies that show the effects of one thermal preconditioning event and how it impacts salmon cellular responses to bacterial infection. It is argued that the preconditioning stimulus must reach a threshold before providing

molecular protection (Overgaard et al., 2004); therefore, it is difficult to determine if a heat shock will provide enough stimulation to aid in combatting *V. anguillarum* infection.

1.8 Knowledge gaps

The stress and metabolic responses of teleosts in response to heat has been well-examined through experimental trials (Anttila et al., 2014; Logan & Somero, 2011; Nakano et al., 2014; Palmisano et al., 2000; Saranyan et al., 2017), and some studies show the upregulation of immune genes after acute heat shock (Dawood et al., 2020; Yu et al., 2020b; Zhou et al., 2021). However, these studies focus mainly on CT_{MAX} experiments and on heat shock lasting no more than a few hours. Some studies have shown that combined heat shock/*V. anguillarum* infection has stressful effects on scallops (Sun et al., 2014; Wang et al., 2012), brine shrimp (Sung et al., 2007), and zebrafish (Bennoit & Craig, 2020). Further, only one study has performed an environmentally realistic heat shock on salmon (Gallant et al., 2017). However, there have been no infection trials showing the combined effects of acute heat shock and live *V. anguillarum* on the salmonid immune system.

This research will be the first time an infection trial using live bacteria has been completed after acute heat shock in Chinook salmon. Further, it will provide several insights into how an experimental heat wave affects the salmonid immune system and stress responses while providing direction for future studies using heat shock trials.

1.9 Thesis structure and objectives

The objective of this thesis was to determine the effect of a 17-hour heat shock on the Chinook salmon immune system and on the organisms' ability to combat a bacterial infection. This 17-hour heat shock is considered environmentally realistic and mimics the temperature fluctuation a shallow freshwater river may undergo during a summer heat wave.

The three specific objectives of this thesis are:

1. To gain an understanding of how of acute heat shock affects the immune system of juvenile Chinook salmon gene and protein expression in key tissues, up to 14 days post-heat shock;
2. To determine the effect of this acute heat shock on the Chinook salmon's ability to combat infection with *Vibrio anguillarum* and assess differences in mortality due to disease and/or heat shock; and
3. To assess the changes in the stress and metabolism responses by measuring glucose and cortisol levels in the plasma.

Based on previous research assessing heat shock and disease in salmonids, my hypothesis is that acute heat shock will stress Chinook salmon, lessening their ability to fight an infection. Additionally, mortality rates due to vibriosis may be higher in heat shocked fish compared to those without heat shock.

Chapter 2

Materials and Methods

2.1 Quinsam River Temperature Trial

During the week of July 18, 2023, 4:00 pm PST to July 25, 2023, 2:00 pm PST, the water temperature of the Quinsam River in British Columbia, Canada (50°01'11.4" N, 125°18'05.8" W) was measured to assess the correlation between stream water temperatures and ambient temperatures. Results from this were used to determine optimal temperature to use for experimental acute heat shock (outlined in Section 2 below).

Water temperature was measured using iButton Thermochrons (DS1921Z-F5; iButtonLink Technology; Wisconsin, USA), which recorded and stored the temperature (range of -5 °C to 26 °C) every 15 minutes. Two iButtons were set up: one approximately 0.30 m from stream surface (termed “Shallow Water”) and one approximately 1.0 m from stream surface (termed “Deep Water”). Prior to placement in the river, each iButton was submerged in a plastic coating and attached to metal stakes using parafilm to maintain integrity in the water. After the recording period, iButtons were removed from the water and temperature data was retrieved and analyzed using ExactLog™ software (iButtonLink Technology). Data from the Government of Canada Hourly Data Report for Campbell River, British Columbia, Canada (49°57'07.000" N, 125°16'23.000" W) was used to determine air temperatures (Government of Canada, 2023). Temperature historical data was recorded every hour.

2.2 Infection Trial

Chinook salmon underwent one of four treatments and are referred to by the treatment names in brackets hereafter: no heat shock and no infection (No HS/PBS), heat shock and no infection (HS/PBS), no heat shock and infection (No HS/*Vibrio*), and heat shock and infection (HS/*Vibrio*). A summary of the tank setup is displayed in Figure 2.1.

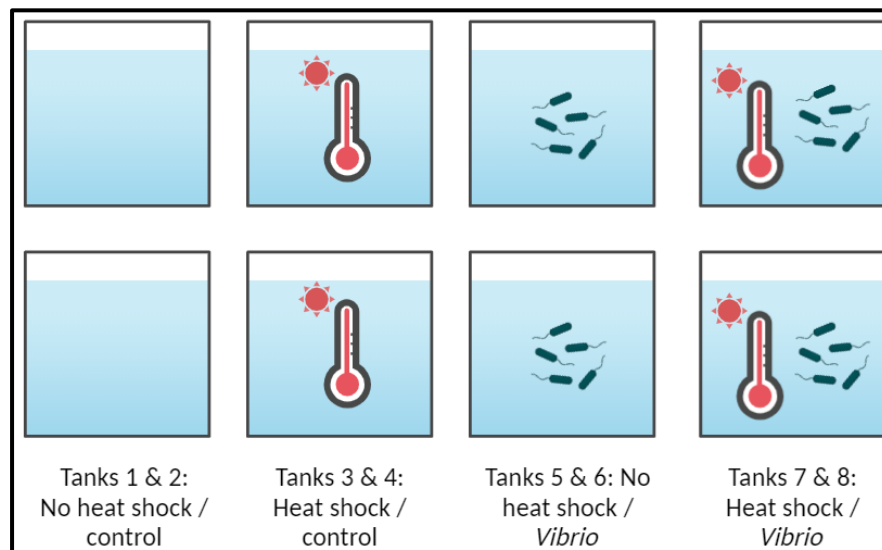


Figure 2.1 Tank setup for acute heat shock/infection trial

Representation of the tank and treatment set up utilized in this heat shock and infection trial. Eight 500 L tanks housed 40 Chinook salmon each at the beginning of this experiment and is labeled with their appropriate treatment (either No heat shock/PBS, Heat shock/PBS, No heat shock/*Vibrio*, or Heat shock/*Vibrio*).

2.2.1 Experimental animals

The fish used in this experiment were provided by Yellow Island Aquaculture Ltd. (YIAL; Quadra Island, British Columbia). 320 Chinook salmon juveniles (104 ± 40 g bodyweight) were housed in freshwater with a 15:9 h (light:dark) photoperiod and were fed twice a day with dry pellets (EWOS Harmony 2 mm) to satiety. Fish were randomly distributed

into one of eight 500 L tanks at a density of 40 fish per tank (fish density = 8.32 kg/m³). Water was maintained at 11.1 ± 0.1 °C and 8.78 ± 0.37 mg/L of dissolved oxygen (measured using the Oxi 3310® handheld dissolved oxygen and temperature meter; D.C., USA) and water flow was maintained at 3 L/min. Salmon were acclimated for 14 days before exposure to treatment. Water outflow from tanks exposed to *V. anguillarum* were treated with a UV sterilizer to prevent pathogenic material from leaving the experimental hatchery.

2.2.2 Culture conditions of *V. anguillarum*

Vibrio anguillarum serotype O1 (J382) (Machimbirike et al., 2023) was used as the infectious agent for this trial. All growth procedures henceforth were performed in triplicate. A loopful of *V. anguillarum* from frozen glycerol stocks was streaked onto tryptic soy agar supplemented with 2% NaCl (TSA 2% NaCl; Multicell Wisent, Quebec, Canada) and was incubated at room temperature for 48 hours. Afterwards, a single colony of *V. anguillarum* was inoculated into 2.5 mL tryptic soy broth supplemented with 2% NaCl (TSB 2% NaCl; Multicell Wisent) and was incubated at room temperature for 24 hours at 200 rpm. 150 mL of TSB 2% NaCl media was inoculated with 150 µL of growth and incubated at room temperature for 20 hours at 200 rpm.

After incubation, 10 mL of the culture was centrifuged at room temperature for 10 minutes at 6,000 rpm to pellet the bacteria. The pellet was washed with phosphate buffered saline (PBS) and recentrifuged according to the parameters above. This was procedure was repeated a total of three times. Afterwards, the pellet was resuspended in 25 mL of PBS for a

final concentration of 8.2×10^7 CFU/mL. This was confirmed by serial dilution and quantification by plating on TSA 2% NaCl, which was incubated for 2 days at room temperature.

2.2.3 Acute heat shock

The thermal cycle used in this experiment (Figure 2.2) was derived from the Quinsam River temperatures collected (as described in Section 2.1) to approximate water temperatures during a summer day in Campbell River. The temperature and dissolved oxygen levels were monitored and recorded every 5 minutes using the Oxi 3310® handheld dissolved oxygen and temperature meter. Fish undergoing heat shock were moved to one of two tubs (Coleman SaluSpa AirJet; Illinois, U.S.A.) at a fish density of 9.08 kg/m^3 . Fish not undergoing heat shock were moved to one of two 1000 L holding troughs at random to mimic handling and treatment stress. Salmon were fasted 24 hours prior to movement and treatment.

The thermal cycle began at 11.5°C and increased to a maximum temperature of 21.4°C over the course of 8.5 hours and at a rate of $1.17^\circ\text{C}/\text{hour}$. To mimic a point at which the temperature would not fluctuate significantly (i.e. mid-day afternoon), fish were held at a temperature of $21.4 - 21.1^\circ\text{C}$ for 1 hour. After this, the temperature was decreased from 21.2°C to 12.2°C over a span of 7.5 hours at a rate of $1.15^\circ\text{C}/\text{hour}$. After heat shock was complete, fish received an injection as described below and returned to one of their two corresponding treatment tanks at random.

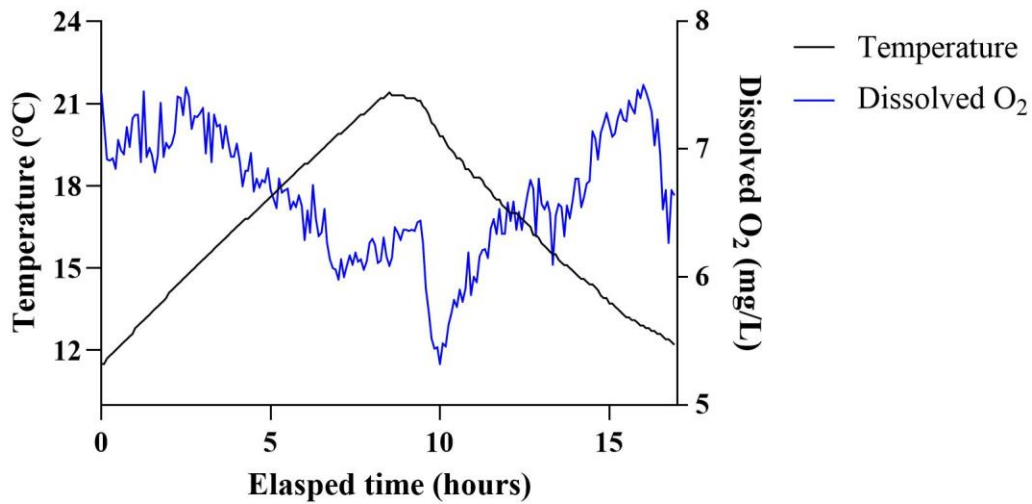


Figure 2.2 Temperature and dissolved oxygen fluctuation of heat bath during experimental heat shock

The temperature and dissolved oxygen levels of the heat bath were monitored and recorded every 5 minutes by a temperature meter and recorded. The temperature of the water, which began at 11.5 °C, was raised by 1.17 °C/hour, held at a peak temperature 21.2 ± 0.2 °C for 1 hour, then lowered by -1.15 °C/hour. The dissolved oxygen averaged 6.64 mg/L through the course of the heat shock.

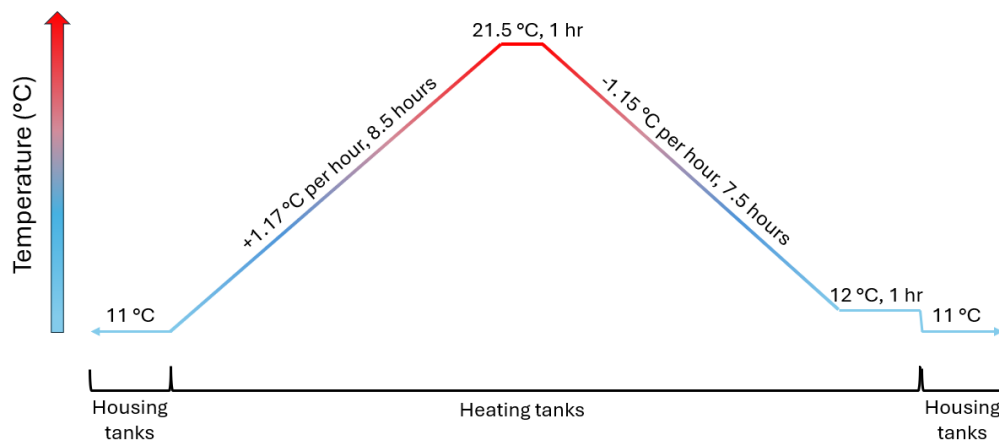
2.2.4 Infection with Live *V. anguillarum*

Fish receiving a bacterial treatment were intraperitoneally (I.P.) injected with 100 µL of live *V. anguillarum* at a final concentration of 8.2×10^6 CFU/mL. Fish that did not receive bacterial treatment were I.P. injected with 100 µL of sterile PBS. 6 fish per treatment, per timepoint (3 fish from each treatment tank) were sampled at timepoints 0, 0.25- (6 hours), 1-, 3-, 7-, and 14- days post-treatment/days post-inoculation (dpt/dpi). Three types of controls were sampled at $t = 0$: absolute controls ($n = 8$), which did not undergo any handling or treatment whatsoever, handling controls ($n = 8$), where fish underwent the same handling as No HS

treatment groups, and heat shock controls (n = 8), where fish underwent heat shock as outlined above, but did not receive an I.P. injection.

To sample, fish were anesthetized with a non-lethal dose of clove oil. Fish weights and fork lengths were measured and a sample of blood from the caudal vein was taken before euthanasia by cervical dislocation. Blood was collected in 3 mL syringes rinsed with heparin, then placed in a 1.5 mL tube containing 100 μ L of heparin to prevent coagulation. Plasma was obtained by centrifuging whole blood at 2,500 x g for 5 minutes, then transferring the plasma to a new 1.5 mL tube. Following euthanasia, spleen, hindgut, and gill tissues were extracted. All tissues and plasma were snap-frozen in liquid nitrogen prior to being stored at -80 °C until utilization.

A.



B.

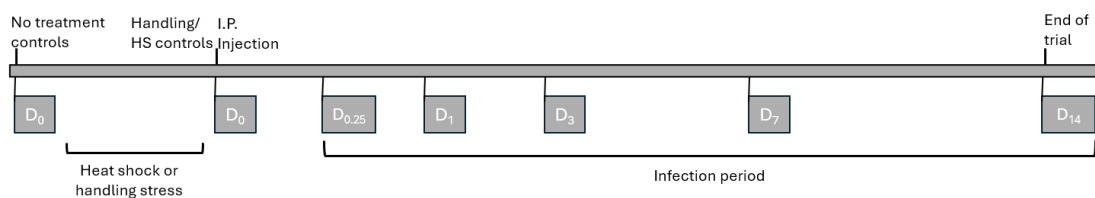


Figure 2.3 Graphical abstracts of experimental timeline

A graphical abstract depicting the acute heat shock timeline and temperatures (A) and the timeline for the experiment (B). Three salmon were sampled from each tank at each timepoint during the infection period, for a total for $n = 6$ for each treatment group.

2.3 qPCR

2.3.1 RNA extraction and cDNA synthesis

Total RNA was isolated from Chinook salmon spleen, hindgut, and gill using 1 mL of TRAZOL reagent following the manufacturer's protocol (GeneBio Systems; Ontario, Canada). After extraction, RNA was quantified using a Take3 plate and Synergy H1 plate reader (BioTek Instruments; Vermont, USA) and 5 µg of RNA was treated using 2 µL of Ambion DNase I and 4 µL of DNase buffer x10 (Invitrogen; Massachusetts, U.S.A.) to remove residual genomic DNA, and the RNA was subsequently washed following the manufacturer's protocol and stored at -80 °C until further use.

Prior to complementary DNA (cDNA) synthesis, DNase-treated RNA samples were quantified. Samples were also assessed for purity using the plate reader, in which a A260/280 ratio of 2.0 ± 0.2 was considered purified RNA. cDNA was synthesized from 250 ng of DNase-treated RNA from each sample using 2 µg of qScript cDNA Supermix (Quanta BioSciences; Massachusetts, U.S.A.) and adjusted to a final volume of 10 µL with RNase-free water. Each sample was incubated at 25 °C for 5 minutes, then 42 °C for 30 minutes, and lastly 85 °C for 5 minutes. Afterwards, samples were stored at -20 °C until further use.

2.3.2 qPCR analysis

The primer sequences of all target genes used in this study are listed in Table 2.1 and include interleukin 1 beta (*il1b*), interleukin 8 (*il8*), tumor necrosis factor alpha (*tnfa*), interleukin-10 (*il10*), transforming growth factor beta (*tgfb*), interferon gamma (*ifng*), interleukin 2 (*il2*), cathelicidin antimicrobial peptide (*camp*), hepcidin antimicrobial peptide (*hamp*), MHC 1 alpha (*mhc1a*), tapasin (*tapasin*), MHC 2 alpha (*mhc2a*), MHC 2 beta (*mhc2b*), heat shock protein (HSP) 47 (*hsp47*), HSP 70 (*hsp70*), and HSP90 (*hsp90*). Additionally, the sequences of five candidate endogenous controls are listed, including beta actin (*actb*), cytochrome c oxidase subunit 6C-1 (*cx6c1*), glyceraldehyde-3-phosphate dehydrogenase (*gadph*), elongation factor 1 alpha (*ef1a*), and 18S ribosomal RNA (*18s*). All tissues used the first sequences listed except for the following deviations, which used Sequence 2: *il10* & *hsp70* in spleen, *hsp90* in gill, & *ifng* in hindgut.

