

Ploidy impacts the response of Chinook salmon (*Oncorhynchus tshawytscha*) to pathogen and thermal stress

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Statement of Contributions

Ivan Cadonic was the sole author for Chapters 1 and 5, which were written under the supervision of Dr. Paul Craig and were not written for publication.

This thesis consists of three chapters, two of which (Chapters 2 & 4) were written for publication and one of which (Chapter 3) was written utilizing data that was published in collaboration with the authors below.

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Dr. Paul Craig was the primary investigator on the Natural Sciences and Engineering Research Council (NSERC) grant which supported conducting this work. Ivan Cadonic was supported by a Queen Elizabeth II Graduate Scholarship in Science and Technology & an NSERC Post-graduate Scholarship (Doctoral). This research was conducted at Yellow Island Aquaculture (BC) and at the University of Waterloo (Ontario) by Ivan Cadonic under supervision from Dr. Craig. Ivan Cadonic conducted the study, collected and analyzed the data, and wrote the manuscript. Dr. Craig, Dr. John Heath, and Dr. Brian Dixon provided input on study design, equipment for analysis, and edited the manuscript. This research is published as:

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Abstract

As climate change continues to reduce wild salmon populations, global reliance on aquaculture will become increasingly essential to sustain food availability. However, the environmental implications of aquaculture in causing Pacific salmon stock declines are becoming more apparent and as such the industry needs to transition to practices that would reduce negative impacts on wild fishes.

Widespread utilization of sterile triploid fishes offers an alternative to normal diploid fish since they are unable to breed with wild population and are less likely to interact with wild fishes. Triploid salmonids have an extra set of chromosomes that prevent them from producing viable gametes, while also maintaining flesh quality for longer due to lack of nutrient deposition from muscle to eggs during sexual maturation.

When farmed under optimal conditions, triploid fish perform similarly to diploids; however, when conditions become suboptimal (e.g. high temperature & infection with pathogens) triploids have reduced survival. The reasons for this reduced performance are mostly unknown, so this thesis aimed to investigate the molecular impacts of increased ploidy in Chinook salmon (*Oncorhynchus tshawytscha*). Specifically, the response of microRNA (miRNA) and protein coding mRNAs were investigated before and after exposure to pathogenic and thermal stress. MiRNAs are small, non-coding RNAs that bind to specific mRNA molecules and reduce protein translation. I hypothesized that diploid and triploid salmonids have subtle differences in molecular (miRNA, mRNA) indices that are exacerbated by changes in the environment (pathogens and temperature), which results in their poorer performance in response to stress.

Chapter 2 investigated the miRNA responses in three immune tissues (hindgut, head kidney, and spleen) before and after exposure to *Vibrio anguillarum*, a common pathogen Chinook experience in open-sea net pens. Overall, ploidy had minimal impacts on miRNA abundances prior to stress. Abundance of miRNAs were altered in both ploidies after *Vibrio* exposure to support immune function. Just prior to *Vibrio*-induced mortality, the spleen of triploid fishes had altered miRNA abundances which identified that in triploids, the spleen is likely associated with their reduced immune competence.

Chapter 3 investigated the immune mRNA responses in gill and heart ventricle before and after recovery from acute thermal stress. Triploid gills likely have altered pathogen sensing based on reduced pattern recognition receptor mRNA abundance. Tissue specific alterations after thermal

stress provided insight into immune dynamics during a cellular heat-shock response. Specifically, pro-inflammatory cytokine mRNA abundance was elevated after recovery from thermal stress. The immune genes measured are likely thermally responsive in a time-dependent manner based on their variability after recovery from thermal stress.

Chapter 4 investigated the cardiorespiratory impacts of increased ploidy before and after a critical thermal maximum (CT_{max}) trial. In agreement with previous studies, triploid hearts became arrhythmic at lower temperatures indicating that their hearts are less resilient to acute changes in temperature. Additionally, ventricle expression of cardiorespiratory genes was altered in triploid fish, implicating that this tissue is associated with their reduced thermal performance.

Overall, this thesis provides evidence for the molecular dysregulation associated with increased ploidy in Chinook salmon. These impacts are minimal under normal conditions but exacerbated by pathogenic and thermal stress. The results here provide targets for aquaculture to develop breeding programs or therapeutics that can be used to improve health and well-being of salmonids, including triploids, in response to climate change.

Acknowledgements

Alright, so I should start off by apologizing to everyone for the length of this document. I've been verbose in some sections, and this acknowledgments section is not spared.

I looked over the acknowledgements for my Master's thesis and realized almost started this one off the same way. However, I think it's still relevant because I really have too many people to thank since I wouldn't be where I am today without all the mentors and peers I've had to this point. Even if you're not specifically named in this section, just know you've helped make me the person I am today.

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List of Abbreviations

-3p	3 prime ('), referring to 3' end of RNA
-5p	5 prime ('), referring to 5' end of RNA
18s rRNA	18S ribosomal RNA
Ago2	Argonaute 2/Slicer
ANOVA	Analysis of variance
b2m	B-2 microglobulin
BC	British Columbia
bp	base pair
BPM	beats per minute
ca4	Carbonic anhydrase 4
CT _{max}	critical thermal maximum
DE	Differentially expressed
dcp1a	mRNA-decapping enzyme 1a
DNA	deoxyribonucleic acid
ECG	electrocardiogram
ef1 α	elongation factor 1 α
FC	Fold change
FDR	false discovery rate
f_H	heart rate
GCRV	grass carp reovirus
HLA-DM	human leukocyte antigen DM
HSP	heat shock protein
IHNV	Infectious hematopoietic necrosis virus
il	Interleukin
IP	intraperitoneal
IPCC	Intergovernmental Panel on Climate Change
ISA	infectious salmon anemia
KEGG	Kyoto encyclopedia of genes and genomes
mb	Myoglobin
MFI	Mean fluorescence intensity
mhc	Major histocompatibility complex
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR
miRNA	MicroRNA
MMC	melano-macrophage centers
mRNA	Messenger RNA
NF- κ B	Nuclear factor κ -light-chain-enhancer of activated B cells
NGS	Next Generation Sequencing
nlrp3	Nucleotide-binding oligomerization (NOD)-like receptor family pyrin containing 3
PAMP	pathogen-associated molecular patterns

PBS	Phosphate-buffered saline
PRR	Pattern-recognition receptor
qPCR	Quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	Reactive oxygen species
SEM	standard error of the mean
ssa	<i>Salmo salar</i>
TAP	Transporter associated with antigen processing
T_{ab}	Arrhenius breakpoint temperature
T_{arr}	Temperature associated with first incidence of cardiac arrhythmia
tgfb	Transforming growth factor β
TLR	Toll-like receptor
UTR	Untranslated region
YIAL	Yellow Island Aquaculture

Quote

“For the Quest is achieved, and now all is over.

I am glad to have you here with me.

Here at the end of all things, Sam.”

-Frodo at Sammath Naur

The Lord of the Rings – The Return of the King

Book 6, Chapter 3

JRR Tolkien, 1955

Chapter 1

General Introduction

1.1 Aquaculture in Canada and around the world

Globally, aquaculture is a critical and rapidly growing industry, and in Canada the overall value is estimated at over \$1.3 billion, with almost \$1.1 billion attributed to salmon farming (Government of Canada, 2024; Statistics Canada, 2023). Canadian salmon exports are predominantly Atlantic salmon (*Salmo salar*; \$806 million in 2021); however, Pacific salmon exports, although lower revenue generated (\$151 million in 2021), have a higher compound annual growth rate from 2017 to 2021 (Agriculture and Agri-Food Canada, 2023). During the same time period, Canadian imports of fresh Pacific salmon fillets have grown by 12.4%, reaching a total of \$270 million in 2021 (Agriculture and Agri-Food Canada, 2023). Wild Pacific salmon stocks are declining (Bendriem et al., 2019; Pauly and Zeller, 2016; Peterman and Dorner, 2012) and therefore, more pressure will be placed on aquaculture to supply food demands in the future.