All qPCR reactions listed below underwent the following sequence: (1) pre-incubation at 95 °C for 10 minutes, (2) 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 63 °C for 5 seconds, and extension at 72 °C for 18 seconds, (3) melt curve generation at 95 °C for 5 seconds, 65 °C for 1 minute, and dissociation at a rate of 0.075 °C/second up to 95 °C. Target specificity was confirmed through one peak on the melt curve.

Primer efficiencies were assessed for each tissue. cDNA from each sample was diluted 1:5, totalling a concentration of 5 ng/mL per sample, and a cDNA pool was created by transferring 4 µL of each sample into one tube. From this pool, a 7-point 1:3 dilution series was created ranging from undiluted cDNA to a dilution factor of 729. For gill and hindgut, due to the

low RNA concentrations of some target genes, primer efficiency was tested using a 7-point 1:2 dilution series instead (indicated in Table 2.1 below). Each primer efficiency was calculated using the formula $E = 10^{(-1/\text{slope})}$ and efficiencies between 90-110% were considered acceptable (Table 2.2).

Table 2.1 Primers used in this study

Gene	Sequence 1	Sequence 2	Reference
<i>il1b</i>	F: CCACAAAGTGCATTTGAAC R: GCAACCTCCTCTAGGTGC		(Semple et al., 2019)
<i>il8</i>	F: ATTGAGACGGAAGCAGACG R: CGCTGACATCCAGACAAATCT		(Méndez et al., 2024)
<i>tnfa</i>	F: CGGACTCCATCGGGGTTAAT R: GACTCAGCATCACCGTAGTTTTG		(Méndez et al., 2024)
<i>tgfb</i>	F: TGTGGGGAGACAACACAAGG R: AAACCAGCGCCATCAAAAAGG		(Méndez et al., 2024)
<i>il10</i>	F: CTACGAGGCTAATGACGAGC R: GATGCTGTCCATAGCGTGAC	F: GCCTTCTCCACCATCAGAGAC R: GATGCTGTCCATAGCGTGAC	1) (Lulijwa et al., 2020) 2) (Semple et al., 2019)
<i>ifng</i>	F: CAACATAGACAAACTGAAAGTCCA R: ACATCCAGAACCACACTCATCA	F: CAACATAGACAAACTGAAAGTCCA R: ACATCCAGAACCACACTCATCA	1) (Bjork et al., 2014) 2) (Lulijwa et al., 2020)
<i>il2</i>	F: AGGAGAACAGCACAACGGAC R: CTTAGACGCTTTGCAGCATGA		(Soto-Dávila et al., 2024a)
<i>camp</i>	F: ATGGGAAACTAATGATGTGC R: CGGTCAGTGTTGAGGGTATT		(Broekman et al., 2013)
<i>hamp</i>	F: GAGGAGGTTGGGCATTGA R: TGACGCTTGAAGTGAATG		(Chettri et al., 2012)
<i>mhc1a</i>	F: ATGCTGTTCTTGTCGTCTTTCT R: AAAGTCCGTCCCTCAGTGTCTCT		(Rogozynski et al., 2024)
<i>tapasin</i>	F: CAAGCTATCGTTTCTCGCCTTTACT R: TGGTTTCTCCTGGACAAACCAGCAC		(Jørgensen et al., 2007)
<i>mhc2a</i>	F: AAGGTCCTCAGCTGGGTCAA R: GCTCAACTGTCTTGTCAGTATGG		(Rogozynski et al., 2024)
<i>mhc2b</i>	F: GCTAACTGTCTTGTCAGTATGG R: AAGGTCCTCAGCTGGGTCAA		(Rogozynski et al., 2024)
<i>hsp47</i>	F: TGGACAGATCGGCCTTGTT R: ACCTGGATGGGCAAGATGGA		(Chetty et al., 2023)

<i>hsp70</i>	F: AGCTAAAGGCCCGTCTATCG R: AACACCCCCACACAGGAGTA	F: GAGACAGGCCACTAAGGACG R: GACATGCCTTTGTCCATGCC	1) (Lewis et al., 2016) 2) (Rogozynski et al., 2024)
<i>hsp90</i>	F: CTTTGAGAACAAGAAGAAGAAGAAC R: CACACCCTTAATGAAGTTGAGGTAC	F: ATGATCGGGCAGTTCGGTG R: AGTGTCAACTTTGACAGTGAA	1) (Marcoli et al., 2023) 2) (von Biela et al., 2020)
<i>actb^{a,b}</i>	F: TGGACTTTGAGCAGGAGATGG R: AGGAAGGAGGGCTGGAAGAG		(Méndez et al., 2024)
<i>cx6cl^{a,b}</i>	F: GCCTGCAATGCGAGGACTCC R: TTCCTTGTTCTGTTACGCCGTAC		(Soto-Dávila, 2024b)
<i>gadph^a</i>	F: GCTGGAATGGGACTCACAC R: GTCAAAACCGTCTCAGTGGG		(Rajanbabu & Chen, 2011)
<i>efla^a</i>	F: CGCACAGTAACACCGAACTAATAAGC R: GCCTCCGCACTTGTAGATCAGATG		(Semple et al., 2019)
<i>18s^a</i>	F: CGTCGTAGTTCCGACCATAAA R: CCACCCACAGAATCGAGAAA		(Soto-Dávila et al., 2024b)

^a indicates a candidate endogenous control gene

^b indicates an endogenous control gene used in this study

Table 2.2 Primer efficiencies for spleen, gill, and hindgut

Gene	% PE (Spleen)	% PE (Gill)	% PE (Hindgut)
<i>actb</i>	109.2	98.7	102.6
<i>cx6c1</i>	100.1	97.2	101.7
<i>il1b</i>	100.4	95.6	111.2
<i>il8</i>	89.5	100.8	109.8
<i>tnfa</i>	105.8	101.6	95.0*
<i>il10</i>	98.3	94.6	110.4*
<i>tgfb</i>	108.8	107.1	104.5
<i>mhc1a</i>	104.1	92.4	97.8
<i>tapasin</i>	108.0	99.0	91.4
<i>mhc2a</i>	101.8	98.9	107.5
<i>mhc2b</i>	93.5	102.7	91.0
<i>ifng</i>	96.2	106.5	97.3*
<i>il2</i>	94.3	102.7	101.6*
<i>camp</i>	99.0	103.8	107.0*
<i>hamp</i>	104.5	96.9	97.5*
<i>hsp47</i>	93.4	93.1*	93.1
<i>hsp70</i>	99.1	106.2	108.7
<i>hsp90</i>	104.4	102.5	94.7

* indicates dilutions were 1:2 series.

Transcript levels of target genes were normalized to two endogenous control genes. cDNA samples were diluted to 2.5 ng for endogenous control testing and qPCR reactions. Transcript levels of five candidate controls (*bact*, *cx6c1*, *gadph*, *18s*, and *ef1a*) were assessed in 50% of samples (3 random samples per treatment and timepoint). Gene stability was calculated using RefFinder, an open-access tool which integrates and ranks gene stability results from

Genorm, NormFinder, BestKeeper, and Delta Ct comparisons (Xie et al., 2023). For all tissues, *bact* and *cx6c1* were the most stable genes and thus were used as endogenous controls.

Transcript levels of target genes were assessed in technical triplicates in each sample, and at a reaction rate of 2.5 ng (7.5 ng total RNA per qPCR reaction). On each plate, three controls were used: a positive control of a cDNA pool was used to confirm the presence of gene transcripts, a negative control using RNase-free water instead of cDNA, and a no-reverse transcriptase control.

2.4 ELISA

Because protein levels are not always correlated with mRNA levels (Frenette et al., 2023), in order to gain a whole level understanding of the immune response to *V. anguillarum*, the protein levels of key cytokines and HSPs were quantified in the plasma of Chinook salmon.

2.4.1 HSP47 indirect ELISA

This ELISA has been previously validated in the Dixon Laboratory. Plasma samples were diluted 1:5 with PBS and 100 µL was added in triplicate wells to a flat-bottomed 96-well plate (2HB, Thermo Fisher Scientific; Massachusetts, U.S.A.). A standard curve was also plated using recombinant HSP47 diluted in PBS to quantify the amount of protein in plasma samples, ranging in concentration from 8 ng/mL to 0.125 ng/mL. 100 µL of PBS was plated and used as a blank. The plate was incubated overnight at 4 °C. The plate was decanted and washed three times with 300 µL of tris-buffered saline (TBS) + 0.01% Tween 20 (TBS-T). This wash step was performed after every step hereafter. Wells were then blocked using 100 µL of TBS + 5% skim milk at 37

°C. After, 100 µL of chicken anti-HSP47 primary antibody in TBS-T 5% skim milk was added to each well and was incubated for 3 hours at room temperature. Anti-chicken antibody conjugated with biotin was added afterwards and incubated for 1 hour at room temperature in the dark. 100 µL of HRP-streptavidin was added to each well and incubated for 1 hour at room temperature in the dark. Then, 100 µL of TMB Sens (Kementec; New Hampshire, U.S.A.) was added to each well for 30 minutes at room temperature in the dark, followed by addition of 100 µL of 0.3 M H₂SO₄ to stop the reaction. The optical density (OD) of each well was read at 450 nm using the Synergy H1 plate reader (BioTek Instruments). The OD of standard curve wells were graphed and the equation of the resulting trendline was used to extrapolate the quantity of HSP47 in plasma samples.

2.4.2 IFN-γ and IL-2 sandwich ELISA

Capture and detection antibodies to cytokines IFN-γ and IL-2 were previously validated in the Dixon Laboratory by western blot and sandwich ELISA. Polystyrene flat-bottomed 96-well plates (4HBX, ThermoFisher Scientific) were coated with 100 µL of 2 µg/mL capture antibody diluted in coating buffer and incubated at room temperature for four hours (goat anti-IFN-γ and goat anti-IL-2). Between this step and all subsequent steps, the plate was decanted and washed three times with 300 µL of TBS-T. After this wash step, wells were blocked using TBS + 5% skim milk (w/v) for 1 hour at 37 °C. Plasma samples were diluted 1:3 with PBS and 100 µL was plated in triplicate wells. IFN-γ and IL-2 recombinant protein standard curves were also plated, with concentrations ranging from 800 pg/mL to 12.5 pg/mL. Additionally, 100 µL of PBS was also plated in triplicate as a blank. The plate was incubated overnight at 4 °C. Next, 100 µL

of the primary detection antibody (chicken anti-IFN- γ and rabbit anti-IL-2) diluted in TBS-T 5% milk was plated and incubated for 3 hours at room temperature. Afterwards, 100 μ L of the corresponding secondary antibody conjugated to biotin (anti-chicken biotin for IFN- γ and anti-rabbit biotin for IL-2) diluted in TBS-T 5% skim milk was added to each well and the plate was incubated for 1 hour in the dark at room temperature. 100 μ L of streptavidin-HRP in TBS-T 5% skim milk was added and incubated for 1 hour at room temperature in the dark. 100 μ L of TMB Sens (Kementec) was added to each well and incubated for 30 minutes at room temperature in the dark, and lastly the reaction was stopped by adding 100 μ L of 0.3 M H₂SO₄. Optical density was read at 450 nm using the Synergy H1 plate reader and quantification of each cytokine was achieved via extrapolation of the standard curve trendline.

2.5 Quantification of *V. anguillarum* in spleen tissues

2.5.1 gDNA Extraction

The genomic DNA (gDNA) of Chinook salmon spleens were extracted using the QIAwave DNA Blood & Tissue Kit following the manufacturer's protocol (Qiagen; Venlo, Germany). gDNA was eluted using the provided buffer, then quantified using a Take3 plate and Synergy H1 plate reader (BioTek Instruments). Each sample was normalized to 250 ng with DEPC water before proceeding with qPCR reactions.

2.5.2 Determining bacterial loads from qPCR C_T values

The sequences for the target genes *V. anguillarum* 16s ribosomal subunit (16s) and Recombinase A (*RecA*) are displayed below in table 2.3. Primer efficiencies were assessed for

both genes as described in Section 2.3.2, and all qPCR reactions were run as outlined in Section 2.3.2 above at a gDNA concentration of 5 ng/μL per each qPCR well. Transcript levels of target genes were assessed in technical triplicates in each sample. Along with assessing the bacterial load in both *V. anguillarum* infected groups (No HS/*Vibrio* & HS/*Vibrio*), the spleens of PBS-injected control fish were also tested for target genes. All C_T values for No HS/PBS and HS/PBS salmon were negative, indicating no *V. anguillarum* contamination in these treatment group.

Table 2.3 *V. anguillarum* primers used in this study

Gene	Sequence	Efficiency %	Reference
<i>16s</i>	F: CCACGCCGTAACGATGTCTA R: CCAGGCGGTCTACTTAACGCGT	98.1	(Crisafi et al., 2014)
<i>RecA</i>	F: CTAGTCGAAATTTTTTCGACACAGC R: GGCTCGTTAAATTTTTATCGACTCTT	96.1	(Crisafi et al., 2014)

2.6 Metabolites

2.6.1 Cortisol ELISA

Plasma cortisol was measured by competitive ELISA using the DetectX® Cortisol Immunoassay Kit (Arbor Assays; Michigan, U.S.A.; Cortisol Ref. KAPDB270) according to the manufacturer's instructions. Serum samples were diluted 1:100 using assay buffer and loaded into 96-well plates in triplicate. Following incubation and washing with the appropriate reagents, plasma cortisol was quantified by measuring the optical density of each well at 450 nm using a Synergy H1 Hybrid plate Reader (BioTek Instruments).

2.6.2 Glucose/Lactate Colorimetric Assays

Plasma glucose and lactate were measured using commercial kits from Sigma-Aldrich (Massachusetts, U.S.A.) following the manufacturer's instructions (Glucose Cat. number MAK263; Lactate Cat. number MAK064). Plasma samples were diluted 1:100 using the provided glucose or lactate assay buffers. To remove the presence of lactate dehydrogenase when measuring lactate, samples were deproteinized with a 10 kDa MWCO spin filter (Avantor; Pennsylvania, U.S.A.) by centrifugation at 13,300 x g for 10 min at 4°C. Samples were then added to 96-well plates in duplicate and metabolite levels were assessed colourimetrically by measuring the absorbance of each well at 570 nm using a Synergy H1 Hybrid plate Reader (BioTek Instruments).

2.7 Statistical Analysis

For temperatures of the Quinsam River (Section 2.1), data are displayed as elapsed time (hours) and temperature (°C) for air, shallow, and deep temperatures. Pearson r values were calculated to determine the correlation between air temperature, shallow temperature, and deep temperature.

The average body weight and fork length of each salmon was taken for each tank at each timepoint. A Kruskal-Wallis test was performed in order to determine if there were any differences between average body weight and fork length of salmon in these groups. Mortalities of salmon due to vibriosis were monitored and recorded each day until the endpoint of the study (t = 14 days) (See Section 2.2). A Log-rank (Mantel Cox) test was performed to determine significant differences in the survival between the four treatment groups.

For qPCR analysis (Section 2.3), gene expression was calculated using the $\Delta\Delta C_q$ method and converted to $\log_2$ fold change. The final value of each sample was the average of the $\log_2$ fold change normalized to each endogenous control (*actb* and *cx6c1*). Each timepoint/treatment is visualized as the mean + standard error (SEM) of 4-6 samples. A two-way ANOVA was performed with a Tukey's post-hoc test to determine significance between treatment groups and timepoints. Controls at $t = 0$ were compared with each other using a one-way ANOVA with a Tukey's post-hoc test. Mann-Whitney tests were also performed between tanks to ensure no significant differences in $\log_2$ fold change values due to "tank effect" (data not shown).

For indirect and sandwich ELISA protocols (Section 2.4), each timepoint/treatment is graphed as the mean + SEM of 4-6 samples. A two-way ANOVA was performed with a Tukey's post-hoc test to determine significance between treatment groups and timepoints. Additionally, for glucose, lactate, and cortisol measurements (Section 2.6), each timepoint/treatment is graphed as the mean + SEM of 3-4 samples. A two-way ANOVA was performed with a Tukey's post-hoc test to determine significance between treatment groups and timepoints for timepoints $t = 0.25, 1, 3, 7,$ and 14 . Controls at $t = 0$ were compared with each other using a one-way ANOVA with a Tukey's post-hoc test. All statistical analyses described above and associated graphs were completed using GraphPad Prism 10.2 (GraphPad Software).

Chapter 3

Results

3.1 Quinsam River temperatures

In order to determine an accurate measurement of the daily fluctuation in temperature of a stream of the Quinsam River, British Columbia during a heat wave (observed from July 18, 2023, until July 21, 2023), temperature sensors were deployed in a shallow (approximately 0.3 m from stream surface) and a deep (approximately 1 m from stream surface) area (Figure 3.1). Unsurprisingly, the temperature of the shallow water was higher than that of the deep temperature and ranged from a low of 18.75 °C to a high of 24.50 °C in the span of 9 hours (40 elapsed hours to 49 elapsed hours). Over the same timespan, the deep stream temperature raised from 15 °C to 18.12 °C. These fluctuations were consistently observed throughout the week until air temperatures cooled and reached a daily maximum of 23.5 °C (94 elapsed hours; July 22, 2023). Over the course of seven days, the average air temperature was 19.55 ± 4.98 °C, while the averages for the stream temperatures were 20.55 ± 1.94 °C for shallow waters and 16.14 ± 1.26 °C for deep waters. The shallow stream temperature was highly positively correlated with the deep stream temperature with a R^2 of 0.889. The air temperature was positively correlated with both the shallow and deep temperatures, with R^2 values of 0.585 and 0.641, respectively.

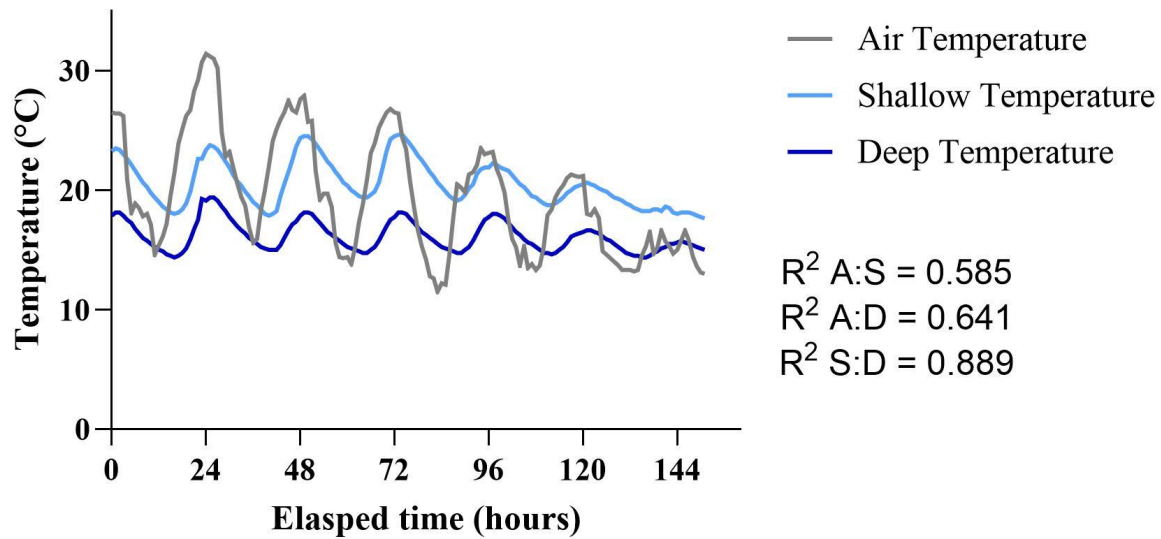


Figure 3.1 Temperature fluctuations of the Quinsam River water and air

The temperature of shallow (approximately 0.3 meter deep; “Shallow Temperature”) and deep (approximately 1 meter deep; “Deep Temperature”) water from the Quinsam River compared to the ambient temperature (“Air Temperature”), presented as elapsed hours from July 18, 2023, at 4:00pm PST to July 25, 2023. Pearson’s correlation was used to determine the relationships between air, shallow, and deep temperatures ($p < 0.0001$).

3.2 Body length and weight

The average weight of Chinook salmon in the $t = 0$ controls was 98.3 ± 23.6 g for the complete controls, 104.8 ± 37.3 g for the handling controls, and 73.2 ± 27.6 g for the heat shock controls. The average weight of Chinook salmon was 108.6 ± 54.9 g for the NHS/PBS groups, 103.8 ± 44.9 g for the HS/PBS groups, 104.7 ± 42.7 g for the HS/*Vibrio* groups, and 99.1 ± 50.0 g for the NHS/*Vibrio* groups. The average fork length in the $t = 0$ controls was 20.3 ± 1.7 cm for the complete controls, 20.8 ± 2.5 cm for the handling controls, and 18.4 ± 2.6 cm for the heat shock controls. The average fork length was 20.6 ± 3.6 cm for the NHS/PBS groups, 20.8 ± 2.8 cm for the HS/PBS groups, 20.6 ± 2.9 cm for the HS/*Vibrio* groups, and 19.9 ± 3.3 cm for the

NHS/*Vibrio* fish. There were no significant differences in fork length or weight across any timepoint and/or group.

3.3 Survival analysis

The survival curves of the four treatment groups are seen in Figure 3.3. Fish infected with *V. anguillarum* without heat shock experienced a 33% mortality rate, while fish that underwent heat shock experienced a 27% mortality rate. Interestingly, mortality in the HS/*Vibrio* group occurred beginning at four dpi and continued until twelve dpi, while mortality in the No HS/*Vibrio* group halted 10 dpi.

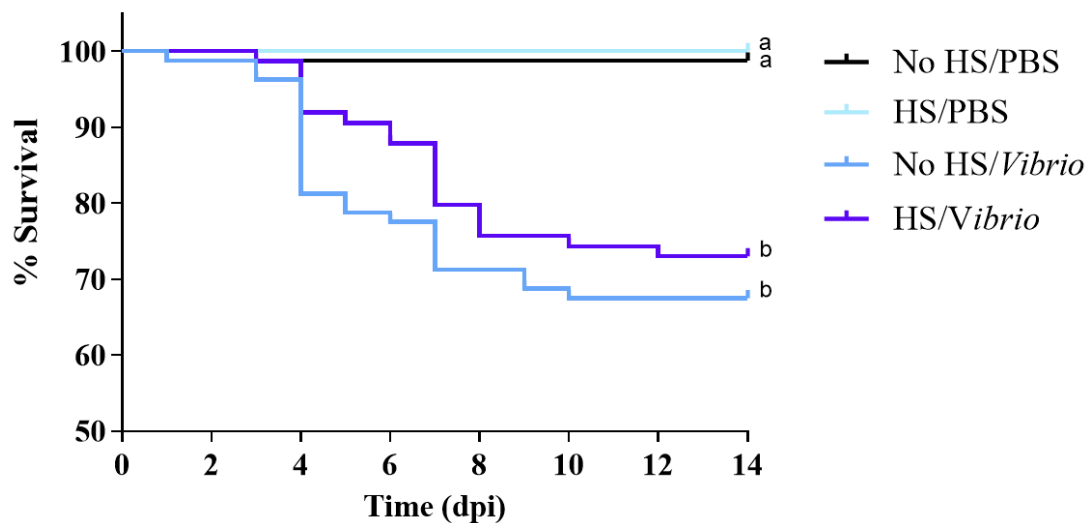


Figure 3.3 Survival Curves of Chinook salmon after treatment with heat shock and/or bacterial injection

Survival of Chinook salmon that underwent no treatment (No HS/PBS), heat shock only (HS/PBS), infection with *V. anguillarum* only (No HS/*Vibrio*), or heat shock and infection (HS/*Vibrio*). Data were analyzed using a log-rank (Mantel-Cox) test and different letters indicate significance in percent survival between treatment groups.

3.4 Gene expression

In order to gain a whole immune system-level understanding of the effect of acute heat shock on Chinook salmon, gene expression was measured using qPCR. These genes are divided into six categories: pro-inflammatory cytokines (3.4.1), anti-inflammatory cytokines (3.4.2), cytokines that cause immune activation (3.4.3), antimicrobial peptides (3.4.4), antigen presentation pathways (3.4.5), and heat shock proteins (3.4.6). A high-level overview of the implications of changes in all genes is presented in the Discussion, Section 4.9.

3.4.1 Pro-inflammatory cytokines

il1b was significantly upregulated in the spleen 0.25 to 7 days after infection with *V. anguillarum* in the non-heat shocked fish, while only being upregulated 1 dpi in heat shock and infected-only fish, despite being significantly upregulated directly after heat shock but before infection compared to controls (Figure 3.4.1 A.; Supplementary Figure 1). *il1b* is significantly upregulated in No HS/*Vibrio* fish compared to both untreated and HS/*Vibrio* groups 3 dpi in hindgut as well (Figure 3.4.1 B). *tnfa* was upregulated 1 dpi in both No HS/*Vibrio* and HS/*Vibrio* groups, however 3 dpi this pro-inflammatory cytokine is downregulated in both groups that received heat shock, significantly different from their No HS/*Vibrio* counterparts in spleen and hindgut (Figure 3.4.1 A, B). *il8* follows a similar trend in spleen, in which all three treatment groups are significantly upregulated 1 dpi. However, by 3 dpi *il8* is only upregulated in No HS/*Vibrio* fish; this difference is seen in hindgut as well (Figure 3.4.1 A, B). All pro-inflammatory responses cease by 14 dpi (Figure 3.4.1 A, B), and there were no significant differences between *il1b*, *tnfa*, and *il8* in gill (Figure 3.4.1 C).

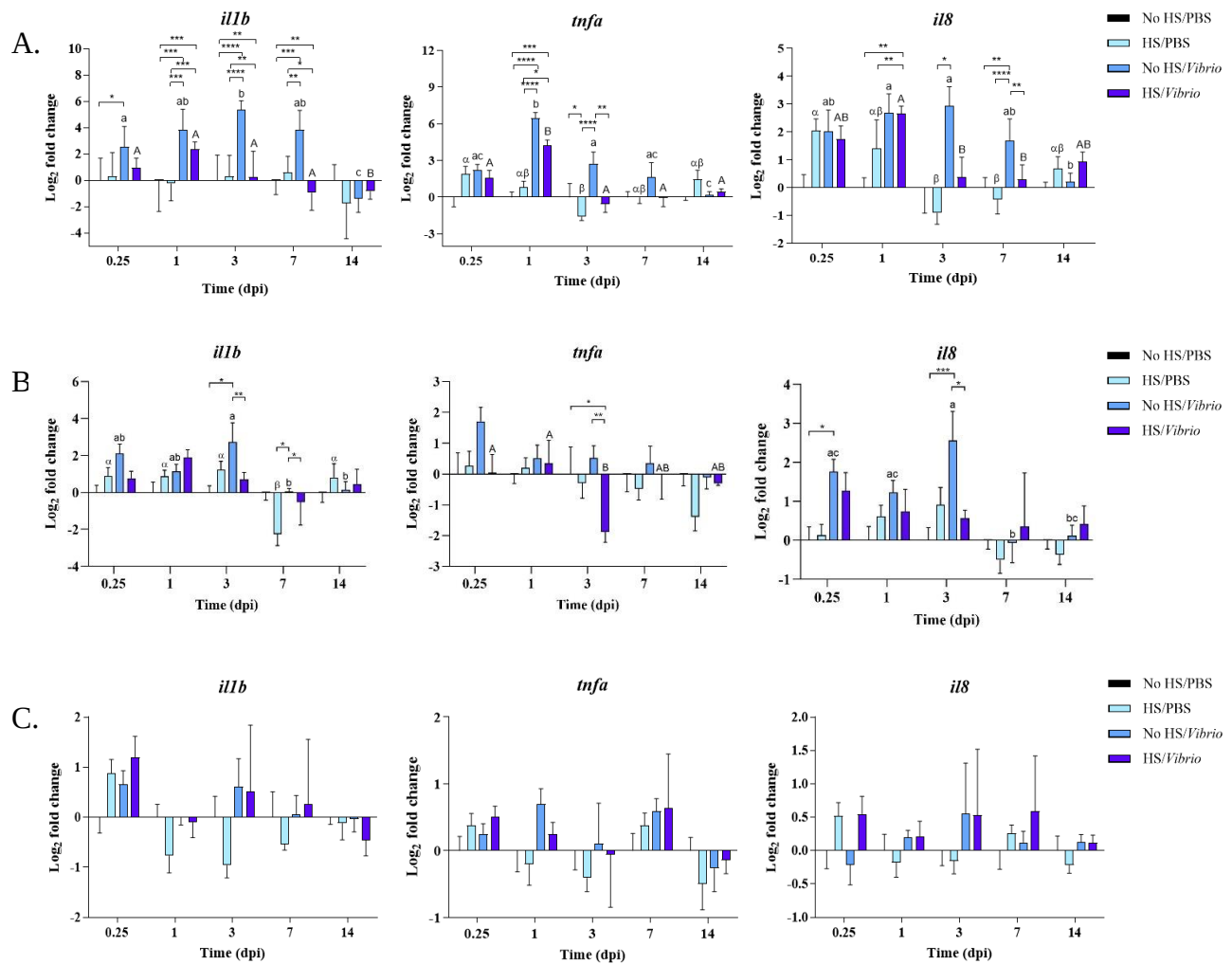


Figure 3.4.1 Pro-inflammatory gene expression in treatment groups after heat shock and/or bacterial injection

Gene expression of IL-1 β (*il1b*), TNF- α (*tnfa*), and IL-8 (*il8*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Each value is the mean $\pm$ S.E.M. (n = 4-6). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint (p < .05 (*), p < .01 (**), p < .001 (***) and p < 0.0001 (****)).

3.4.2 Anti-inflammatory cytokines

To assess the anti-inflammatory response of either heat shock, infection, or both, the transcripts of *tgfb* and *il10* were assessed (Figure 3.4.2). No significant changes were detected in *tgfb* in spleen or gill (Figure 3.4.2 A., C.). *il10* transcripts in the spleens of No HS/*Vibrio* salmon were significantly upregulated 3 dpi compared to all other treatment groups and remained upregulated 7 dpi (Figure 3.4.2 A), suggesting a large anti-inflammatory response corresponding with the upregulation of pro-inflammatory cytokines (see Figure 3.4.1 A.). Heat shock with no bacterial infection does not upregulate *tgfb* or *il10* directly after treatment (Supplementary Figure 2) but triggers a significant upregulation of *tgfb* in the hindgut after 1 day. *tgfb* is significantly downregulated in HS/*Vibrio* fish compared to their HS/PBS counterparts 1 dpi, however *il10* is significantly upregulated at the same timepoint (Figure 3.4.2 B.).

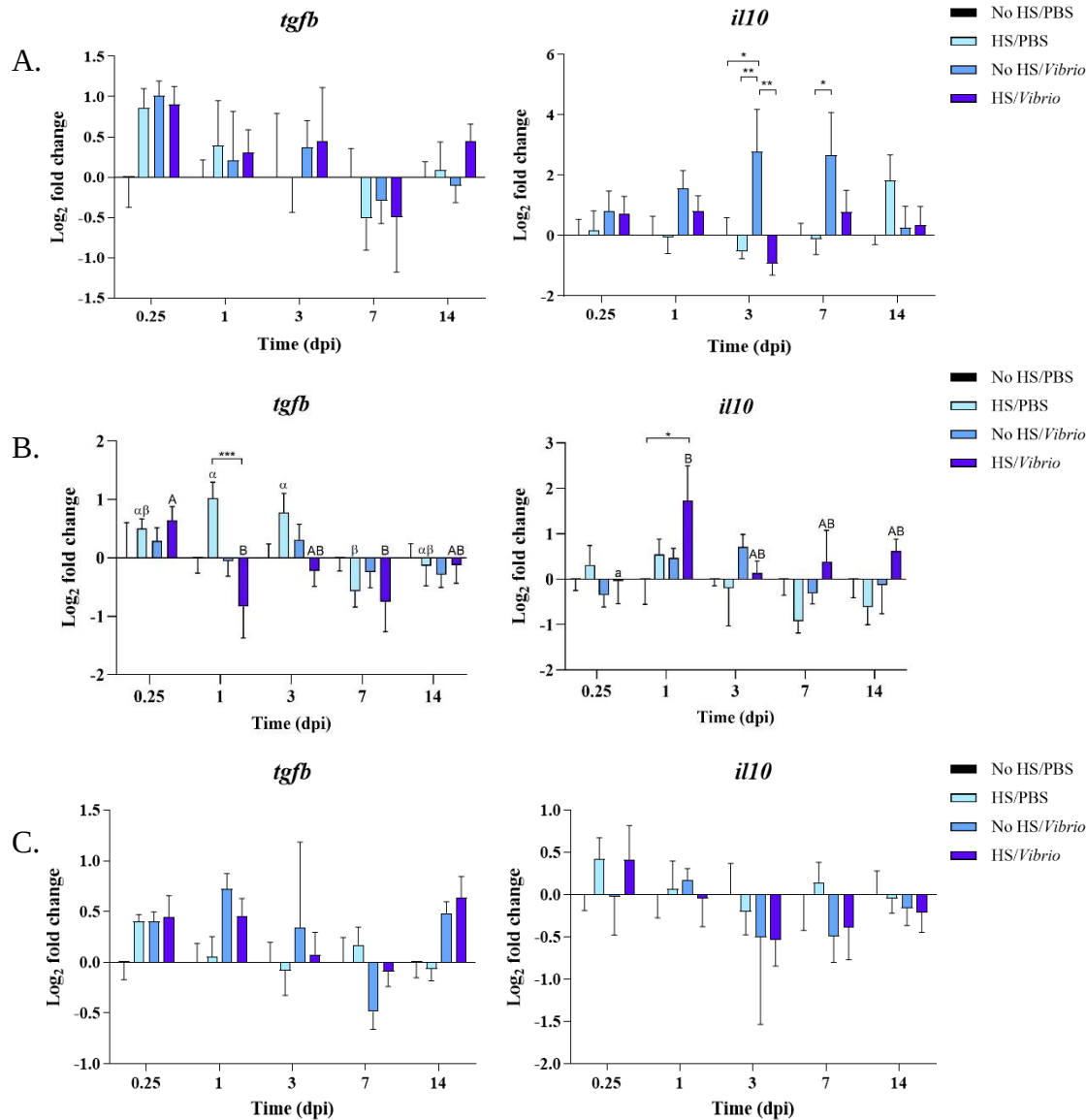


Figure 3.4.2 Anti-inflammatory gene expression in treatment groups after heat shock and/or bacterial injection

Gene expression of TGF- β (*tgfb*) and IL-10 (*il10*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Each value is the mean $\pm$ S.E.M. (n = 4-6). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint (p < .05 (*), p < .01 (**), and p < .001 (***)).

3.4.3 Immune system-activating cytokines

The patterns of cytokines transcripts of *ifng*, which activates macrophages and contributes to T_H1 and T_H2 differentiation (Ignatz et al., 2020), and *il2*, which is a marker of T cell activation (Wang et al., 2018) were measured to assess cytokine-led activation of the immune system after treatment (Figure 3.4.3). *ifng* was significantly upregulated compared to No HS/PBS fish in both treatment groups infected with *V. anguillarum* (No HS/*Vibrio* and HS/*Vibrio*) 1 dpi in the spleen (Figure 3.4.3 A). *ifng* expression varied in the hindgut in fish that only received heat shock, first upregulating 3-days post-treatment then downregulating 7- and 14-days post-treatment (Figure 3.4.3 B).

il2 was significantly upregulated 6 h after infection in the No HS/*Vibrio* treatment group compared to controls. Interestingly, in the HS/*Vibrio* treatment group, there is upregulation of *il2* at 0.25 dpi, and a significant upregulation 14 dpi, insinuating two incidences of T cell differentiation that is not seen in No HS/*Vibrio* fish (Figure 3.4.3 A.). In the hindguts of HS/PBS salmon, heat shock resulted in an initial downregulation (Supplementary Figure 3), followed by upregulation of *il2* 1-day post-treatment and subsequent downregulation 7-days post-treatment (Figure 3.4.3 B.). This fluctuation is not seen in other treatment groups or in other tissues.