The environmental implications of aquaculture practices are becoming more apparent, and increasing pressure is being placed on the industry to reduce impacts on wild fishes. Introduction of non-native aquatic organisms for the purpose of aquaculture has been associated with disease outbreaks in wild populations (Peeler et al., 2011). The Canadian government has recently mandated an end to open-sea net pen culturing by 2029 in British Columbia (BC) to protect wild Pacific salmon (Fisheries and Oceans Canada, 2024). Although this could reduce pressure on wild fishes, transitioning farming in BC from (predominantly) Atlantic salmon to Pacific salmon could provide a more profitable and sustainable practice since they are endemic to the west coast. Farming of Pacific salmon, specifically Chinook salmon (*Oncorhynchus tshawytscha*), are an economically competitive option to Atlantic salmon for the BC aquaculture industry (British Columbia Ministry of Agriculture, 2017). Wild BC Chinook salmon in 2018 had a wholesale value of \$23 million and have the fastest growth rate of all Pacific salmon species (BC Ministry of Agriculture, 2020; Gislason et al., 2017). Evolutionarily, Pacific salmon have a complicated life cycle that involves juvenile development in freshwater streams, transition to saltwater (smoltification) to increase mass, and finally transition back to freshwater streams to reproduce and die (Groot and Margolis, 1991). Chinook salmon have been naturalized in the Great Lakes through extensive stocking programs to act as the primary fishery in Lake Ontario, initially as population control of alewife (*Alosa pseudoharengus*) but now an important sport fishing industry (Brandt, 1986; Legard et al., 2022). This success story provides hope for rearing land-based Chinook salmon using freshwater systems on the west coast as open-sea net pens are removed.

Although transitioning to an endemic species would provide more sustainable aquaculture practices, there are still issues that have the potential to impact wild salmon populations. Populations that are declining (such as Pacific salmon) have a greater chance of experiencing introgression from aquaculture stocks (Diserud et al., 2022). When fish escape from farms, they can impact wild population resilience by competing for resources and interbreeding with wild fishes (Glover et al., 2012; reviewed by Jensen et al., 2010; Lura and Sægrov, 1991). Breeding between wild and farmed fish populations can impact many traits critical for survival, including developmental success, genetic diversity, and overall size (Bolstad et al., 2021; Fleming et al., 2000; San Román et al., 2023; Skaala et al., 2006; Skaala et al., 2019). Salmon runs transfer nutrients from marine to terrestrial habitats so declines in wild salmonid populations will have far reaching consequences on ecosystem health on the west coast (Cederholm et al., 1999; Helfield and Naiman, 2001). Developing technologies and infrastructure to reduce these stressors is vital, especially as climate change continues to negatively impact wild salmon populations.

1.2 Triploid fish

Many problems associated with aquaculture can be solved through widespread utilization of triploid fishes. Triploid fish offer a sustainable alternative to farming normal diploid fish since they are physiologically sterile (reviewed by Benfey, 1999; Benfey et al., 1989). Triploidy can be induced by artificial retention of the second polar body just after fertilization (autopolyploidy) or by interbreeding between closely related species (allopolyploidy; reviewed by Piferrer et al., 2009a). For this thesis, autopolyploids are utilized since they can be easily produced by exposing zygotes to either high temperature or high pressure to induce three chromosome copies in salmon (Johnson et al., 2004). As triploids age, gonadal development is inhibited since abnormal chromosomal alignment during meiosis results in aneuploid gametes (Cimino, 1972; Huang et al., 2021; Xu et al., 2016). However, male triploids can produce viable gametes (at a lower concentration relative to diploids) and depict spawning-like behaviours while female triploids have highly reduced gonad mass and produce eggs with low viability (do Nascimento et al., 2017; Fjelldal et al., 2014; Lahnsteiner and Dünser, 2024; Yang et al., 2023). Therefore, aquaculture facilities (such as Yellow Island Aquaculture) utilize sperm from sex-reversed females during fertilization to maintain entire stocks of genetically female (XXX) triploids.

Culturing triploid fishes offers a more profitable and sustainable alternative to diploid fish. Sexual maturation in salmonids reduces flesh quality due to substrate deposition from muscle into eggs (Cleveland et al., 2012; McClure et al., 2007). Therefore, sterile fishes are able to grow larger and maintain flesh quality for production, which would increase economic value of stocks (Bao et al., 2023, 202; Crouse et al., 2023; Gomes et al., 2020; Manor et al., 2014). In addition to sterility improving flesh quality, culturing triploid fishes would benefit the wellbeing of wild salmonids. Their sterility means that triploids would have reduced impact on wild genetic diversity should they escape from farms. Triploid Atlantic salmon escapees are less likely to migrate into natural salmon spawning habitats, which would reduce pressure on wild fishes during a stressful life history event (Cotter et al., 2000; Glover et al., 2016). Behaviourally, triploid salmon are less aggressive and have poorer foraging performance relative to diploid conspecifics (Carter et al., 1994; Garner et al., 2008). Therefore, escapees are less likely to survive outside of farms and should have reduced impact on the at-risk natural populations.

Triploids have distinct physiological differences from diploid fishes. They have larger cells compared to diploids due to the increase in nuclear DNA, which maintains the cellular nuclear-to-cytoplasm ratio (Benfey, 1999). In general, this results in less cells per tissue since tissue size remains the same. However, specific modifications can occur to maintain tissue function, such as the maintenance of myofiber size in triploid zebrafish (*Danio rerio*; Small et al., 2024). The altered cellular profile has implications for tissues that rely on distinct cellular machinery for proper function. For example, endothelial thickness and gill surface area is greater in triploid fish which could impact oxygen diffusion (Sadler et al., 2001; Small et al., 2024). Triploids have fewer circulating erythrocytes since teleost erythrocytes are nucleated; thus, blood smears are often used to validate ploidy (Graham et al., 1985; Sadler et al., 2000; Small et al., 2024). Despite their increased size, triploid rainbow trout (*Oncorhynchus mykiss*) erythrocytes have similar oxygen affinity compared to diploids (Verhille and Farrell, 2012). When investigating ploidy differences, triploids have similar characteristics under optimal conditions. Salmonid studies have found that increased ploidy has little effect on aerobic capacity based on similar metabolic rate and maximum swimming speeds (Benfey et al., 1997; Bernier et al., 2004; Bowden et al., 2018a; Cotterell and Wardle, 2004; Small and Randall, 1989). Freshwater-phase Atlantic salmon diploids and triploids have similar proteomes when reared under ideal conditions (Nuez-Ortín et al., 2017). Additionally, in the absence of any stressors, triploid Brook trout (*Salvelinus fontinalis*) have similar anaerobic capacities based on their muscle lactate and

glycogen content (Hyndman et al., 2003a; Hyndman et al., 2003b). This consistent performance at baseline is validated in zebrafish, where housekeeping gene expression, developmental stage progression, and overall larval growth is not impacted by increased ploidy (Van De Pol et al., 2020). Therefore, rearing triploid fish would be more beneficial if farms can maintain constant conditions within their facilities.

However, there are major issues with triploid fishes that make them less profitable for aquaculture, especially as climate change progresses. Triploids have lower overall survival during early development (Cotter et al., 2002; Howard et al., 2023; O’Flynn et al., 1997), and higher incidences of skeletal deformities (Chalmers et al., 2020; Fjelldal and Hansen, 2010; Fraser et al., 2013; Sadler et al., 2001; Vera et al., 2019). Additionally, Triploids have reduced survival during saltwater transfer, an important life history event in salmonid aquaculture to produce market-quality fillets (Cotter et al., 2002; Johnson et al., 2019; O’Flynn et al., 1997; Shrimpton et al., 2012). Chronic high temperatures result in higher mortality in triploid stocks, which will only worsen in response to climate change (Hyndman et al., 2003b; Ojolick et al., 1995). With increases in temperature, hypoxic events will become more frequent and triploid fish have reduced survival in response to low oxygen concentrations (Ellis et al., 2013; Hansen et al., 2015; Jensen and Benfey, 2022; Sambafras et al., 2017; Scott et al., 2015). Triploid salmonids also have higher infection and mortality rates compared to diploids after bacterial, viral, and parasite exposure (Aunsmo et al., 2022; Ching et al., 2010; Jhingan et al., 2003; Ozerov et al., 2010; Weber et al., 2013). These issues make triploids less likely to be utilized in aquaculture since they are less likely to survive through the production cycle and the quality of fillets at the end will result in decreased profits for farmers (Madaro et al., 2021). Identifying potential molecular reasons for poor triploid performance can allow for development of therapeutics that will improve their survival and wellbeing. For the purpose of this thesis, triploid responses to pathogenic and thermal stress were focused on.