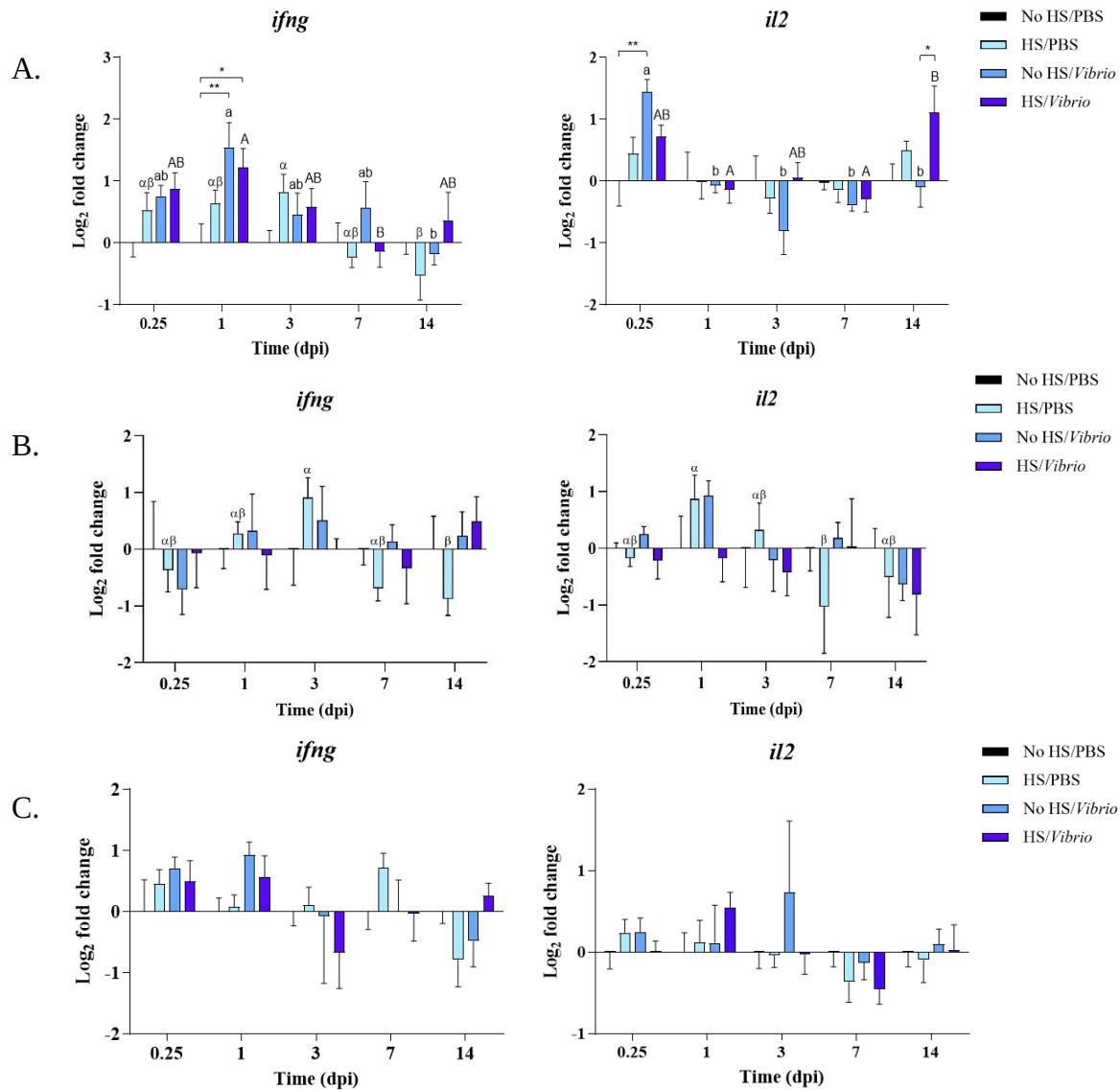


Figure 3.4.3 Immune activating cytokines gene expression in treatment groups after heat shock and/or bacterial injection

Gene expression of IFN- γ (*ifng*) and IL-2 (*il2*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Each value is the mean $\pm$ S.E.M. (n = 4-6). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint (p < .05 (*)) and p < .01 (**)).

3.4.4 Antimicrobial peptides

The transcripts of antimicrobial peptides cathelicidin (*camp*) and hepcidin (*hamp*) varied in spleen, gill, and hindgut (Figure 3.4.4). In spleen and hindgut, fish that received an injection of *V. anguillarum* follow a similar pattern of upregulation regardless of the presence of heat shock. 1, 3, and 7 dpi, *camp* was significantly upregulated in both infected groups compared to both uninfected groups (Figure 3.4.4 A., B.). Interestingly, *camp* had the largest effect on the HS/PBS group in gill, as it was upregulated 1 dpi and 14 dpi, but downregulated 7 dpi (Figure 3.4.4 C). However, the stress of handling was enough to cause significant upregulation of *camp* in both the gills and spleen compared to controls (Supplementary Figure 4). In both treatment groups receiving *V. anguillarum*, *hamp* was significantly upregulated compared to both No HS/PBS and HS/PBS groups in hindgut and gill 1 dpi (Figure 3.4.4 B, C). Interestingly, *hamp* expression in No HS/*Vibrio* fish is significantly higher than in HS/*Vibrio* fish in hindgut and gill 3 dpi, and in spleen and gill 7 dpi (Figure 3.4.4 A, B, C). Additionally, fish that received heat shock and a sham injection had a significant upregulation of *hamp* compared to NHS/*Vibrio* after 14 days in the spleen (Figure 3.4.4 A).

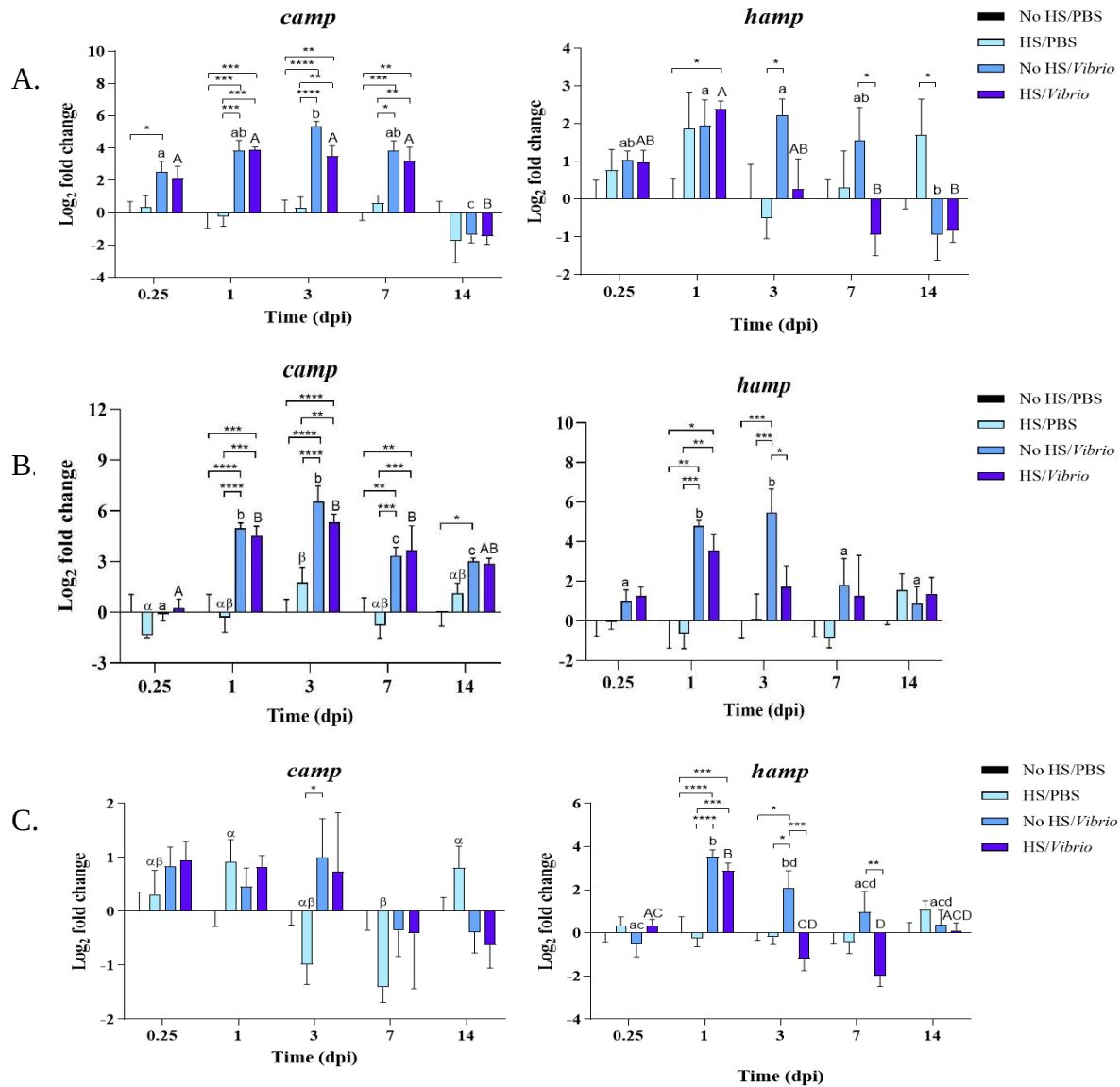


Figure 3.4.4 Antimicrobial peptide gene expression in treatment groups after heat shock and/or bacterial injection

Gene expression of cathelicidin (*camp*) and hepcidin (*hamp*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Each value is the mean $\pm$ S.E.M. ($n = 4-6$). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint ($p < .05$ (*), $p < .01$ (**), $p < .001$ (***) and $p < .0001$ (****)).

3.4.5 Antigen presentation pathways

The transcripts of *mhc1a* and *tapasin* were measured over 14 days and the results are shown in Figure 3.4.5. In the spleen, *mhc1a* begins upregulated 1 dpi, then decreased 3 dpi and was significantly downregulated in HS/*Vibrio* samples compared to No HS/*Vibrio* samples (Figure 3.4.5 A). *tapasin* in the spleen increased in No HS/*Vibrio* salmon 1 dpi compared to no treatment controls. Interestingly, spleen *tapasin* transcripts in all treatment groups were significantly downregulated by 3 dpi and lasting until 14 dpi compared to 0.25 dpi and 1 dpi for HS/PBS and both infected groups, respectively (Figure 3.4.5 A). In the hindgut, this same downregulation is observed in the HS/PBS treatment group at 3 dpi. Opposingly, groups receiving *V. anguillarum* decreased, then either returned to base levels or increased by 7 dpi (Figure 3.4.5 B). *tapasin* did not differ significantly across timepoints or treatment groups in the gills (Figure 3.4.5 C).

The transcript levels for *mhc2a* and *mhc2b* are displayed in Figure 3.4.6. Though there are no significant differences in *mhc2a* in the spleen, in fish that received only bacterial infection, *mhc2b* follows a similar trend to the endogenous pathway of downregulation 1, 3, and 7 dpi. Particularly, *mhc2b* is significantly downregulated 3 dpi in No HS/*Vibrio* salmon compared to all other treatment groups at this timepoint (Figure 3.4.6 A). Opposingly, in the hindgut, both *mhc2a* and *mhc2b* were significantly downregulated in HS/*Vibrio* fish compared to PBS control groups 1 dpi. Heat shock also caused a downregulation in these transcripts in HS/PBS fish 7 dpi (Figure 3.4.6 B). There were no significant differences in *mhc2a* or *mhc2b* in the gills (Figure 3.4.6 C).

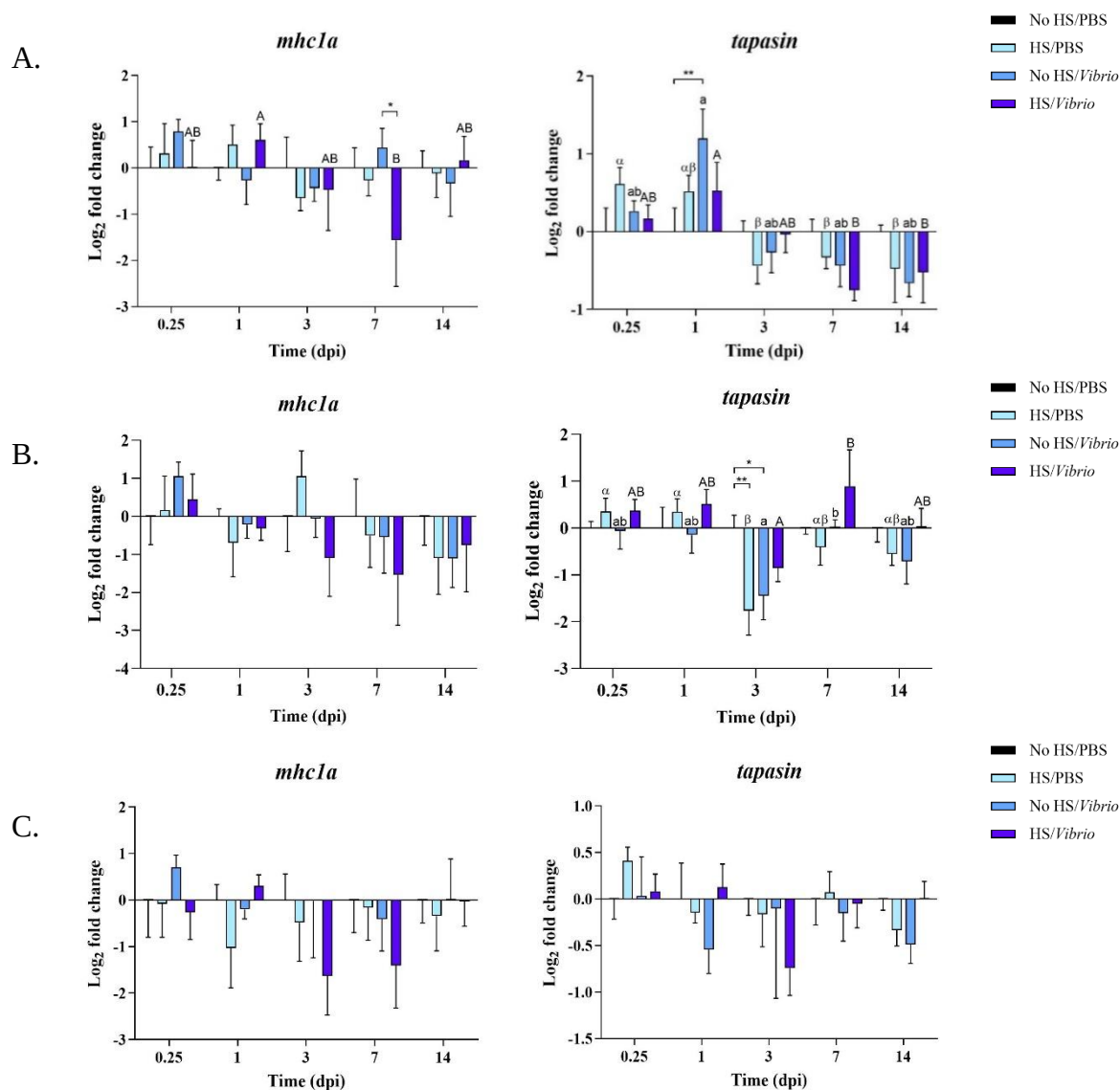


Figure 3.4.5 Endogenous antigen presentation pathway gene expression in treatment groups after heat shock and/or bacterial injection

Gene expression of MHC Ia (*mhc1a*) and tapasin (*tapasin*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Each value is the mean $\pm$ S.E.M. ($n = 4-6$). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint ($p < .05$ (*) and $p < .01$ (**)).

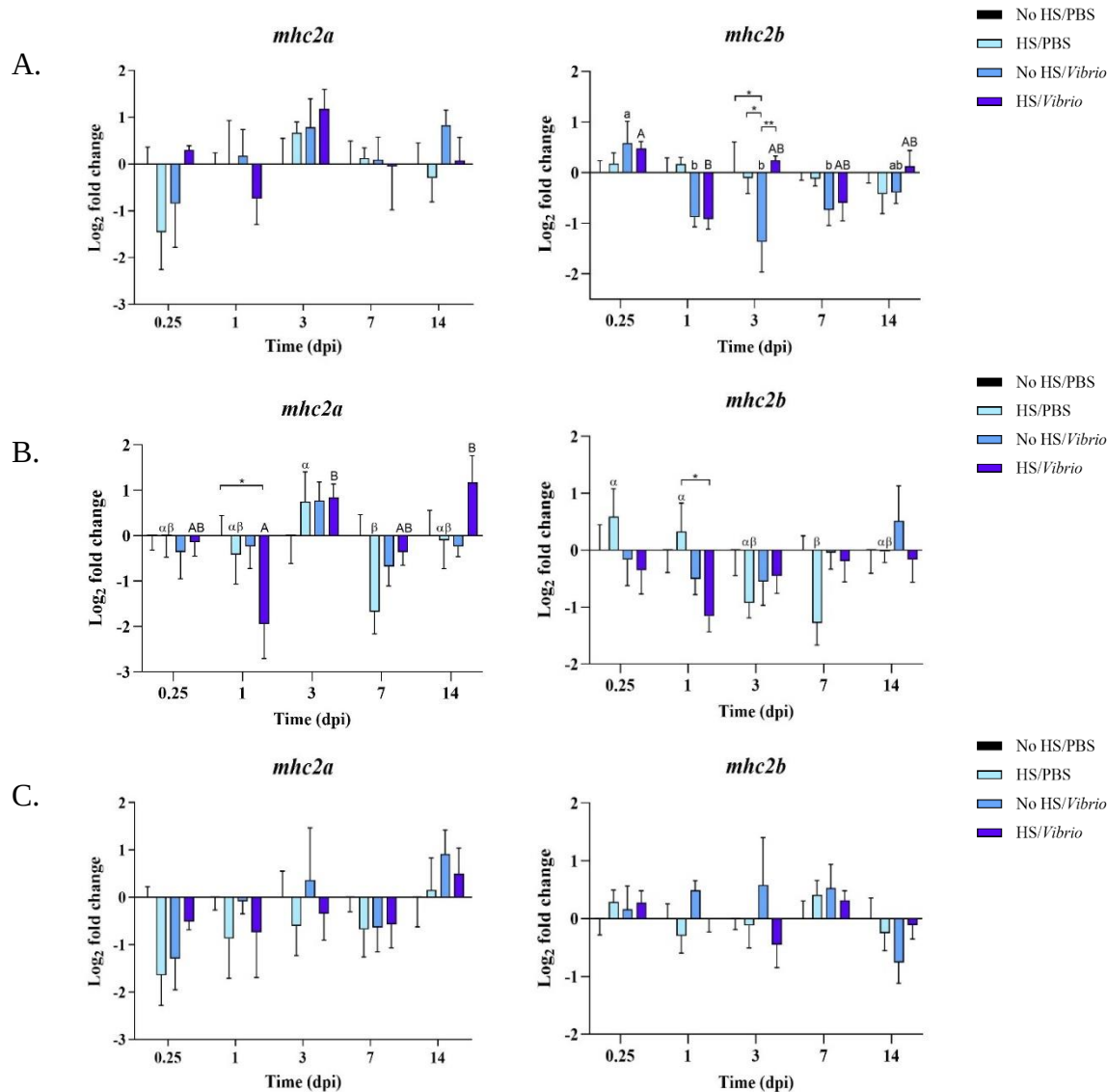


Figure 3.4.6 Exogenous antigen presentation pathway gene expression in treatment groups after heat shock and/or bacterial injection

Gene expression of MHC II α (*mhc2a*) and MHC II β (*mhc2b*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/Vibrio), or both heat shock and I.P. injection (HS/Vibrio). Each value is the mean $\pm$ S.E.M. (n = 4-6). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint (p < .05 (*) and p < .01 (**)).

3.4.6 Heat shock proteins

The mRNA transcript levels for HSP47, HSP70, and HSP90 are shown in Figure 3.4.7. Unsurprisingly, there is a significant upregulation of *hsp47* immediately after heat shock (Supplementary Figure 7). This gene remains upregulated 0.25 and 1 dpi in both heat shock groups in all tissues, then returns to control levels 3 days after heat shock. Interestingly, in the gill, No HS/*Vibrio* treatment causes an increase in *hsp47* 0.25 and 7 dpi (Figure 3.4.7 C). Similar trends can be seen in *hsp90* for the heat shock treatment groups, however in the spleen 1 dpi and onwards, *hsp90* in both heat treatment groups do not differ significant from any other treatment. Contrastingly, *hsp90* is significantly different in salmon that received *V. anguillarum* only compared to non-infected, non-heated controls (Figure 3.4.7. A). Surprisingly, *hsp70* was only upregulated in the gills immediately after heat shock and did not change significantly in either the HS/PBS or HS/*Vibrio* salmon in the spleen or gills over 14 days (Supplementary Figure 7, Figure 3.4.7 A,C). In the hindgut, *hsp70* is downregulated in heat shocked treatment groups compared to controls 3 dpi, followed by an upregulation in HS/*Vibrio* fish (Figure 3.4.7 B).

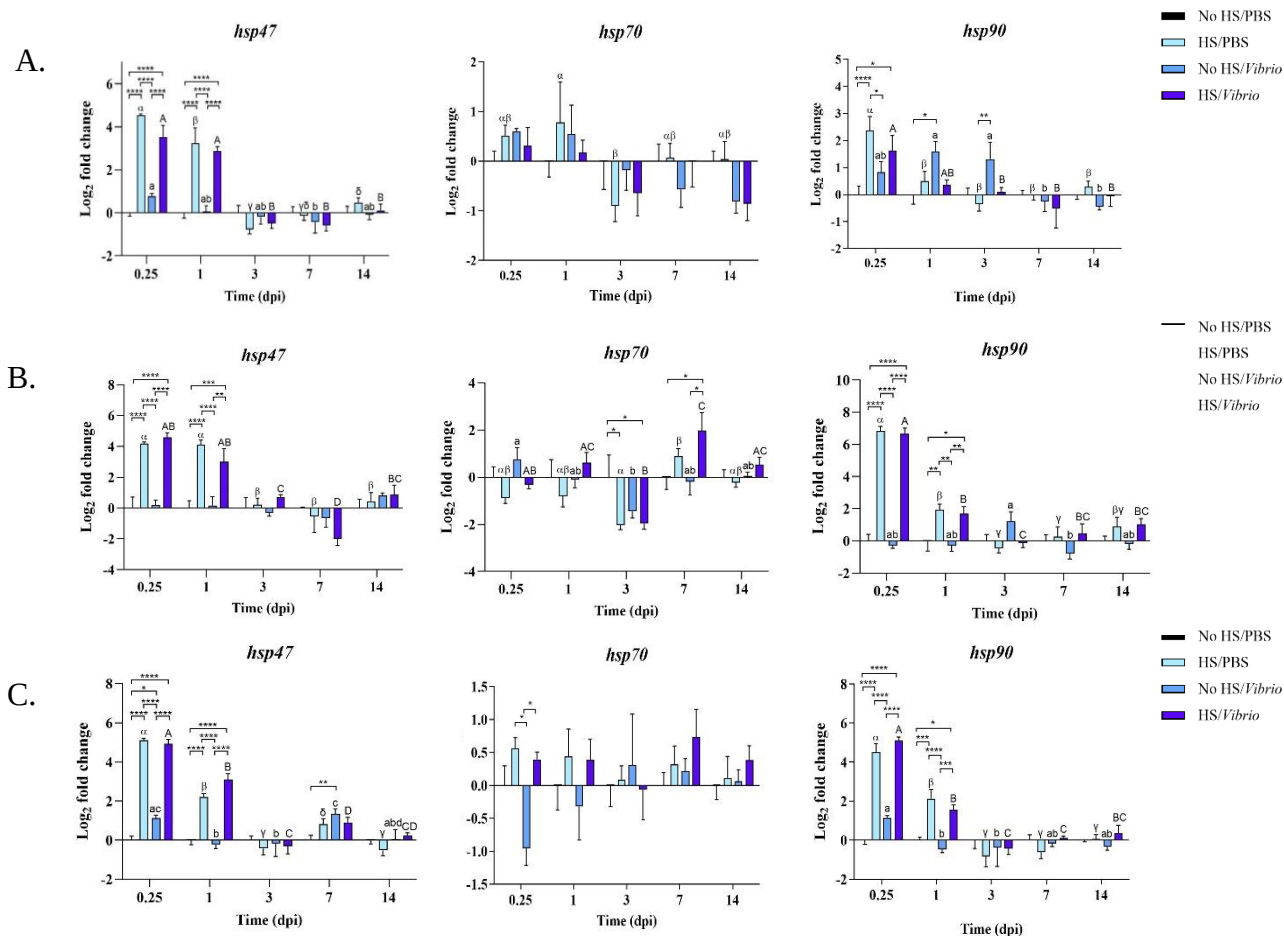


Figure 3.4.7 Heat shock protein gene expression in treatment groups after heat shock and/or bacterial injection

Gene expression of HSP47 (*hsp47*), HSP70 (*hsp70*), and HSP90 (*hsp90*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Each value is the mean $\pm$ S.E.M. ($n = 4-6$). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint ($p < .05$ (*), $p < .01$ (**), $p < .001$ (***) and $p < 0.0001$ (****)).

3.5 *V. anguillarum* in spleen tissues

Bacterial loads were quantified by C_T values of the *V. anguillarum* genes for the 16S ribosomal subunit and the DNA repair protein RecA, in order to ensure that differences in mortality and in immune response measured by qPCR are not due to a reduced bacterial load in either *V. anguillarum* infected groups. Further, non-infected control samples were also assessed to ensure no contamination between treatment groups occurred during the experiment. The C_T values for *RecA* in both infected groups did not differ significantly across treatment or time, indicating a similar bacterial load in both the heated and unheated salmon (Figure 3.5 A). For 16S, there is a significant difference between the C_T values at 1 dpi for No HS/*Vibrio* and 7 dpi for HS/*Vibrio* treatments. All uninfected samples tested were either negative (for *RecA*) for the target genes or had C_T values above 35.00 (for 16S), indicating a negative result.

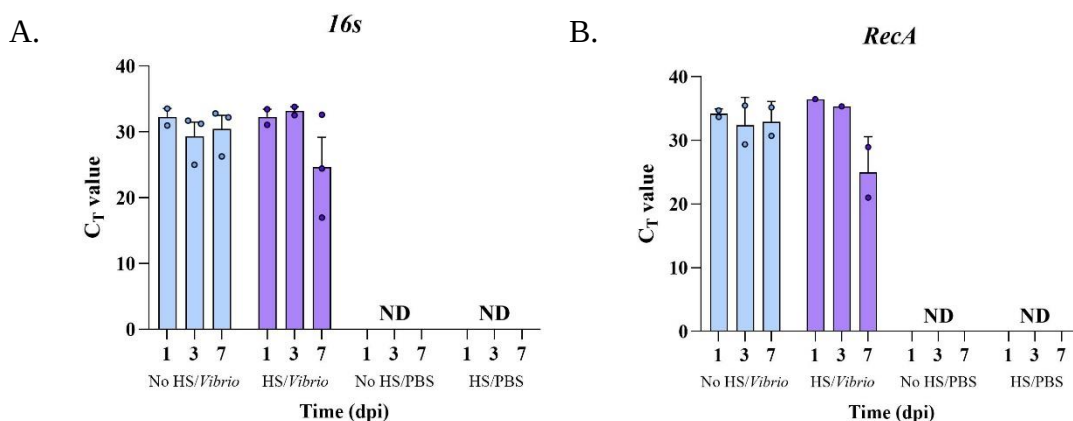


Figure 3.5 Gene expression of *V. anguillarum* bacterial loads from spleen tissues

The C_T values for the target genes 16S (A) and RecA (B) specific for *V. anguillarum* in spleen tissues of infected Chinook salmon. “ND” indicates C_T values for that treatment were not detectable. Values were measured by qPCR and are displayed as the mean ± S.E.M. values from n = 2-3 salmon.

3.6 Protein quantification

The protein levels of HSP47, IL-2, and IFN- γ in the plasma and head kidney were assessed using ELISA. HSP47 protein was consistent across groups and there were no significant differences across treatments or timepoints (Figure 4.6). IL-2 was not detectable in any plasma samples. HSP47, IL-2, and IFN- γ was also not detectable in head kidney.

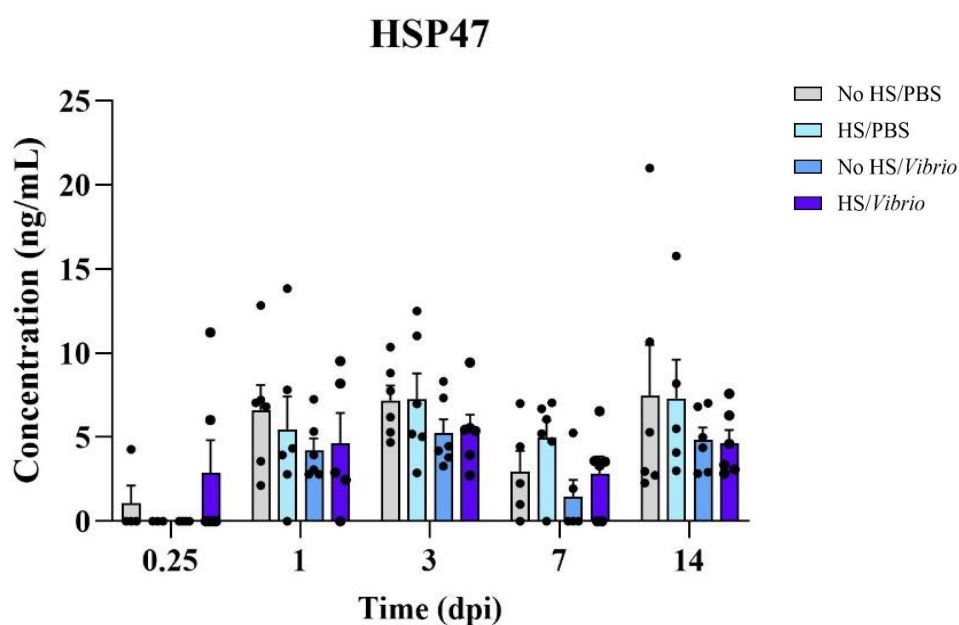


Figure 3 Indirect ELISA of total HSP47 protein in plasma

Protein levels of HSP47 in plasma of juvenile Chinook salmon undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Concentration was calculated by interpolation of standard curve from recombinant HSP47, measured at 450 nm and ranging in concentration from 0.125-8 ng/mL. Each value is the mean $\pm$ S.E.M. (n = 3-6). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test.

3.7 Metabolites and cortisol

3.7.1 Plasma glucose and lactate

The concentrations of glucose Chinook salmon plasma are displayed in Figure 3.7.1 A. Heat shock did not cause a significant change in glucose concentration compared to handling controls. However, glucose increased 6 hours post-treatment for all groups, including the No HS/PBS group, which experienced a significant increase compared to handling controls. Glucose concentrations stabilized in all treatment groups 1 dpi. Heat shock caused a significant increase in lactate concentrations in plasma compared to handling controls (Figure 3.7.1 B). However, this increase returns to levels similar to other treatments for the HS/PBS treatment group 6 hours after heat shock. There were no significant differences observed for salmon infected with *V. anguillarum* regardless of heat shock.

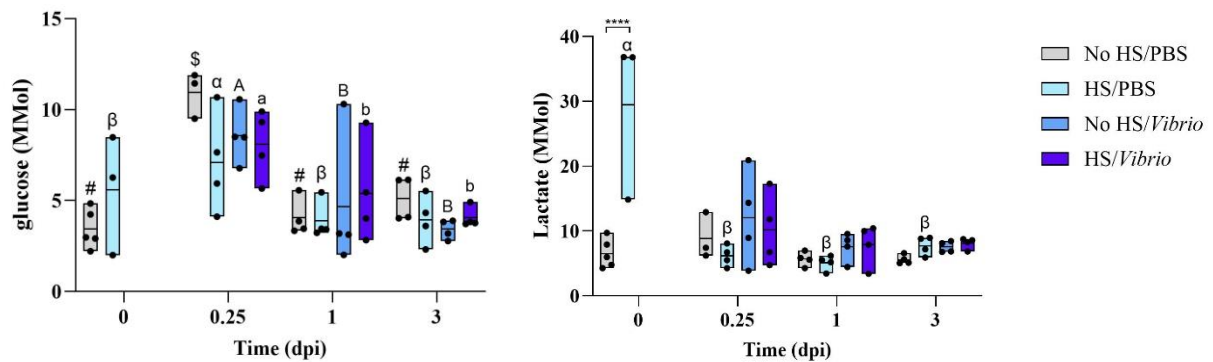


Figure 4.1 Plasma glucose and lactate concentrations in treatment groups after heat shock and/or bacterial injection

Concentrations of glucose and lactate (in MMol) in plasma samples of Chinook salmon undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Concentration was calculated by interpolation of standard curve from recombinant glucose or lactate measured at 570 nm. Each value is a boxplot ranging from the minimum and maximum values in each treatment group. (n = 3-5). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint (p < .0001 (****)).

3.7.2 Plasma cortisol

Concentrations of cortisol in the plasma was quantified for each treatment group and timepoint (Figure 3.7.2). Similarly to lactate, heat shock caused a significant increase in cortisol compared to handling controls. 6 hours after heat shock and onwards, cortisol in HS/PBS salmon did not differ significantly between treatment groups. Cortisol concentration seems to fluctuate in both No HS/PBS and No HS/*Vibrio* salmon, with concentrations at their highest 7 days after handling and/or after infection. The greatest variation amongst individual salmon was seen in the HS/*Vibrio* treatment group as displayed by the min/max points in each boxplot. However, significant differences are only seen between 1 and 14 dpi.

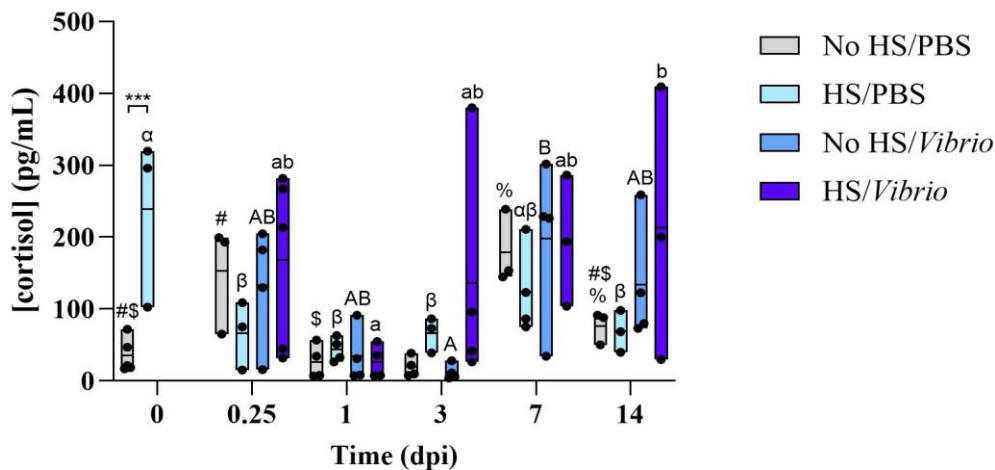


Figure 3.7.2 Plasma cortisol concentrations in treatment groups after heat shock and/or bacterial injection

Concentrations of cortisol (in pg/mL) in plasma samples of Chinook salmon undergoing no treatment (No HS/PBS), heat shock treatment only (HS/PBS), I.P. injection of *V. anguillarum* only (No HS/*Vibrio*), or both heat shock and I.P. injection (HS/*Vibrio*). Concentration was calculated by interpolation of standard curve from recombinant cortisol measured at 450 nm ranging from 50 – 3,200 pg/mL. Each value is a boxplot ranging from the minimum and maximum values in each treatment group. (n = 3-5). Samples were compared between time points and treatments using a 2-way ANOVA with Tukey post-hoc test. Different letters represent significant differences in the same treatment between time points. Asterisks represent significant differences between treatments within the same timepoint ($p < .001$ (***)).