1.3 Teleost Immune Response

Water-borne pathogens are prevalent in marine habitats which can result in salmonids having to contend with both osmotic and pathogen stress during smoltification (Mauduit et al., 2022; Wang et al., 2023). The teleost immune response is robust and vigilant due to the constant assault from aquatic pathogens. Physical barriers act as the primary defense against infection through physical (i.e. mucus), chemical (i.e. altered pH) and biological (i.e. commensal bacteria) deterrents to pathogen

establishment (Fast et al., 2002; reviewed by Gomez et al., 2013). However, infection can occur if barrier integrity is impacted, either by damage to the organism itself or by pathogen-associated infection mechanisms (Chen et al., 2008; Evensen and Lorenzen, 1996; Sundh et al., 2019). When this occurs, cells can sense extracellular and intracellular pathogen associated molecular patterns (PAMPs) using pattern-recognition receptors (PRRs; reviewed by Poynter et al., 2015; Zhang et al., 2014). Signals, in the form of secreted extracellular proteins called cytokines, are recognized by immune cells to communicate the type of infection occurring (reviewed by Zou and Secombes, 2016). Chemokines are also secreted in a concentration-dependent manner to guide specific immune cells to infection sites (Lally et al., 2003; Tian et al., 2009). Humoral mediators (e.g. antibodies) and recruited immune cells then act to neutralize and clear (respectively) pathogens (Nuñez-Ortiz et al., 2018; Sakai, 1984). Repeated infections by the same pathogen can occur, so the adaptive immune response can be activated to provide a more specific response. In this system, major histocompatibility complex (MHC) cell surface proteins are used to communicate the specific type of pathogen present (Kandil et al., 1996; reviewed by Yamaguchi and Dijkstra, 2019). MHCs are loaded with either endogenous (class I) or exogenous (class II) peptides and presented to effector immune cells, allowing for more efficient clearing of pathogens (Chen et al., 2017; Dijkstra et al., 2013; reviewed by Wong-Benito et al., 2023). It is important to note that one of the proteins involved in MHC class II presentation (HLA-DM) in humans is absent in teleosts, so current understanding of how exogenous antigen presentation occurs is limited (Dijkstra et al., 2013). In addition, the adaptive immune response is less effective in fishes due to their lack of efficient antibody affinity maturation and reduced lymphocyte proliferation (Magnadóttir, 2006; Zapata et al., 1995). Adaptive antibody generation is delayed relative to other vertebrates, taking up to 4-6 weeks in salmonids while pathogen-associated mortality can occur within days of exposure (Ellis, 2001). Therefore, teleosts in general rely more extensively on their innate immune response to clear pathogens (Bromage et al., 2004; Ellis, 2001; Vigliano et al., 2006).

In teleosts, most tissues have a role in immune function. Nonetheless, there are specialized tissues that play similar roles to the well-studied mammalian immune system. The hindgut in teleosts harbors commensal bacteria that act to reduce pathogenic bacterial activity (reviewed by Langlois et al., 2021; Sadeghi et al., 2023). Additionally, some evidence exists for antigen sampling in hindgut similar to M cells in mammals and hosts resident lymphocytes (e.g. B & T cells) that are involved in mucosal immunity (Fuglem et al., 2010; reviewed by Stosik et al., 2023). The teleost head (or

anterior) kidney is the primary hematopoietic tissue that is analogous to the mammalian bone marrow (Zapata and Amemiya, 2000). This tissue also functions to clear pathogens and damaged cells from circulation and contains melano-macrophage centers (MMCs) which, in fish, contain antibody-producing cells (Ellis, 1980; Ferguson et al., 1982; Vigliano et al., 2006). Similarly, the teleost spleen contains resident macrophages that clear damaged erythrocytes and pathogens, to present antigens to T & B cells (Agius and Agbede, 1984; Ellis, 1980; Koppang et al., 2003; Koppang et al., 2010). Splenic MMCs have been found in other fish species, although salmonid spleens lack discrete centers and instead contain diffuse melano-macrophages (Agius and Roberts, 2003; Vigliano et al., 2006). The teleost gill, although providing similar respiratory function to the lung, is in constant contact with the pathogen-abundant external environment. T cells are present throughout the respiratory epithelium but also reside in aggregates called interbranchial lymphoid tissue which are involved in immune tolerance (Aas et al., 2017; Haugarvoll et al., 2008; Koppang et al., 2010). The teleost thymus is located within the opercular cavity and, similar to the mammalian thymus, is involved in T cell development (Koppang et al., 2010). Recent studies have identified that salmonids, similar to birds, have a bursa near their urogenital pore that likely functions in sampling and presenting water-borne pathogens (Løken et al., 2020). Nasopharynx-associated lymphoid tissue functions similarly by sampling antigens and producing antibodies against potential pathogens, and therefore is a potential site for immunization administration (Magadan et al., 2019).

It has been well established that triploid fishes have lower survival after infection compared to diploid fishes. Triploid Pacific salmon exposed to *Vibrio anguillarum*, a common infection in sea pens that causes vibriosis, showed increased mortality compared to diploid siblings (Ching et al., 2010; Jhingan et al., 2003). Similarly, triploid rainbow trout have higher mortality after exposure to bacterial cold-water disease (*Flavobacterium psychrophilum*; Weber et al., 2013a). Natural outbreaks of infectious salmonid anemia (ISA) caused higher mortality in triploid Atlantic salmon net pens and, at the infection peak, triploids had more viral RNA copies (Aunsmo et al., 2022). Triploids have also been found to be more susceptible to Salmon fluke (*Gyrodactylus salaris*), a parasite that reproduces quickly and can compromise osmoregulation in Atlantic salmon (Ozerov et al., 2010). This natural susceptibility of triploids has been observed within the intestinal microbiome, where triploid goldfish (*Carassius auratus*) had higher abundance *Vibrio* bacteria compared to diploids (Cai and Wei, 2023). Overall, these decreases in survival are due to triploids having compromised immune responses, as indicated by altered cellular mediators and transcriptional responses, which have been summarized in

Table 1.1. Further investigation of the transcriptional impacts of increased ploidy is necessary to understand why these fishes are more susceptible to disease-associated mortality.

Table 1.1: Compromised immune function in triploid fishes.

Summary table of studies that have shown altered immune function in triploids relative to diploid fishes. Rearing temperature (if reported in the study) is indicated. If fish were uninfected, a “-” was used in place of any infection. Acronyms: IP = intraperitoneal, LPS = lipopolysaccharide, il = interleukin, $\text{tgf}\beta$ = transforming growth factor β , IgM = immunoglobulin M, MHC = major histocompatibility complex.