Chapter 4

Discussion

The purpose of this study was to assess the effects of acute heat shock on the Chinook salmon immune system and on their ability to combat *V. anguillarum* infection. To do this, salmon underwent a 17-hour heat shock, with temperatures increasing from 11 °C – 21.5 °C. Afterwards, salmon were I.P. injected with *V. anguillarum* and their immune responses were assessed over the span of two weeks. Salmon were divided into four treatment groups: No HS/PBS, HS/PBS, No HS/*Vibrio*, and HS/*Vibrio*.

4.1 Quinsam River temperatures

In order to ensure accuracy with the heat shock temperatures, iButton® temperature sensors were deployed in the Quinsam River in British Columbia to assess shallow and deep stream temperature patterns over the course of one week. The correlation between shallow river/deep river temperatures and air temperatures was found to be 0.585 and 0.641, respectively. Correlation studies of similar streams have shown a higher relationship ($R^2 > 0.8$) but has fluctuated between 0.61-0.95. (Graf, 2019; Morrill et al., 2005). The present study has further confirmed this positive relationship between air and water temperature. Further, the Quinsam River, a common river in which Chinook salmon use to spawn (Government of Canada, 2014), is susceptible to increasing temperatures as the number of heat waves continues to rise (Tassone et al., 2022).

River temperatures fluctuated almost 6 °C over the course of 9 hours (Figure 3.1), indicating that there is a considerable temperature range that salmon must ensure in one day

alone. Further, there was a significant positive correlation of 0.889 between shallow and deep-water temperatures, insinuating that even with a lower depth (and thus cooler water) available to them, salmon will still experience a range of temperatures that may be outside of their preferred temperature. It has been previously demonstrated that month-long exposure of salmon from the Quinsam River to temperatures above 17.0 °C causes increased adult mortality and egg mortality, as well as delayed spawning (Jensen et al., 2003). Therefore, it is crucial to continue research in this area in order to fully understand the possible effects on Chinook salmon and related species.

4.2 Pro-inflammatory cytokines

Pro-inflammatory cytokines are crucial to the coordination of the immune response and are often upregulated early in bacterial infection (Semple & Dixon, 2020). However, the ability of heat stress to initiate a pro-inflammatory response is debatable (Esam et al., 2022; Guo & Dixon, 2021). Some studies display a pro-inflammatory response after heat stress in tilapia (Esam et al., 2022), Atlantic salmon (Zanuzzo et al., 2020), and Chinook salmon (Guo et al., 2023), while others on zebrafish (Weber et al., 2023) show no effects of heat on innate immunity. In this study, we found that heat shock increased *il1b* and *il8* transcripts, however, this response ceased by 6 hours post-infection (Supplementary Figure 1; Figure 4.1). Chinook salmon that received *V. anguillarum* with no heat shock showed a pro-inflammatory response up to 7 dpi. Interestingly, the same pattern was not observed for HS/*Vibrio* fish and cytokines were only upregulated up to 1 dpi (Figure 4.1). Based on these data, *V. anguillarum* was the key driver of pro-inflammatory responses rather than heat shock. This pattern is consistent with one study in

Atlantic salmon, in which fish were exposed to heat stress then injected with a multi-valent vaccine. *il1b* levels were higher in vaccinated/non-heated fish than in vaccinated/heated fish 24 hours post-infection (Zanuzzo et al., 2020). Further, several studies have shown an anti-bacterial pro-inflammatory response in salmonids towards *V. anguillarum* (Ching et al., 2010; Hou et al., 2021; Semple et al., 2022).

In this trial, it's possible that the heat shock was able to prepare the immune system for *V. anguillarum* infection as a method of thermal preconditioning. This is further confirmed by the similar bacterial loads in both the heat shocked and non-heat shocked infection groups (Figure 3.4), indicating that these salmon were fighting an infection of a similar magnitude (thus, the immune responses seen are due to the other main experimental variable – heat shock). Guo et al. (2023) also found that preconditioning Chinook salmon with an initial heat shock, followed by a secondary thermal challenge, lowered the transcript levels of *il1b* and *tnfa* in the spleen and gills, respectively. In this context, experiencing a heat wave may give Chinook salmon a slight advantage against vibriosis due to the stimulation of inflammatory responses before exposure to *V. anguillarum*.

4.3 Anti-inflammatory cytokines

Anti-inflammatory cytokines are considered a key regulator of the immune system due to their role in tissue homeostasis (Sanjabi et al., 2009). *il10* follows a similar pattern to pro-inflammatory cytokines, increasing to a much larger degree in No HS/*Vibrio* fish than in HS/*Vibrio* fish (Figure 4.2). This is likely a corresponding cytokine release to prevent tissue damage from excessive inflammation. This correlated mechanism is shown by Wei et al. (2015),

who showed that both IL-10 and TGF- β attenuated LPS-induced pro-inflammatory response in grass carp. Further, in the Chinook spleen cell line CHST, IL-10 transcripts were increased 96 hours after exposure to heat-killed *V. anguillarum*, after a peak of IL-1 β and TNF- α 24 hours post-exposure (Semple et al., 2024).

Few studies have assessed the anti-inflammatory effects of acute heat shock. However, there is evidence that temperatures ranging from 15-25 °C aid in anti-inflammatory responses to vaccination (Raida & Buchmann, 2007) and viral infection (Sanhueza et al., 2021). In this study, we found *tgfb* increased from 6 hours to 3 days post-heat shock (Figure 4.2). This is somewhat consistent with Dittmar et al. (2014) who found that performing experimental heat wave induces *tgfb* gene expression in three-spined sticklebacks 2 weeks after temperature change. Overall, it seems that the anti-inflammatory cytokines *tgfb* and *il10* were able to effectively regulate immune responses to *V. anguillarum* and provide protection in the face of heat stress. However, in order to strengthen these conclusions about anti-inflammatory responses to heat shock, further research is needed.

4.4 Immune system-activating cytokines

The transcript levels of the cytokines *ifng* and *il2* were measured to assess their role in activation of a further immune response to heat, bacteria, or both stressors. *ifng* was found to be upregulated early in only the *V. anguillarum* treatment groups (Figure 3.4.3). As a major activator of macrophages (Ignatz et al., 2020), the early release of *ifng* allows for a larger immune response to be mounted. IFN- γ has been shown previously to increase at 24 hours after injection with a bacterial vaccine (Raida & Buchmann, 2007). Heat shock did not appear to alter

ifng in a significant manner, though expression is slightly lower in HS/*Vibrio* fish (Figure 3.4.3 A). One study by Ignatz et al. (2020) found *ifng* to have an inverse relationship with temperature, with lower transcript levels at 16.5 °C compared to 10.5 °C 24 hours after injection with the immunostimulant pIC in Atlantic salmon.

il2 transcripts were only increased in No HS/*Vibrio* salmon 6 hours post-infection (Figure 3.4.3 A). This is not surprising, as the participation of IL-2 in antibacterial immunity is well-known (J. Zhang et al., 2024). Further, Buonocore et al. (2020) showed that treatment of sea bass with a *V. anguillarum* vaccine shows significant induction of IL-2 6 hours post-injection. However, the response of IL-2 to heat is less well-reported. There is evidence that under times of stress, IL-2 and IFN- γ is produced by glucocorticoids (i.e. cortisol) (Castro et al., 2011; Elenkov & Chrousos, 1999). Also, a main function of IL-2 is the differentiation of naïve T cells into T_H1 and T_H2 cells (Wang et al., 2018). Based on these two factors, it would not be expected for this cytokine to be upregulated by acute heat stress alone, due to the short nature of the stressor and the absence of cortisol after heat shock (see Results Section 3.7.2). However, at 14 dpi, we found *il2* increased in HS/*Vibrio* salmon. This may be due to a late adaptive response to *V. anguillarum*; however, it is difficult to tell only after 14 days.

4.5 Antimicrobial peptides

Antimicrobial peptides play a key role in mediating the innate immune response in salmonids. Both cathelicidin and hepcidin have well-known anti-bacterial properties (Katzenback, 2015). In studies using the Chinook salmon embryonic cell line CHSE-214, production of *camp* transcripts was induced 24 hours after *V. anguillarum* stimulation

(Broekman et al., 2013; Maier et al., 2008). In our study, AMPs was increased in all three tissues up to 7 days post-infection (spleen, hindgut, and gill; Figure 3.4.4). Other *in vivo* studies also show that *V. anguillarum* and other Gram-negative pathogens increases cathelicidin transcript levels in several organs in ayu (Lu et al., 2011) and rainbow trout (Chang et al., 2006), solidifying its role in anti-bacterial innate immunity.

Acute heat shock had varying effects on AMP transcripts. In *camp*, bacterial infection seems to be the main driver of antimicrobial activity. Fold changes in both infected groups follow a similar pattern to each other and is not altered in the heat shock control group. In *hamp*, heat shock had a more significant effect on Chinook salmon. Interestingly, these transcript levels follow a trend similar to those of pro-inflammatory cytokines – increased immediately after heat shock and increased to a lesser degree with *V. anguillarum* compared to fish that were not heated (Figure 3.4.4, Supplementary Figure 4). In this sense, heat shock either had little effect on, or improved, AMP transcripts before *V. anguillarum* exposure. There are several links between hepcidin and inflammatory responses (Alvarez et al., 2014; Shi & Camus, 2006), which relates to our findings in the present study (see Section 4.2). Further, when carp were challenged with *V. anguillarum*, hepcidin was produced in the spleen, hindgut, and head kidney as early as three hours post-infection (Li et al., 2013). There are few studies that have assessed AMP transcripts after heat shock but have differing results. In Atlantic cod, both *camp* and *hamp* transcript levels decreased immediately after acute heat stress but returned to basal levels three days post-stressor (Ruangsri et al., 2014). However, in another study, hepcidin increases in turbot skin in response to gradual water temperature increase from 16 °C to 19 – 21 °C (Huang et al., 2011). Hepcidin is

also upregulated in salmon undergoing stressful circumstances such as starvation (Martin et al., 2010) and hypoxia (Zanuzzo et al., 2020), and thus its role during heat stress needs to be further understood.

4.6 Antigen presentation

The antigen presentation pathways are essential in coordinating the adaptive immune response after initial contact with a pathogen. The endogenous antigen presentation pathway transcripts *mhc1a* and *tapasin* showed only slight differences between treatments. Both heat stress and bacterial infection resulted in a decrease in *tapasin* transcripts compared to controls. This downregulation is only seen in *mhc1a* transcripts from the HS/*Vibrio* treatment group (Figure 3.4.5), indicating that heat shock provides a correlation of endogenous antigen presentation components that is not seen in non-heat shocked, infected salmon. Again, research thus far on the effects of heat on the endogenous pathway has shown opposing results. While increases in water temperature show increases in *mhc1* in Atlantic cod (Pérez-Casanova et al., 2008), *mhc1a* transcripts decrease at high temperatures in walleye skin cells (Abram et al., 2020) and in rainbow trout (Guo et al., 2023).

Transcripts involved in the MHC II pathway only changed slightly after heat shock and/or infection with *V. anguillarum*. Interestingly, there was a decrease in *mhc2b* transcripts observed in the No HS/*Vibrio* treatment group (Figure 3.4.6). This is surprising, as *V. anguillarum* is an extracellular pathogen and has shown to increase activity in the MHC II pathway (Lim & Hong, 2021; Xing et al., 2020). It is possible that in this instance, *V. anguillarum* was able to downregulate MHC II genes to evade the immune system. This concept

was demonstrated previously in *mhc2a* transcripts in gilthead seabreams infected with a parasite (Johnstone & Chaves-Pozo, 2022), and the ability to repress genes to evade the immune system has been proposed in other *Vibrio* spp. (Hsiao et al., 2006). Heat shock combined with *V. anguillarum* infection appears to decrease MHC II pathway transcripts (Figure 3.4.6 B). There have been few studies to date that examine the MHC II pathway in response to heat with conflicting results. Dittmar et al. (2013) saw no differences in MHC IIb gene expression 2 weeks after an experimental heat wave. Another study showed that MHC II genes in rainbow trout are downregulated at low temperatures, and the authors concluded that these genes are temperature dependent (Nath et al., 2006). Applying this theory to the present study emphasizes the influence of temperature on the exogenous presentation pathway. However, further research using upper thermal tolerances is needed to strengthen this conclusion.

4.7 Heat shock proteins

Heat shock proteins are essential cell signaling molecules and maintain correct protein conformation when the host experiences stressful conditions, such as acute heat stress or bacterial infection (Mohanty et al., 2018). Unsurprisingly, in our study, heat shock protein transcripts increased immediately after heat shock and remained upregulated for several hours afterwards (Supplementary Figure 7, Figure 3.4.7). The HSP response has been well-examined, and results consistently show that these transcripts increase in teleosts after heat shock (Palmisano et al., 2000; Purohit et al., 2014; Weber et al., 2023). There is also an interesting link between HSPs and the immune response in teleosts, in which HSPs are involved in innate immunity and T-cell mediated responses (Roberts et al., 2010). In juvenile Chinook salmon,

HSPs were upregulated in response to infectious hematopoietic necrosis virus (IHNV) (Eder et al., 2007). In the present study, *V. anguillarum* treatment alone caused an increase in *hsp90* and *hsp47* transcripts, further solidifying the relationship between immunity and HSPs.

Interestingly, *hsp70* did not play a significant role in either heat shock or bacterial infection. These results were not expected, as *hsp70* is often used as a reliable biomarker of environmental stress (Roberts et al., 2010; Wong & Do, 2017). Further, Ackerman & Iwama (2001) observed an upregulation of *hsp70* in response to *V. anguillarum* in rainbow trout, providing evidence for HSP70's role in immunity. One study, however, showed no significant changes in the gut of rainbow trout after injection with *V. anguillarum* bacterin or stress by air exposure (Khansari et al., 2018), similar to what we observed in the present study. Therefore, due to these conflicting results, it is crucial in future studies to include several HSP transcripts in order to fully understand the role of HSPs during heat stress and/or infection.

In order to assess correlations between mRNA and protein levels, an ELISA was used to quantify levels of HSP47 in the plasma of Chinook salmon. No significant differences could be detected in any treatment or timepoints (Figure 4.6). As salmonid antibodies and subsequent diagnostic assays are difficult to develop and perform, limited comparative studies are available. One study, however, found the expression of *hsp47* not to be an effective biomarker of temperature change in the plasma of Atlantic salmon (Vadboncoeur et al., 2023). However, as this study and other research groups have found, *hsp47* expression is highly induced in tissues such as spleen, gill, liver, and muscle in response to thermal stress (Akbarzadeh et al., 2018). HSP47 plays a relatively specific role as a molecular chaperone for fibril-forming collagens and

is also localized in the endoplasmic reticulum of cells (Wang et al., 2015). This being said, HSP47 may have stronger differences in other tissues not measured in the present research. However, our research group presents the first reported ELISA usable for detecting HSP47 in Chinook salmon tissues and plasma.

4.8 Metabolites and cortisol

In salmonids, a cellular and endocrine stress response results from exposure to stressors, such as heat stress or bacterial infection (Chadwick et al., 2015; Fast et al., 2008). Liver glycogenolysis converts stored glycogen into free glucose to be circulated to tissues for energy use (Mauduit et al., 2022). In this study, we measured plasma glucose and lactate concentrations to assess the effect of heat or *V. anguillarum* on the metabolism of Chinook salmon (Figure 3.7.1). Though there was no difference in glucose levels between handling and heat shock controls, glucose levels increased in all treatment groups 6 hours post-treatment. It is possible that both handling and heat stress caused a delayed glucose increase that explains the spike in plasma levels 6 hours post-treatment. One study found that glucose increased 3 hours after a 15-second handling stress (Fast et al., 2008). Further, during an environmentally realistic heat shock, Gallant et al. (2017) found glucose to peak in Atlantic salmon plasma when temperature was at its maximum, then return to levels similar to controls (pre-heat shock) during recovery, 8 hours after thermal peak. Therefore, it is possible that peak glucose levels for this study would be seen during the heat shock itself rather than afterwards. In future studies, it would be helpful to measure plasma metabolite levels during the thermal peak in order to confirm this.

Secondly, initial heat shock caused an increase in lactate compared to handling controls, then remained stable in all treatment groups for the remainder of the experiment. It is debatable whether heat shock has an effect on lactate levels in salmonids (Forgati et al., 2017; Lulijwa et al., 2021). There are several factors that may affect lactate levels and the need for anaerobic metabolism, for example, available oxygen (Vianen et al., 2002), lactate used in muscle metabolism (Weber et al., 1986), and fish cortisol levels (Laiz-Carrión et al., 2002). Salmon undergoing heat shock did experience hypoxic conditions, with dO₂ levels as low as 5.32 mg/L at high temperatures (See Figure 2.2), which may explain the spike in lactate. When lactate did increase in response to stress, plasma levels prove to recover quickly (between 2 to 4 hours post-stress) in other studies (Galloway & Kieffer, 2003) as well as in the present study. The presence of *V. anguillarum* did not affect lactate plasma levels. However, plasma was only tested up to 3 dpi, as lactate concentrations were expected to fluctuate the most immediately after heat shock. One study found that infection with the bacterial pathogen *Aeromonas salmonicida* did not affect lactate concentrations until six dpi (Yi et al., 2016), thus, the connection between metabolism and disease should be further examined.

Cortisol is a well-known biomarker for stress in salmon (Lulijwa et al., 2021). Immediately after heat shock, cortisol was increased in heat shock controls compared to handling controls (Figure 3.7.2). This is unsurprising, as several studies have shown the immediate effect of heat on plasma cortisol levels (Barton et al., 1985; Guo et al., 2023; Lulijwa et al., 2021). 6 hours post-treatment and through the duration of the trial, cortisol levels did not differ between treatment groups. Previous work shows that raised cortisol levels in the plasma of stressed fish

return to levels similar to controls in as little as 3 hours (Fast et al., 2008). Further, studies have shown that cortisol suppresses immune system, and specifically the gene expression of pro-inflammatory cytokine such as IL-1 β (Carrizo et al., 2021; Shepherd et al., 2018), so it is possible that our results showed a preference towards the immune response rather than a stress response to fight *V. anguillarum*. What was most intriguing was the variation of cortisol levels in individual fish part of the same treatment group and timepoint (for example, HS/*Vibrio* at 3- and 14-dpi). The corticosteroid stress response in salmon can vary and is influenced by genetic variation, sex, and size (Sopinka et al., 2016). Furthermore, cortisol can begin to circulate in as little as 3-5 minutes after handling stress (Sopinka et al., 2016), which may also explain a small spike in cortisol during sampling in this trial. Of course, the high levels of cortisol may be in response to the pathogen. However, previous studies show varying results between cortisol levels and disease. In juvenile rainbow trout, there was a progressive increase in bacterial load of *V. anguillarum* in the blood and plasma cortisol concentration (Ackerman & Iwama, 2001). In another study, plasma cortisol remained unchanged in Atlantic salmon infected with *V. salmonicida* (Damsgård et al., 2004). Due to these conflicting results and results in the present study, further investigation is needed regarding the role of bacterial infection on plasma cortisol levels.

4.9 Conclusions and future directions

In past research, it is generally accepted that heat shock negatively affects salmon due to the stress response that results. Our research examined this using a 17-hour, environmentally realistic heat wave; however, it was discovered that exposing Chinook salmon to this stressor

had few long-term and negative effects to the salmon stress and immune responses, given ample time to recover from said heat. Surprisingly, experiencing a heat wave may give Chinook salmon a slight advantage against vibriosis due to the incidental preconditioning effects of heat before exposure to *V. anguillarum*. Specifically, exposure to heat, then *V. anguillarum*, delayed mortality due to vibriosis by up to 4 days, lessened the inflammatory (and corresponding anti-inflammatory) response necessary to combat this pathogen and modulated the antimicrobial peptide and heat shock protein responses needed during infection. This is well-demonstrated in Table 4.1 below, which summarizes the gene expression observed throughout the trial. Lastly, the heat wave caused an initial increase in plasma lactate concentrations, but neither heat stress nor *V. anguillarum* impacted either glucose or lactate in the days following treatment.

Table 4.1 Summary of gene expression measured by qPCR

	Heat shock only (HS/PBS)					<i>V. anguillarum</i> only (No HS/ <i>Vibrio</i>)					Heat shock/ <i>V. anguillarum</i> (HS/ <i>Vibrio</i>)				
Treatment → Effect ↓	0.25	1	3	7	14	0.25	1	3	7	14	0.25	1	3	7	14
Pro-inflammatory						↑	↑	↑	↑			↑			
Anti-inflammatory		↑						↑	↑			↑↓			
Immune activation						↑	↑					↑			↑
AMPs					↑-	↑	↑	↑	↑			↑	↑-	↑-	
MHC I			↓				↑	↓						↓	
MHC II								↓				↓			
HSPs	↑	↑					↑	↑	↑-		↑	↑		↑-	

An overview of the changes in gene expression grouped into one of seven categories (leftmost row). Red arrows indicate an upregulation of genes compared to non-heat shocked, non-infected controls, blue arrows indicate a downregulation, and grey dashes & dark grey squares with no symbol indicate no change in gene expression. Treatments & timepoints with two symbols indicate that two or more genes in that category have differing effects.

It has been suggested previously that multiple stressors may provide Chinook salmon with a cumulative or even synergistic stress response (Barton et al., 1986). Our conclusions from the current study not only support this to be true, but also applies this theory to a realistic scenario which Chinook salmon may need to combat while in shallow, freshwater environments. In this sense, thermal preconditioning seemed to have a positive effect on Chinook salmon. The idea of a “cellular buffer” resulting from preconditioning seems most appropriate given these conclusions – though mortality due to vibriosis did not change, there was a 3–4-day delay in mortality rates in heat shocked fish. However, given the lower immune transcript levels in surviving fish after 14 days, it may still be appropriate to say that acute heat shock provided a molecular response strong enough to aid in combatting vibriosis infection.

This study is the first time that preconditioning has been performed prior to a bacterial pathogenic exposure. There has only been one other study to examine this concept in salmon using a viral pathogen after hypoxia preconditioning (Lund et al., 2017). Our conclusions generally agree that preconditioning mitigated the effects of the pathogen. However, more studies need to be conducted both in order to strengthen these ideas and to broaden the scope of this research to other fish pathogens of interest. Further, this study mainly focused on gene expression using qPCR in conjunction with protein quantification by ELISA. The immune system is a complex organization of signalling cascades and cell interactions that cannot be

defined by cytokine transcript and protein levels alone. Therefore, future studies should also consider measuring elements such as complement, lysozyme, and phagocytic assays to assess innate responses in immune cells, as well as the long-term effects of heat shock/*V. anguillarum*, such as antibody production.

This research is also the first time that a day-long experimental heat wave was performed on salmon and the resulting immune effects have been analyzed. The temperatures used for our heat wave were based on data collected from the Quinsam River in British Columbia, Canada, to accurately mimic a heat stress that salmon may face outside of a laboratory setting. River temperatures in Western and Northern Canada will continue to rise in the coming years and has already shown to negatively impact wild salmon populations (Parkinson et al., 2016; von Biela et al., 2020). It is intriguing to envision how heat stress has already impacted wild salmon and if exposure to temperatures outside of their optimal range changes their ability to combat future stressors, such as disease. This research provides a solid baseline of how changing river temperatures can affect salmon, however, using salmon directly from these streams may help researchers gain the most accurate impacts of heat stress. Further, it is important to note that most salmonids, including Chinook salmon, are anadromous, and develop in saltwater before returning to freshwater to spawn. Further, the Chinook salmon used in this study were juvenile (i.e. not yet of spawning age) and raised in freshwater. Therefore, it is crucial to also assess if the type of water would have an impact on either preconditioning or on heat stress alone.

The change in global temperature, proportion of heat waves, and increase in stream temperature are arguably inevitable events that will impact several aspects of aquatic ecosystems

in coming years. Therefore, it is crucial to turn our attention and research focuses to understanding the biological impacts of warming water on salmonid populations and disease prevalence in these species. Canadian governmental strategies that emphasize stabilizing and restoring Pacific salmon populations have been implemented thus far (DFO, 2019). This research can build upon these initiatives and inform the design strategies of habitat management in rivers less susceptible to temperature fluctuation (Corey et al., 2017), fisheries management (Guo et al., 2023), and population management for sustainable populations for generations to come (DFO, 2019). Overall, this research successfully showed the physiological and immunological consequences of thermal and pathogenic stress in salmonids and provides a step in understanding the implications of warming temperatures on these species.

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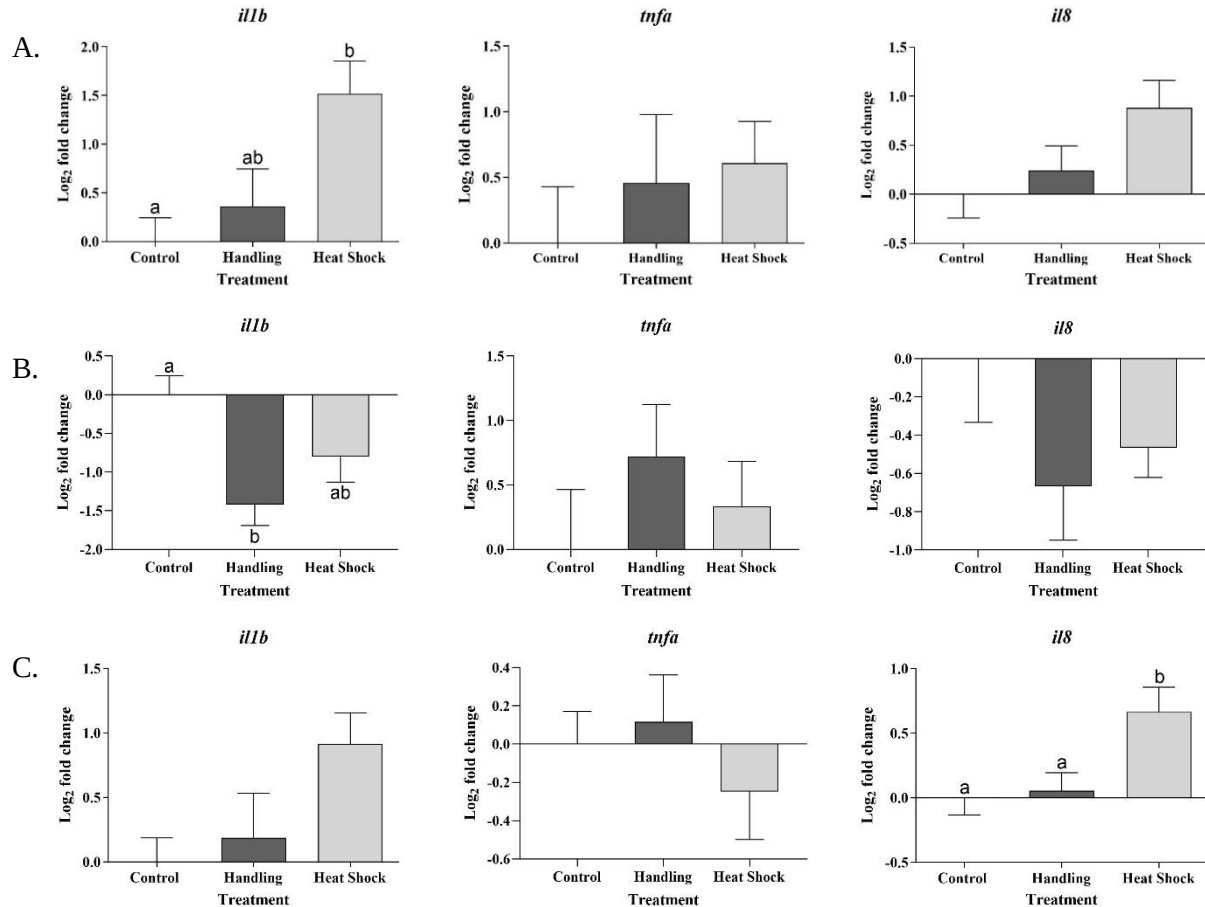
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Appendices

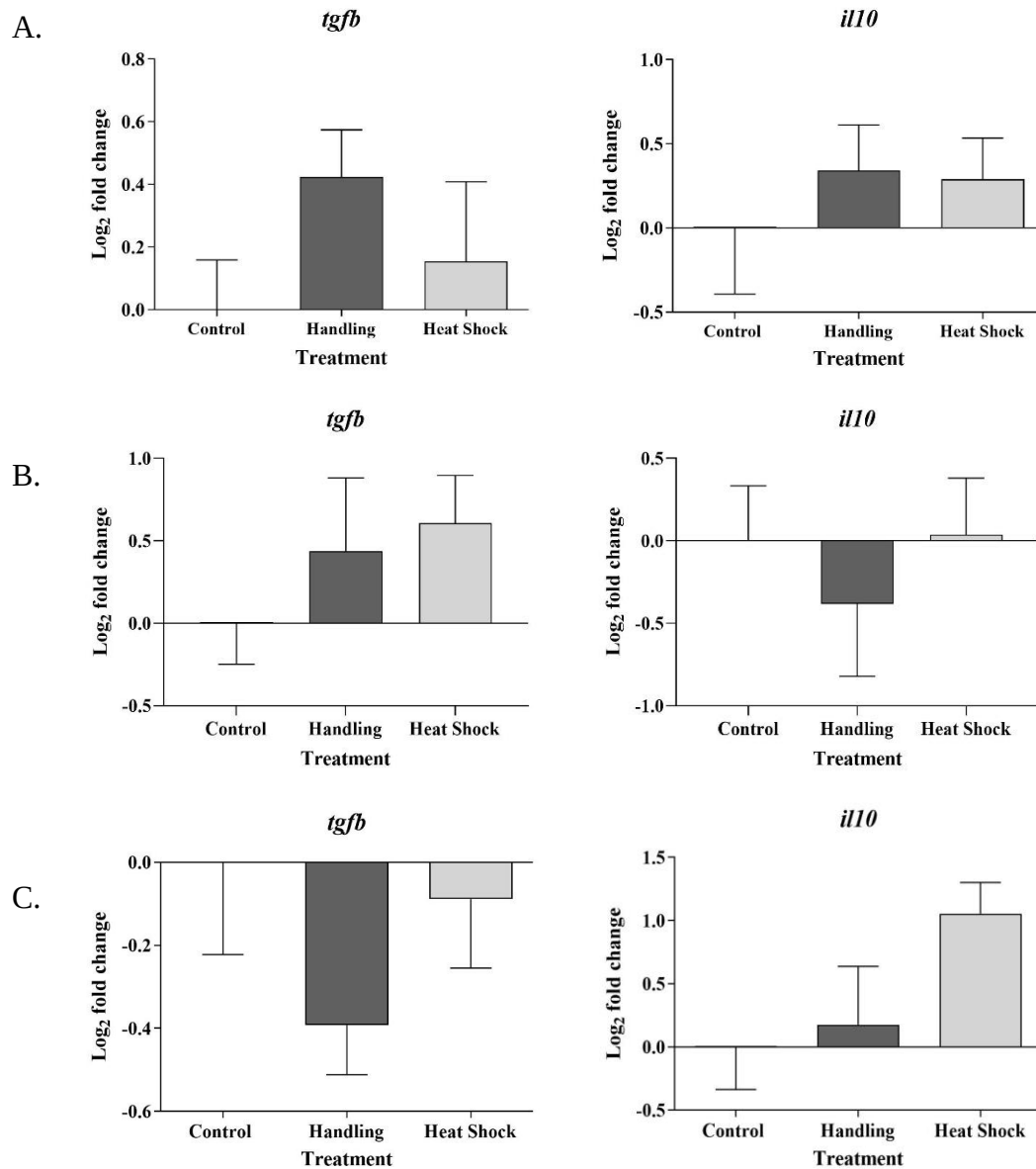
Appendix A

Supplementary Figures



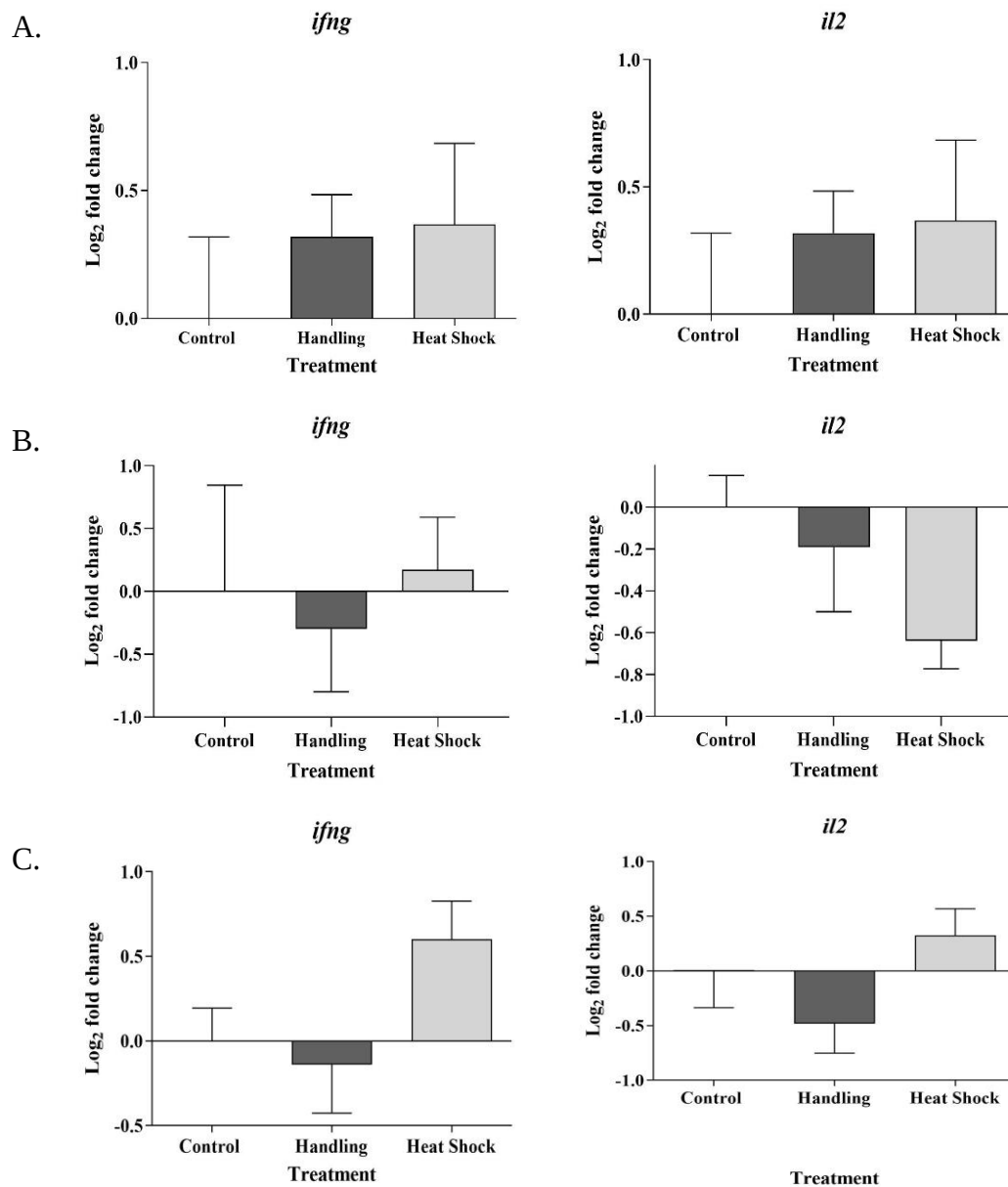
Supplementary Figure 5 Pro-inflammatory gene expression in t=0 controls

Gene expression of IL-1 β (*il1b*), TNF- α (*tnfa*), and IL-8 (*il8*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing either no heat shock or handling stress (Control), handling stress (Handling), or heat shock (Heat Shock). Each value is the mean $\pm$ S.E.M. (n = 8). Samples were compared between treatments using a one-way ANOVA with Tukey post-hoc test. Different letters represent significant differences between treatments.