Species	Rearing Temperature	Infection	Tissue	Immune impact in triploids	Reference
<i>Astyanax lacustris</i>	28°C	IP injection of <i>Aeromonas jandaei</i>	Spleen	Lower splenic lysozyme C and lysozyme G expression after infection	Meira et al., 2023
		-	Blood, head kidney, liver, and spleen	Lower circulating neutrophil counts; Lower splenic, head kidney, and liver expression of il-1 β , lower splenic expression of $\text{tgf}\beta$	Levy-Pereira et al., 2021
<i>Carassius auratus</i>	Not reported	-	Intestine	Reduced intestinal bacterial diversity, higher intestinal <i>Vibrio</i> abundance; altered tissue immune gene expression	Cai and Wei, 2023
<i>Oncorhynchus kisutch</i>	Not reported	IP injection of <i>Piscirickettsia salmonis</i>	Head kidney	Elevated il-10 expression early in infection	Correa et al., 2015
<i>Oncorhynchus mykiss</i>	12°C	-	Blood	Lower circulating neutrophil counts; Reduced thrombocyte aggregation capacity	Iunes et al., 2021a&b
	Not reported	-	Blood	Lower total number of leucocytes, lower neutrophil and monocyte counts	Yamamoto and Iida, 1994
<i>Oncorhynchus tshawytscha</i>	11.3°C	IP injection of <i>Vibrio anguillarum</i>	Head kidney	Reduced IgM and MHC class II expression; altered immune and inflammatory mRNA abundance	Ching et al., 2010
<i>Salmo salar</i>	12°C	IP injection of LPS	Serum	Reduced complement activity; delayed hypoferrremic response	Langston et al., 2001
<i>Salmo trutta caspius</i>	12-13°C	-	Blood	Lower proportion of circulating eosinophils, neutrophil, and large lymphocytes; Higher proportion of lymphocytes	Jamalzadeh et al., 2008

1.4 Teleost Thermal Responses

Climate change is impacting global temperatures, and all organisms are going to have to cope with more frequent thermal stress, especially ectothermic poikilotherms such as teleost fishes (Fry, 1971). Extreme weather events, like heat waves, are predicted to become more frequent unless atmospheric carbon levels and fossil fuel usage are reduced (Frölicher et al., 2018; Intergovernmental Panel on Climate Change (IPCC), 2022; Meehl and Tebaldi, 2004; Oliver et al., 2018). Aquatic organisms are able to adapt to gradual changes in temperature (Fry, 1947); therefore, yearly increases in mean temperature are slow enough for aquatic organisms to acclimatize to. However, acute changes in temperature are quick enough that acclimatization is not possible and can push fish past their physiological thermal tolerance (Schulte, 2024; Van Der Walt et al., 2021). Identifying the maximum water temperature associated with acute changes is ecologically relevant since this thermal point would be associated with organismal death should it be reached in the wild (Rezende et al., 2014). Critical thermal maximum (CT_{max}) studies utilize consistent increases in temperature to quantify the dynamic thermal tolerance of organisms, as indicated by the temperature associated with loss of equilibrium (Lutterschmidt and Hutchison, 1997). Additionally, fishes can recover from heat shock if returned to cooler waters, making this non-lethal endpoint ideal for reducing animal usage (Morgan et al., 2018).

The current scientific discourse on what governs this endpoint is heated (excuse the pun), but is thought to be species-specific, with some fish (including salmonids) CT_{max} being limited by oxygen delivery (Eliason et al., 2011; Pörtner and Knust, 2007). Despite these discrepancies, the physiological responses to acute changes in temperature are being clarified to understand how ectotherms maintain homeostasis in response to acute thermal swings. As temperatures increase, reaction rates for metabolic processes increase which results in elevated tissue oxygen demand (Fry, 1947; Somero, 1978). Fish can respond to this increased demand by increasing oxygen uptake through opercular ventilation rate and increasing tissue oxygen delivery by elevating heart rate (Farrell, 2009; Farrell et al., 1996; Gamperl et al., 2022; Heath and Hughes, 1973). Heart rate increases linearly with temperature until reaching a maximum heart rate, at which point cardiac function decreases before becoming arrhythmic prior to CT_{max} (Farrell et al., 1996; Heath and Hughes, 1973). The endpoint for CT_{max} is believed to be governed by this cardiac failure in addition to brain being unable to maintain neuronal function at high temperatures (Farrell, 2002; Friedlander et al.,

1976; Jutfelt et al., 2019; Robertson, 2004). In conjunction with these tissue responses, molecular dynamics are altered by temperature. Cells have to maintain function in spite of changes to protein structure, membrane fluidity, and chemical reaction rates (reviewed by Ern et al., 2023). Protein misfolding is particularly detrimental since, as they misfold at higher temperatures, they can produce aggregates which interfere with normal cellular function (Nguyen and Bensaude, 1994). Heat shock proteins (HSPs) are molecular chaperones upregulated as temperatures increase to correct misfolded proteins, prevent further protein denaturation, signal the presence of cellular damage, and potentially induce apoptosis if temperature-induced damage is too far progressed (reviewed by Roberts et al., 2010).

Considerable work has been conducted on thermal performance of triploids, although not as much investigation on the molecular responses of triploids to thermal stress. Most studies that investigate the effects of acute changes in temperature on triploids have identified that ploidy has little impact on CT_{max} (Benfey et al., 1997; Bowden et al., 2018b; Ellis et al., 2013; Galbreath et al., 2006; Scott et al., 2015). However, when oxygen levels decrease, triploid salmonids have reduced acute thermal tolerance and survival (Ellis et al., 2013; Hansen et al., 2015; Sambaous et al., 2017). Triploid salmonids also have higher mortality when exposed to chronic increases in temperature (Hyndman et al., 2003b; Ojolic et al., 1995) which is believed to be partially attributable to their lower thermal optima (Altimiras et al., 2002; Atkins and Benfey, 2008; O'Donnell et al., 2017). Another potential source of this reduced performance occurs at the cardiac level. Triploid rainbow trout hearts exposed to an acute thermal challenge became arrhythmic at lower temperatures compared to diploids (Verhille et al., 2013). The reasons for this reduced performance at high temperature are still unclear but work on the molecular mechanisms underlying these thermal challenges could provide insight. For example, triploid erythrocytes have lower heat shock protein abundance and, when exposed to an acute heat stress, experienced higher protein breakdown relative to diploids (Saranyan et al., 2017). Further investigation of the molecular impacts of increased ploidy after thermal stress will offer clarity on the reasons for poor thermal performance.

1.5 MicroRNA

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate translation of specific messenger RNA (mRNA) targets. The primary miRNA is transcribed and forms a stem-loop that is cleaved within the nucleus by the enzyme Droscha to produce the precursor miRNA, before being

transported to the cytosol where the enzyme Dicer produces the miRNA duplex (Hutvágner et al., 2001; Kim et al., 2016; Lee et al., 2003). Finally, one strand of this duplex gets degraded (called the miRNA* or passenger strand) while the other (called the mature miRNA or guide strand) is maintained in the cytosol (Khvorova et al., 2003; Lau et al., 2001; Schwarz et al., 2003). This mature miRNA is ~22 nucleotides-long and associates with proteins to become an RNA-induced silencing complex (RISC) which can bind to 3' untranslated regions (UTRs) of specific mRNA molecules and either degrade the mRNA or repress translation (**Figure 1.1**; Bartel, 2004; Lee et al., 1993; Wightman et al., 1993). One of these proteins is argonaut 2 (Ago2; also called slicer) which both binds to the miRNA and acts as the catalytic subunit involved in mRNA degradation (Krol et al., 2010; Lee et al., 2002; Peters and Meister, 2007). This machinery can act as a powerful inducible regulatory mechanism since miRNAs only require 7-8 nucleotide complementarity with the mRNA 3' UTR to inhibit translation (Bartel, 2009; Eichhorn et al., 2014; Kehl et al., 2017). MicroRNAs can be produced rapidly and are more stable than other RNA molecules, making them important regulators of protein synthesis when conditions are unfavourable (Reichholf et al., 2019). Additionally, this stability means that they can act as a translational buffer for their targets where mRNA abundance needs to surpass a threshold before proteins can be produced (Mukherji et al., 2011). Therefore, they offer a method to fine tune translational mechanics when organisms are experiencing stress.

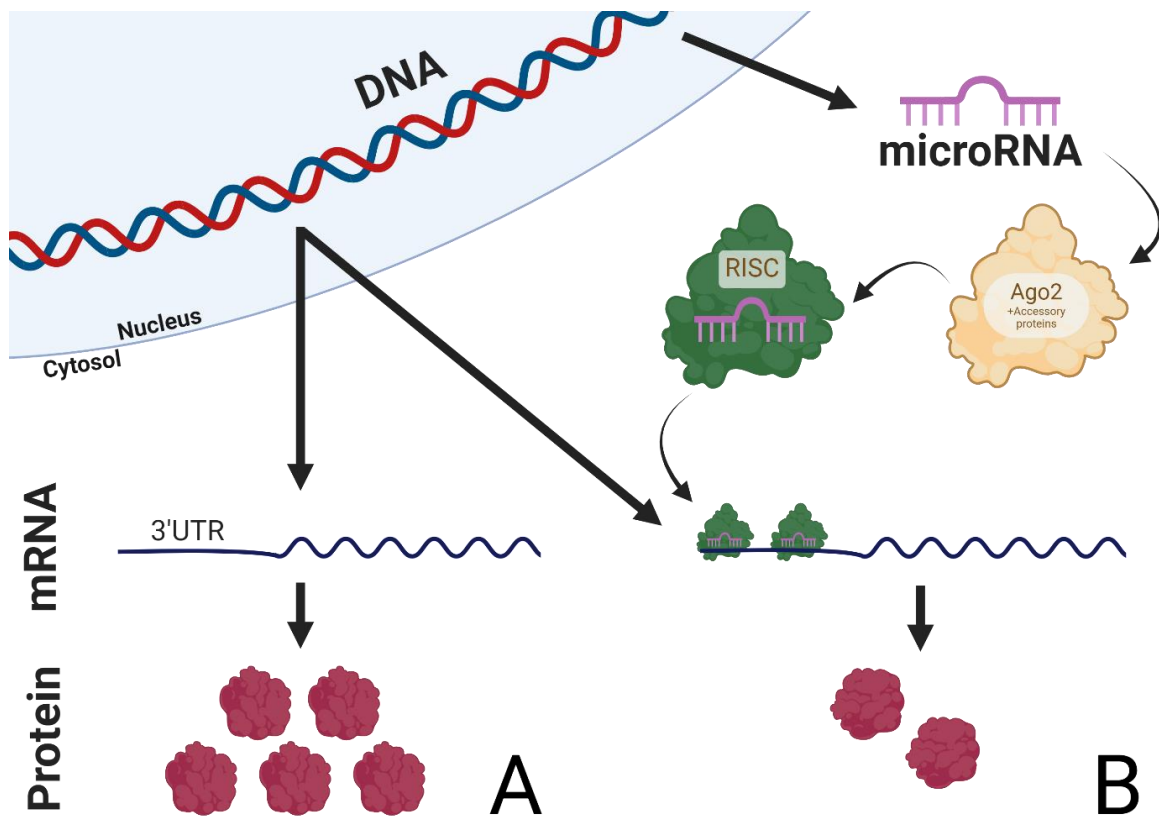


Figure 1.1: Regulation of mRNA translation by microRNA.

Within the nucleus, DNA is transcribed into messenger RNA (mRNA) which is shuttled to the cytosol where it is translated into a functional protein (A). Translation can be impacted by microRNA (miRNA), which are also transcribed by DNA and, after moving to the cytosol and being incorporated into the RNA induced silencing complex (RISC), bind to the 3' untranslated region of specific mRNA molecules (B). This results in either reduced translational efficiency or degradation of the mRNA transcript. Figure generated in BioRender.

Mature miRNA sequences are highly conserved across evolution, although the 3' UTRs that they target tend to change more readily between species (Hertel and Stadler, 2015; Johnston et al., 2019; Simkin et al., 2020; Xu et al., 2013). Therefore, relationships between miRNA and their target mRNAs have to be predicted and validated for non-model species to understand what changes in their abundance could mean physiologically. MicroRNAs have important roles in regulating differentiation during teleost development as indicated by the model organism zebrafish (Wienholds et al., 2005). Rearing temperature can expedite teleost development, and in European Sea bass (*Dicentrarchus labrax*) miRNA involved in reproduction and immune responses are dysregulated at higher

temperatures (Papadaki et al., 2022). Most importantly for this thesis, miRNAs are established regulators of teleost stress responses. Recovery after air exposure and handling, common stressors experienced during aquaculture when moving fish between systems, alter miRNA abundances in stress responsive tissue, blood plasma, mucus, and the surrounding water (Cadonic et al., 2020; Ikert et al., 2021). Chronic exposure of zebrafish to thermal, hypoxic, and pharmaceutical stress causes altered miRNA and mRNA abundances two generations after exposure (Luu et al., 2021). Hypoxic stress alters liver miRNA abundances in largemouth bass (*Micropterus salmoides*) and rainbow trout which act to support the cellular response to low oxygen (Sun et al., 2020; Wu et al., 2024). Overall, these studies validate the importance of small non-coding RNA in stress responses; however, few studies have investigated whether the poor stress tolerance of triploid fishes is related to altered miRNA abundances. Indeed, prior to this thesis, the only studies investigating miRNA in triploid fishes have investigated their altered profile in gonads or the epigenetic impacts of increased ploidy in the absence of stress (Huang et al., 2021; Li et al., 2022; Ren et al., 2022; Tao et al., 2018; Wang et al., 2020b; Zhou et al., 2021b). Investigation of whether altered abundance in response to pathogen or thermal stress would provide clarity on why these fishes perform worse when conditions are poor.

1.5.1 MicroRNAs and Immune Function

MicroRNAs are important regulators of immune function across evolution since their ability to negatively regulate protein abundance can support the activation and deactivation of immune responses. Processes such as inflammation are controlled by miRNAs to support the return to homeostasis after removal of pathogens (reviewed by Alam and O'Neill, 2011). MicroRNAs also regulate innate and adaptive immune responses through pathways such as toll-like receptor (TLR) signalling, cytokine signalling, and stress-induced cell signals (Fayyad-Kazan et al., 2014; Ma et al., 2011; Perry et al., 2008; Quinn and O'Neill, 2011; Sheedy et al., 2010; Stern-Ginossar et al., 2008). Nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), a transcription factor that activates inflammatory gene expression, upregulates miR-146 to prevent excessive inflammation (Perry et al., 2008; Taganov et al., 2006). Immune cell fate is regulated by miRNAs, supporting the specificity of the immune response (Cobb et al., 2005; Muljo et al., 2005; O'Connell et al., 2010; Shapouri-Moghaddam et al., 2018; Vigorito et al., 2007). Finally, miRNAs can act as direct effectors of immune function by communicating between cells and directly targeting pathogenic transcripts (Jopling et al., 2005; Mittelbrunn et al., 2011; Rezende et al., 2023; Saçar Demirci and Adan, 2020;

Zhang et al., 2018). These immune networks are tightly controlled, and dysregulation of miRNAs has been identified as causing human autoimmune diseases (Kalla et al., 2015; Rodriguez et al., 2007).

Studies in fish have validated that infection alters miRNA abundance to manipulate the immune response. The development of transcriptomic studies has been paramount in identifying specific miRNAs that are altered by infection. These techniques can be utilized on non-model species since they only require a sequenced genome. As such, pathogen challenge studies that utilize transcriptomics have shown that miRNA abundances are altered after infection in a time- and organ-dependent manner (Eslamloo et al., 2018; Gao et al., 2019; Gong et al., 2015; Najib et al., 2016; Ning and Sun, 2020; Salazar et al., 2020; Sha et al., 2014; Wang et al., 2020a; Zhang et al., 2023; Zhou et al., 2021a). Sequencing of miRNA has been combined with other transcriptomic techniques (mRNA, long non-coding RNA, circular RNA) to get a more complete view of the molecular changes involved in regulating immune function in teleosts (Gao et al., 2022; Ramberg et al., 2022; Sun et al., 2023; Wang et al., 2020a; Zhou et al., 2020). In general, transcriptomic studies have shown that specific microRNAs downregulated early in the infection are involved in activating immune function (inflammation, TLR signalling, cytokine and chemokine production, etc.), while miRNAs downregulated late in the infection are involved in regulating inhibitory factors important for the return to homeostasis. However, in another study, Salmonid alphavirus infection in Atlantic salmon demonstrated an increase in 20 different miRNAs that target inflammatory response genes (Andreassen et al., 2017). This study showed that immune regulation by miRNA is complex, infection dependent, and likely involved in preventing excessive inflammation. Recent studies have started to validate the transcriptomic-predicted immune role of miRNAs by confirming direct transcript 3'UTR regulation. These studies have shown that teleost miRNAs regulate TLR signalling (Bi et al., 2017; Chu et al., 2017b; Wang et al., 2016), complement activity (Liu et al., 2023), cytokine signalling (Chu and Xu, 2016; Guan et al., 2022; Nie et al., 2019; Sun et al., 2022; Yu et al., 2024), and different points in the NF- κ B signalling pathway (Chu et al., 2017a; Chu et al., 2019; Cui et al., 2022; Lv et al., 2022; Sun et al., 2021; Wang et al., 2024; Yang et al., 2022a; Yang et al., 2022b). These validation studies are important to allow for mapping out immune networks and the complex interactions that are involved in combatting pathogens. However, similar to mammals, teleost pathogens have evolved ways to utilize aspects of this network. Infectious hematopoietic necrosis virus (IHNV), grass carp reovirus (GCRV), and *Edwardsiella tarda* alter miRNA abundances in teleost tissues to support their pathogenicity (Huang et al., 2024; Li et al., 2020a; Li et

al., 2020b; Li et al., 2023a). Even so, this extensive species-specific validation of miRNA function provides insight into regulation of immune response and potential targets for therapeutics.