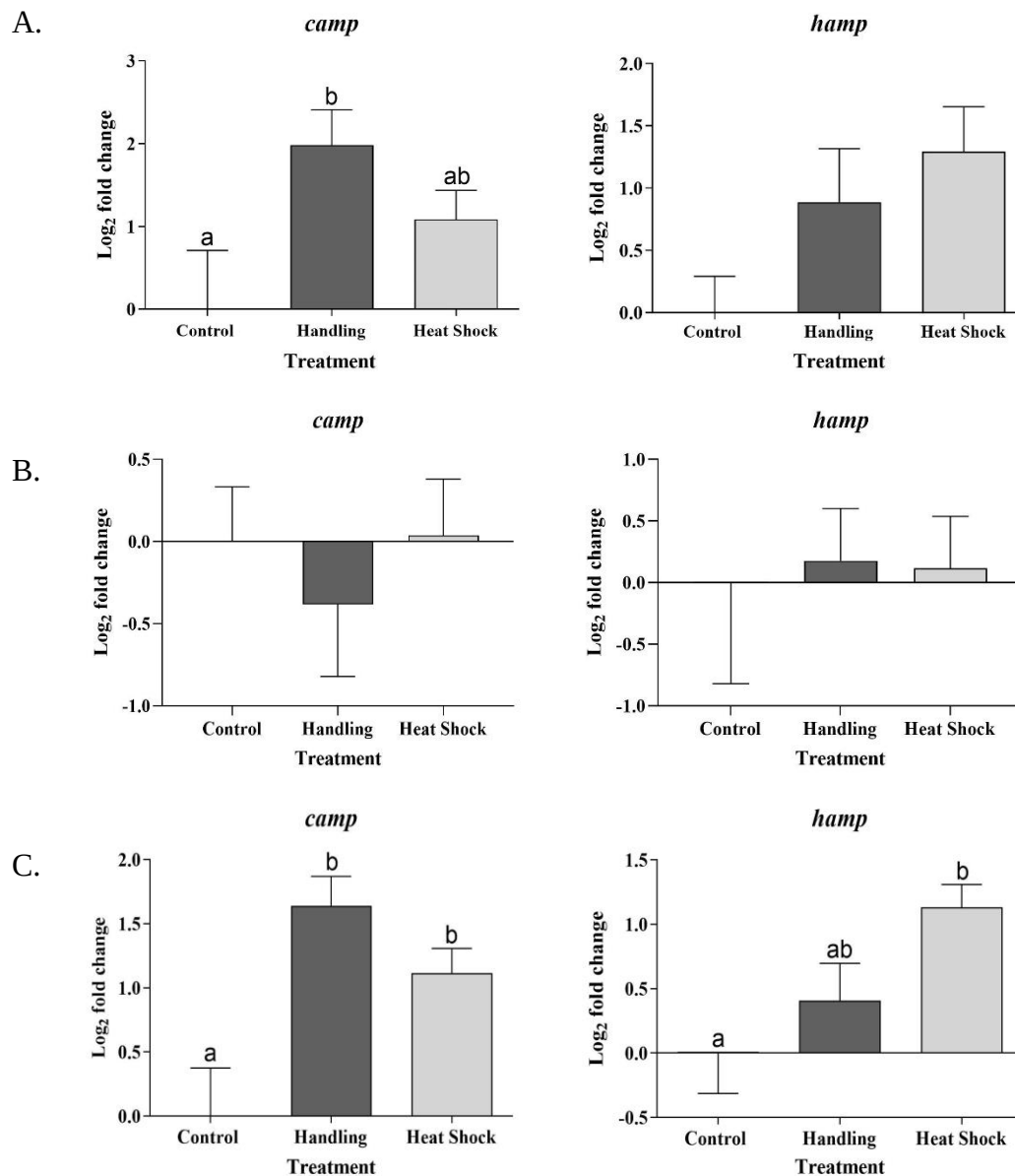
Supplementary Figure 7 Anti-inflammatory gene expression of t=0 controls

Gene expression of IL-10 (*il10*) and TGF- β (*tgfb*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing either no heat shock or handling stress (Control), handling stress (Handling), or heat shock (Heat Shock). Each value is the mean $\pm$ S.E.M. (n = 7-8). Samples were compared between treatments using a one-way ANOVA with Tukey post-hoc test.



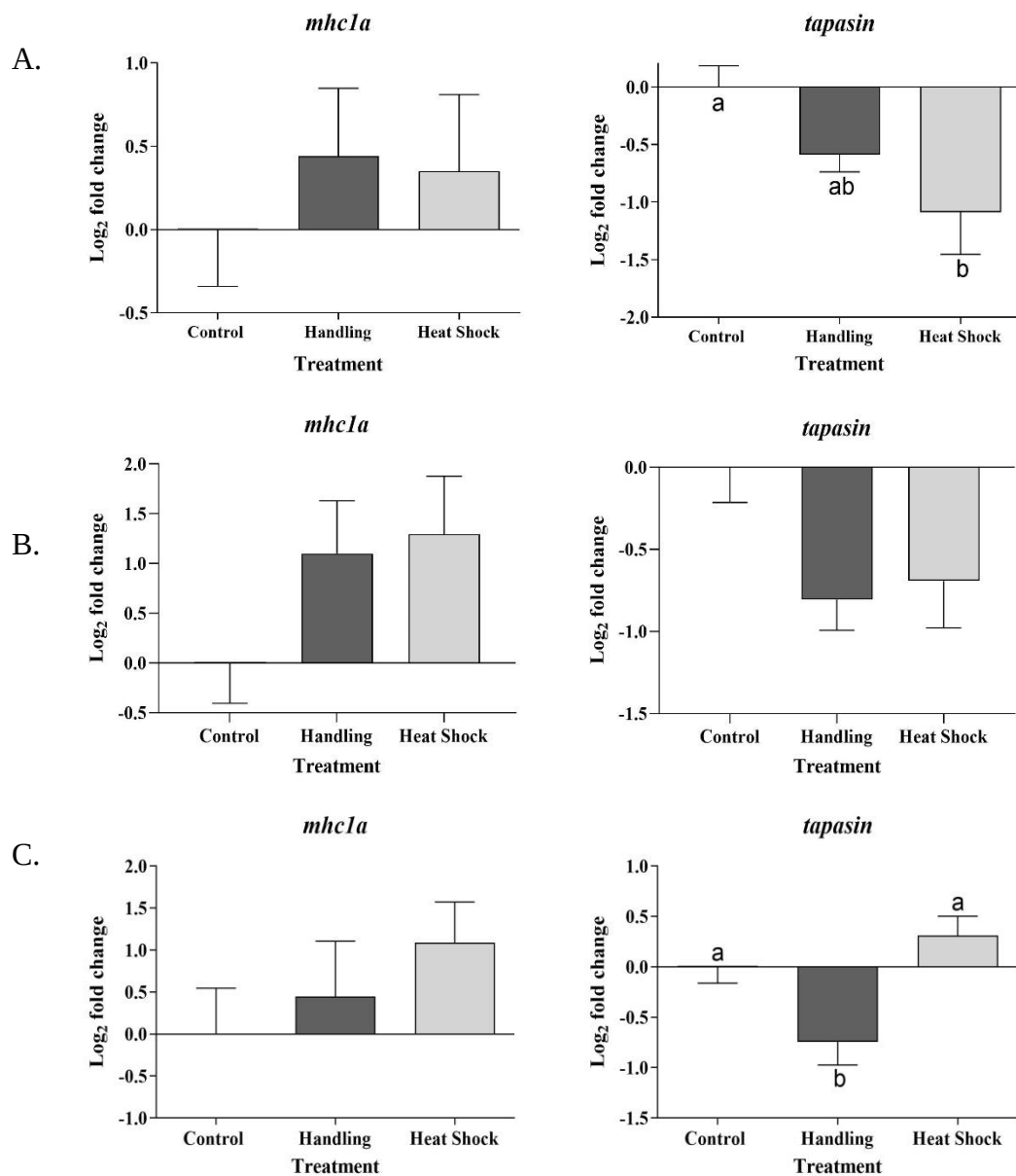
Supplementary Figure 3 Immune activation gene expression of t=0 controls

Gene expression of IFN- γ (*ifng*) and IL-2 (*il2*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing either no heat shock or handling stress (Control), handling stress (Handling), or heat shock (Heat Shock). Each value is the mean $\pm$ S.E.M. (n = 7-8). Samples were compared between treatments using a one-way ANOVA with Tukey post-hoc test.



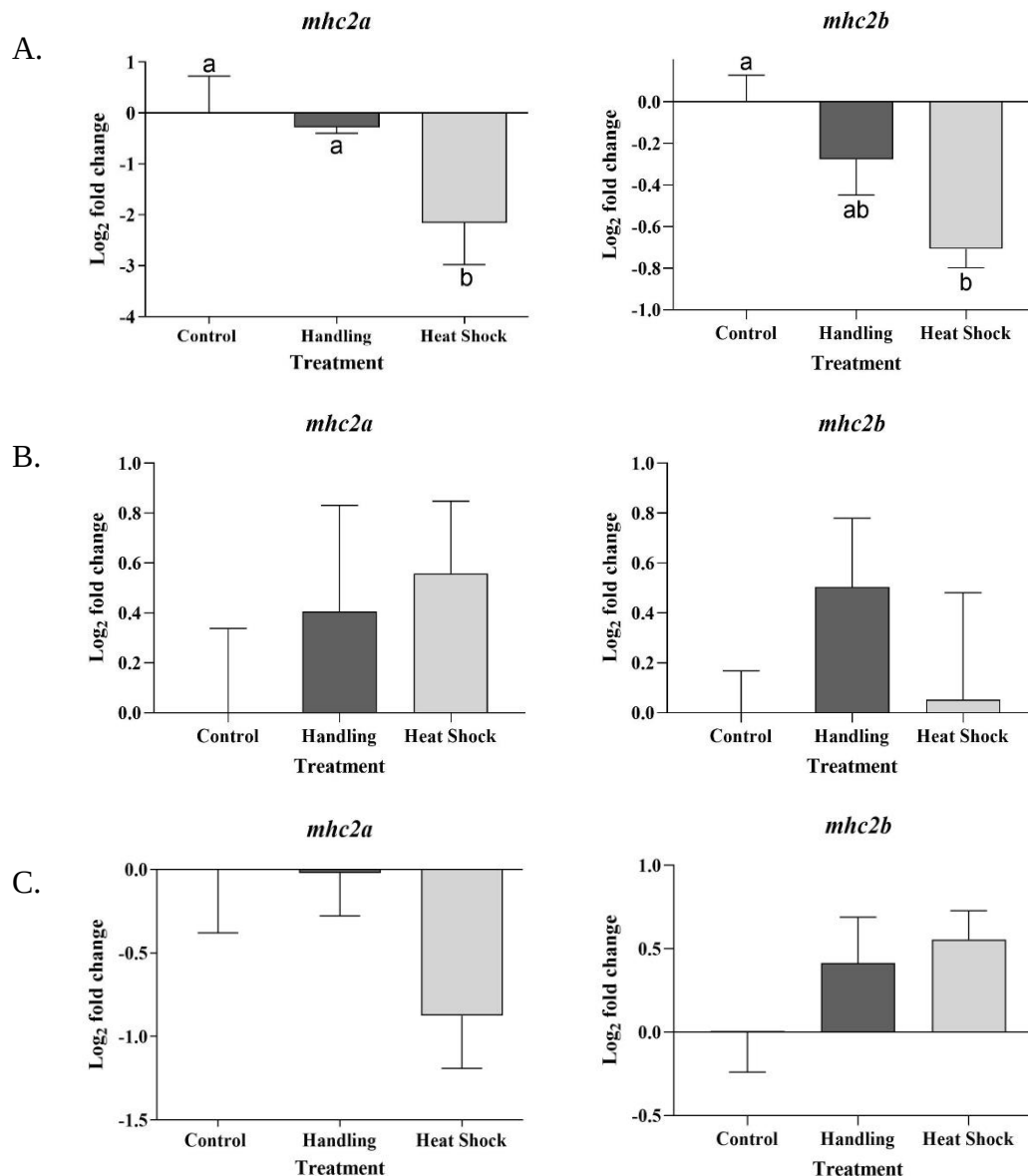
Supplementary Figure 4 Antimicrobial peptide gene expression of t=0 controls

Gene expression of cathelicidin (*camp*) and hepcidin (*hamp*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing either no heat shock or handling stress (Control), handling stress (Handling), or heat shock (Heat Shock). Each value is the mean $\pm$ S.E.M. (n = 6-8). Samples were compared between treatments using a one-way ANOVA with Tukey post-hoc test. Different letters represent significant differences between treatments.



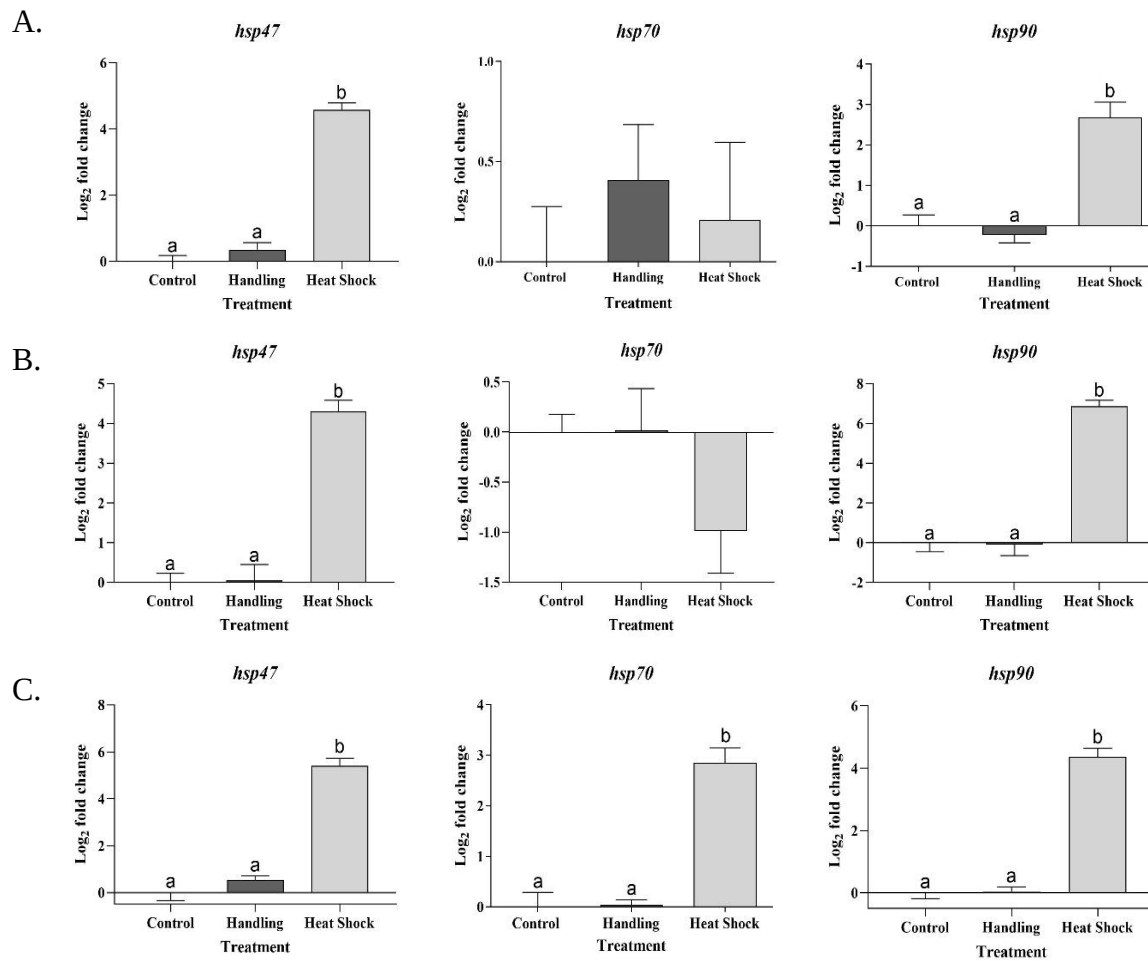
Supplementary Figure 5 Endogenous antigen presentation pathway gene expression of t=0 controls

Gene expression of MHC I- α (*mhc1a*) and tapasin (*tapasin*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing either no heat shock or handling stress (Control), handling stress (Handling), or heat shock (Heat Shock). Each value is the mean $\pm$ S.E.M. (n = 6-8). Samples were compared between treatments using a one-way ANOVA with Tukey post-hoc test. Different letters represent significant differences between treatments.



Supplementary Figure 6 Exogenous antigen presentation pathway gene expression of t=0 controls

Gene expression of MHC II- α (*mhc2a*) and MHC II- β (*mhc2b*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing either no heat shock or handling stress (Control), handling stress (Handling), or heat shock (Heat Shock). Each value is the mean $\pm$ S.E.M. (n = 6-8). Samples were compared between treatments using a one-way ANOVA with Tukey post-hoc test. Different letters represent significant differences between treatments.



Supplementary Figure 7 Heat shock protein gene expression of t=0 controls

Gene expression of HSP47 (*hsp47*), HSP70 (*hsp70*), and HSP90 (*hsp90*) in juvenile Chinook salmon spleen (A), hindgut (B), and gill (C) undergoing either no heat shock or handling stress (Control), handling stress (Handling), or heat shock (Heat Shock). Each value is the mean $\pm$ S.E.M. (n = 6-8). Samples were compared between treatments using a one-way ANOVA with Tukey post-hoc test. Different letters represent significant differences between treatments.