MicroRNA-based therapy has already been utilized to improve health and survival in grass carp (*Ctenopharyngodon idella*; Lu et al., 2021). Therefore, identification of how ploidy impacts miRNA abundances during infection could provide targets for therapeutic development, eventually resulting in improved health for aquaculture stocking of these fishes.

1.5.2 MicroRNAs and Thermal Exposure

Temperature is an important regulator of nucleotide stability as high temperatures cause denaturation of nucleic acid duplexes. Nucleotide binding is impacted by temperature and therefore ectothermic poikilotherms have altered miRNA sequence parameters to improve thermodynamic base-pairing across temperature ranges (Biggar and Storey, 2015; Carmel et al., 2012; Hibio et al., 2012).

Although this can complicate how miRNAs function, their role in regulating cellular responses is well characterized. The cellular response to heat is also regulated by miRNA, as has been shown across taxonomic groups encompassing ectothermic and endothermic organisms (Funikov et al., 2016; Hao et al., 2016; Nehammer et al., 2015; Okusha et al., 2022; Permenter et al., 2019). Transcriptomic studies have supported this role as miRNA abundances are altered during thermal acclimation and after thermal stress in teleosts (Huang et al., 2018; Li et al., 2017; Sun et al., 2018; Zhang et al., 2017). Additionally, chronic heat exposure (4°C) of the Emerald rockcod (*Trematomus bernacchii*), a stenothermic Antarctic fish that is used to seawater temperatures -1.5°C, resulted in altered gill expression of miRNA associated with cellular stress (Vasadia et al., 2019). Similar to the immune role of miRNAs, recent work has begun to validate direct miRNA-mRNA relationships. Rainbow trout and Japanese flounder (*Paralichthys olivaceus*) microRNAs can also directly target Hsp90 transcripts, making them important regulators of the cellular heat shock response (Li et al., 2023b; Liu et al., 2022; Zhao et al., 2023). As described, cardiac function is involved in teleost thermal tolerance and miRNAs have been confirmed to regulate heart gene expression. The gene regulatory networks between miRNA and core cardiac genes are conserved across evolution (Nachtigall et al., 2021). Teleost cardiac plasticity to temperature may also be regulated by miRNAs. Specifically, rainbow trout miR-29b-3p regulates heart collagen abundance (Johnston et al., 2019). Collagen content in fish hearts changes with thermal acclimation, implicating this miRNA in thermal adaptation for its involvement in collagen remodeling (Johnston and Gillis, 2022). Overall, it is clear that miRNA act as regulators of thermal stress and cardiac function but currently no studies have

investigated whether their altered abundance could be involved in reduced thermal performance of triploids.

1.6 Objectives & Hypotheses

The overall objective of this thesis was to investigate the molecular dysregulation associated with different stressors in diploid and triploid Chinook salmon. Specifically, I hypothesize that **diploid and triploid salmonids have subtle differences in molecular (miRNA, mRNA) indices that are exacerbated by changes in the environment (pathogens and temperature), which results in their poorer performance in response to stress.** I chose two stressors that were easy to conduct in the field setting where our fish were held – a pathogen challenge (*Vibrio anguillarum*) and an acute thermal challenge.

The objective for **Chapter 2** was to identify which immune tissue and which miRNA could be involved in poor immune performance of diploid and triploids. Hindgut, head kidney, and spleen were collected before infection and after 3-days of *Vibrio anguillarum* exposure to assess whether miRNAs were altered by ploidy or infection. The phenotypic effects of miRNA changes were predicted bioinformatically to understand how gene regulation changed between the groups. This chapter provides evidence for the role miRNA play in the triploid immunocompromised phenotype.

The objective for **Chapter 3** was to investigate immune gene expression in diploids and triploids before and after an acute temperature exposure. Gill and cardiac ventricle tissue were collected before and after 1-hour of recovery from CT_{max} to assess whether ploidy impacted immune gene expression during a cellular heat shock response. This chapter provides evidence that recovery from acute thermal stress impacts immune gene expression, and that altered expression in triploid fishes could implicate them having reduced survival after acute changes in temperature.

Finally, the objective for **Chapter 4** was to examine the cardiac response to acute temperature exposure in diploid and triploid Chinook salmon to provide insight regarding why they fail. Thermal tolerance of diploid and triploid salmon was assessed and the cardiac performance during thermal challenge reported. Additionally, cardiac atrium and ventricle gene expression was assessed to investigate the cardiorespiratory repercussions of increased ploidy. This chapter provides evidence that triploid hearts have hindered function in response to acute increases in temperature and provides molecular evidence for why this cardiac failure occurs.

Chapter 2

Diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha*) have altered microRNA responses in immune tissues after infection with *Vibrio anguillarum*

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2.1 Introduction

The usage of sterile fish in farming is one method to reduce the impact that aquaculture has on the natural environment. For salmon, sterility can be induced through production of triploid offspring using high pressure or high temperature to induce retention of a second set of maternal chromosomes (Benfey, 1999; Johnson et al., 2004). These progeny will have a reduced genetic impact on wild populations should they escape and are less likely to successfully migrate into spawning habitats (Benfey, 1999; Glover et al., 2016). When environmental conditions are maintained consistently, triploid fish perform similarly to diploid fish (Nuez-Ortín et al., 2017; O’Flynn, 1997). However, triploids overall have reduced developmental success and poor performance in stressful environments, making their widespread implementation in aquaculture less profitable (Cotter et al., 2002; Fjelldal and Hansen, 2010; Johnson et al., 2019; Madaro et al., 2021; O’Flynn, 1997; Ozerov et al., 2010; Shrimpton et al., 2012).

Maintaining fish health in aquaculture is vital since diseases can spread rapidly and decimate stocks. Teleost species that have marine life stages, such as Chinook salmon (*Oncorhynchus tshawytscha*), are particularly susceptible due to the high prevalence of pathogens in saltwater habitats (Emmenegger et al., 2013; Mauduit et al., 2022; Thompson et al., 2004). Therefore, salmon are vaccinated prior to net pen transfer to protect them from high mortality during this stressful event. Vibriosis, caused by *Vibrio anguillarum*, is one such disease that aquaculture stocks are vaccinated against due to its high mortality rates (Frans et al., 2011). *Vibrio anguillarum* is a gram-negative opportunistic bacterium that causes hemorrhagic septicemia, diagnosed through symptoms such as skin lesions and inflammation along ventral and lateral surfaces, and eventual mortality (Frans et al., 2011; Thompson et al., 2004; Toranzo et al., 2005). The teleost immune system responds to infection by secreting cytokines to activate cells that can clear bacteria and infected cells directly or by secreting humoral mediators (Mashoof and Criscitiello, 2016; Nakanishi et al., 2015; Uribe et al., 2011; Whitehouse and Dixon, 2019). Vibriosis is problematic for triploid fish since bacterial infections have been associated with reduced survival compared to diploids (Ching et al., 2010; Jhingan et al., 2003; Weber et al., 2013). Increased ploidy has been associated with altered complement activity, antioxidant capacity, and leukocyte numbers which could explain observed differences in survival (Iunes et al., 2021a; Langston et al., 2001; Rodrigues et al., 2020; Xiong et al., 2021). Transcriptional differences have been observed in response to other pathogens, indicating that triploids have altered expression of immune genes during infection (Cai and Wei, 2023; Correa et al.,

2015). Additionally, modest transcriptional differences in triploids within the first three days of infection have been associated with higher *Vibrio*-induced mortality (Ching et al., 2010). Therefore, elucidating molecular dysregulation in triploids would inform potential therapeutic options to prevent infection-associated loss in triploid stocks.

The immune system is tightly regulated to prevent excessive tissue damage through inflammation and cellular mediators. MicroRNA (miRNA) are well-established epigenetic effectors of the immune system that regulate host and pathogen gene expression in a time dependant manner and manipulate cellular fate to produce effector cells ideal for specific infections (Cobb et al., 2005; Jopling et al., 2005; Lu et al., 2010; Vigorito et al., 2007). These small, 22-base pair (bp) RNA molecules regulate protein expression by binding to the 3' untranslated region (UTR) of mRNA transcripts to inhibit translation or induce mRNA degradation (Lee et al., 1993; Wightman et al., 1993; Zeng et al., 2003). Studies in teleosts concur with the human and mammalian literature, indicating that the immune role of miRNA is evolutionarily conserved (Chu and Xu, 2016; Guan et al., 2022; Liu et al., 2019; Zhang et al., 2022). Sequencing-based studies have shown that changes in miRNA abundance are time- and tissue-dependent, with changes in miRNA abundance identified from hours to weeks after pathogen exposure (Andreassen et al., 2017; Bai et al., 2020; Eslamloo et al., 2018; Gao et al., 2019; Ning and Sun, 2020; Sha et al., 2014). Currently, the only studies in teleosts investigating the impact of increased ploidy on miRNA expression have shown that gonadal expression is altered between diploids and triploids (Huang et al., 2021; Wang et al., 2020). It is unknown whether miRNA regulatory networks vital for proper immune function are altered in triploid fish, and how this relates to established reduced immune performance of triploid fishes.

The objective of this study was to compare miRNA expression between diploid and triploid Chinook salmon after infection with *Vibrio anguillarum*. Additionally, this study compared expression between immune tissues to identify miRNAs that respond to infection and whether changes in expression relate to established immune deficiencies in triploids. We hypothesized that increased ploidy results in altered miRNA profiles in immune tissues and the miRNA identified regulate the immune response. We further hypothesized that diploids and triploids have greater differences in miRNA expression just prior to mortality compared to uninfected fish. To test these hypotheses, Next Generation Sequencing (NGS) was used to quantify miRNA abundance from hindgut, head kidney, and spleen before (Day 0) and after infection, but prior to initial mortalities (Day 3). Full-sibling diploid and triploid fish were used to reduce the effect of genotype on immune

function. Comparisons in miRNA gene expression were made between ploidies as well as how each ploidy responded over the three days of infection. Differentially expressed miRNA were identified bioinformatically and validated using qPCR. Finally, mRNA targets of differentially expressed miRNA were analyzed to predict the phenotypic impacts on KEGG pathways.

2.2 Methods

2.2.1 Ploidy induction and rearing

For this study, full-sibling diploid and triploid Chinook salmon were produced at Yellow Island Aquaculture (YIAL, British Columbia, Canada) following the protocol as previously described (Johnson et al., 2004). Briefly, eggs from female Chinook were artificially inseminated from a phenotypic male (hormonally masculinized genotypic female). At 2 minutes post-fertilization, hatchery water (7.5°C) was added and at 30 minutes post-fertilization, half of the eggs were directly transferred to vertical incubation trays (Heath Tecna) while the other half was subjected to 10 000 psi of pressure for 5 minutes to induce retention of the second polar body. This triploid subset was transferred to an adjacent vertical incubation tray to prevent mixing with diploid siblings. Individual trays in the stack were separated into 12 compartments to allow for family identification until the alevin stage, at which point diploids and triploids from each family were transferred to separate 200 L barrels at 8°C. The maximum density for this stage was 200 fish/barrel (0.05 kg/m³), but actual barrel densities depended on family survival to parr stage. However, this density is far lower than those expected to cause negative impacts to growth (Rosburg & Barnes, 2024). To reduce genotype impacts on immune responses, only one family (full-sibling diploids and triploids) was utilized for this infection trial. Tanks were checked daily for mortalities and fish were hand fed twice daily using commercial feed (Taplow Feeds, British Columbia, Canada). For this trial, sampled diploids had a mean weight of 12.30 ± 0.44 g and length of 10.8 ± 0.1 cm. Sampled triploids had a mean weight of 13.73 ± 1.06 g and length of 11.3 ± 0.3 cm. All animals use and handling protocols followed the Canadian Council of Animal Care guidelines and were approved by the University of Waterloo's Animal Care Committee (AUPP# 43212).

2.2.2 *Vibrio anguillarum* infection

Full-sibling diploid (n = 37) and triploid (n = 41) fish were transferred to separate 200 L tanks (8°C) and allowed to acclimate for 4 weeks prior to the start of the trial. Unequal numbers within each ploidy were due to human error during transfer. Ten fish per ploidy were sampled to collect tissues (head kidney, spleen, and hindgut) prior to infection. Diluted clove oil (0.4 mL/L of water; Sigma-Aldrich, C8392) was used to terminally anesthetize fish and blood collected via caudal vein using heparinized capillary tubes. Blood was transferred to a cold 1.5 mL tube containing 4 U of heparin (Sigma-Aldrich, H3149) and kept on ice for ploidy confirmation. Hindgut, spleen, and head kidney tissue were placed in individual tin foil packets before flash freezing at approximately -200°C in a portable dry shipper (VWR, 55709-232).

The rest of the cohort of fish were then exposed to the pathogen to simulate an outbreak of *Vibrio anguillarum*. Individuals were sedated using diluted clove oil (0.1 mL/L of water) and received a 100 µL intraperitoneal injection (IP) of 4.0×10^5 CFU/mL of *V. anguillarum* resuspended in sterile phosphate buffered saline (PBS). Fish were then moved to a recovery bath of aerated hatchery water (10°C) before transfer back to the original barrels. Concurrent with this infection, a mixed subset of diploid (n = 100) and triploid (n = 100) fish were IP injected with sterile PBS to account for injection associated mortality. These injection control fish included extra families since they were also utilized for a subsequent, larger scale study but were maintained under similar rearing conditions within the same facility. Barrels were monitored for 17 days and mortalities recorded twice per day. Over the 17-day infection, no mortalities occurred in the injection control group. IP injection of the *Vibrio* strain used in this study causes mortalities around day 4 (Semple et al., 2022). Therefore, 10 diploid and 10 triploid fish were sampled on day 3 using the same protocol described above to assess molecular differences prior to *Vibrio*-induced mortality.

2.2.3 Ploidy confirmation

Fish ploidy was assessed by measuring the DNA content and erythrocyte cell size using flow cytometry and an adapted protocol (Glover et al., 2016). Briefly, blood from eight fish per ploidy at each timepoint (total n = 16 per ploidy) was treated with heparin and kept on ice until sampling was complete. After sampling, heparin-treated blood was fixed using 4% paraformaldehyde solution for 60 minutes before washing cells with sterile PBS three times. Erythrocyte DNA was then stained overnight at room temperature using a PBS buffer containing 50 µg/mL propidium iodide (Sigma,

P4864), 50 µg/mL RNase A (Millipore-Sigma, 70856), 0.3% Tween-20 (Fisher Scientific, BP337-100). Stained erythrocytes were diluted 1:30 and media measured using the Moxi Flow cytometer (ORFLO, MXF002) and MF-S cassettes (MXC020). Each sample was gated to remove cell debris and unstained cells to generate a median fluorescence intensity (MFI) and an average cell size (µm) for each fish. This analysis only included the fish within the infection trial (either Day 0 or Day 3) and did not include a subset of fish from the PBS injected group due to insufficient flow cytometry cassettes.

2.2.4 RNA extraction and small RNA sequencing

RNA was extracted from the hindgut, head kidney, and spleen of diploids and triploids before (Day 0) and after (Day 3) infection using the *mirVana* miRNA Isolation Kit (Ambion, AM1560/1561). Tissue was ground to a powder over liquid nitrogen before following the manufacturer's protocol without deviation. RNA integrity numbers (RIN) values for each sample was assessed using the Agilent 2200 TapeStation (Agilent) and only samples with RIN values ≥ 7.0 were used in this study.

Library preparation and sequencing was conducted by The Centre for Applied Genomics (TCAG, The Hospital for Sick Children, Toronto, Ontario). In total, hindgut (n=6), head kidney (n=4), and spleen (n=4) samples for each ploidy at each timepoint were chosen to continue on to library prep based on highest RIN values. Head kidney and spleen had lower sample sizes because they were run within the same sequencing run and therefore less samples for each group were utilized to maintain sequencing depth within each sample. The NEBNext Small RNA Library Prep (New England Biolabs, E7330) was used to generate libraries for each sample with RNA inputs depending on sample concentration (300 ng for hindgut and 100 ng for spleen/head kidney). Single-end sequencing was conducted on Novaseq 6000 (Illumina) with a minimum coverage of 10 million reads per sample. Raw read datafiles for all samples have been submitted to the NCBI Expression Omnibus ([GSE230701](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE230701)).

2.2.5 Bioinformatics analysis of small RNA sequencing

Analysis of reads was carried out in a similar protocol to Ikert et al., 2021. Initially, raw reads underwent quality control using FastQC (version 0.11.8) to confirm that files had high quality small RNA sequencing reads (Andrews, 2010). Reads were then run through Cutadapt (version 3.4 in Python 3.6.7) to remove the sequencing adapter, trim low-quality bases and reads, and remove reads larger than 35 bp and smaller than 18 bp.

Quality trimmed reads were then analyzed using the miARma-seq pipeline (v1.7.2) to generate read counts for each miRNA genome coordinate (Andrés-León et al., 2016). The Known miRNA approach was used for this study since the newest Ensembl genome assembly (Otsh_v1.0; GCA_002872995.1) has a .gff3 file (Oncorhynchus_tshawytscha.Otsh_v1.0.105.gff3.gz) that contains Chinook salmon genome coordinates for miRNAs. Therefore, Bowtie1 (version 1.1.2) aligned reads to the generated index for Ensembl release 105, Otsh_v1.0 (<https://doi.org/10.6084/m9.figshare.22802639>). FeatureCounts (version 1.5.0-pl) was then used to generate a read count matrix containing the number of reads at each miRNA genome coordinate for each individual (Liao et al., 2014).

Differential expression of miRNAs was completed using DESeq2 (version 1.34.0) since it has been shown to be more accurate for low replicate values when compared to other tools (Love et al., 2014; Schurch et al., 2016). All code was run in R (version 4.1.3) and each tissue (hindgut, head kidney, spleen) was run independently to establish differential expression profiles over the infection. Hindgut, spleen, and head kidney files for comparison A (diploid day 0 compared to triploid day 0), comparison B (diploid day 0 compared to diploid day 3), comparison C (triploid day 0 compared to triploid day 3), and comparison D (diploid day 3 compared to triploid day 3) can be found in supplemental materials.

MicroRNA genome coordinates with adjusted-*p* values < 0.05 for each comparison were identified as differentially expressed and their identity manually confirmed. Mature miRNA are generated from a precursor miRNA transcript stem loop that is cleaved by Dicer to produce two mature transcripts, named -3p or -5p depending on their origin on the precursor transcript (Hutvágner et al., 2001). One of these transcripts will be loaded into the RNA induced silencing complex (RISC) and become the mature miRNA (also called the guide strand) while the other (termed the miRNA* or passenger strand) is usually degraded (Bartel, 2004; Ha and Kim, 2014; Khvorova et al., 2003; Lau et al., 2001; Schwarz et al., 2003). Therefore, the reads associated with differentially expressed miRNA coordinates were parsed to identify the biologically active mature miRNA using Samtools (version 0.1.19; Li et al., 2009). Finally, the identity of the mature miRNA was identified by blasting the highest expressed read in miRbase for *Salmo salar* (Ambros et al., 2003; Griffiths-Jones et al., 2008, 2006).

The predicted phenotypic effects of these changes in miRNA were then analyzed by investigating the genes that are expected to be regulated by the identified mature miRNA. For this

analysis, *Salmo salar* genes were analyzed since they are the closest related teleost species compatible with the KEGG database. The canonical method that miRNA use to regulate protein expression is through binding to the binding sites in the 3' UTR of specific gene transcripts (Bartel, 2004). Therefore, differentially expressed miRNA for each comparison in each tissue were grouped and compared against the *Salmo salar* 3' UTR transcriptome acquired from Ensembl Biomart (Cunningham et al., 2022). Each miRNA list was analyzed using miRanda (version v3.3a), an algorithm that is able to predict miRNA binding sites based on sequence complementarity and energetically favourable binding between the RNA transcripts (Enright et al., 2003). Stringent cut-offs for alignment score (140) and minimum free energy (-20 kcal/mol) based on previous studies were used to reduce false positive predictions (Cadonic et al., 2020; Kostyniuk et al., 2019). Gene lists for each comparison in each immune tissue were then analyzed using KOBAS (version 3.0.3) to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways significantly impacted by changes in miRNA abundance (Bu et al., 2021; Kanehisa and Sato, 2020).

Examples of the miARma-seq configuration file, logfiles and stats files for each tissue, R code for DESeq2, the *Salmo salar* 3' UTR file, and a more in-depth description of the bioinformatics analysis can be found here (<https://doi.org/10.6084/m9.figshare.22802816>).

2.2.6 qPCR validation of small RNA sequencing

Six samples from each treatment for each tissue were used to validate the sequencing results and increase sample size for the head kidney and spleen samples. RNA samples were thawed over ice and cDNA generated using the Agilent miRNA 1st-Strand cDNA Synthesis Kit (Agilent, 600036). The protocol was followed without deviation using 500 ng of RNA for the polyadenylation reaction. A pooled sample was generated to test primer efficiency and diluted to generate a 7-point standard curve. Each cDNA sample was diluted 15x per manufacturer protocol and stored at -20°C until subsequent analysis. All qPCR reactions were conducted using a CFX96 Touch Real-Time PCR Detection System (Biorad). Each 10 µL PCR reaction contained 3 µL of cDNA, 1 µL of 3.125 µM forward primer, 1 µL of 3.125 µM reverse primer or Universal Reverse Primer (Agilent, 600037), and 5 µL of SsoAdvanced SYBR Green Master Mix (Biorad, 1725274). qPCR runs were initiated by polymerase activation at 95°C for 30 seconds, before running 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 15 seconds. Non-template controls were run on each plate to detect potential contamination. Each cycle ended with a plate read and after 40 cycles a melt curve (65°C to 95°C in 0.5°C increments) was conducted to confirm that only one PCR product was

generated. Relative abundance was calculated using the normalized expression ($\Delta\Delta C_q$ equation) in CFX Maestro 2.3 (Biorad, version 5.3.022.1030) with 18s rRNA and elongation factor 1 α (EF1 α) as housekeeping genes. For all three tissues, housekeeping gene stability was assessed using the same software and determined to be stable. Primer sequences, M scores, primer efficiencies, and r^2 values are presented in **Table 2.1**.

2.2.7 Statistical analysis

Differential expression of sequencing results was completed using R (version 4.1.3) and DESeq2 (version 1.34.0) as described above. Graphpad Prism (version 9.5.0) was used for all other statistical analysis and graph generation. Figures represent mean $\pm$ standard error of the mean (SEM) and the standard significance level of $p \leq 0.05$ used for all statistical tests. Normality of residuals was assessed for all statistical tests and if found to be not normal, initial values transformed to allow for parametric analysis. Flow cytometry results were analyzed using a two-tailed T test to determine whether cell size (μm) and DNA content (MFI) was different between diploids and triploids. Mortality curves were generated to account for sampling by subtracting half of the withdrawals from the total number of fish initially infected (Martin et al., 1987). A Mantel-Cox test was then used to assess whether there were differences between diploid and triploid survival and Fisher's exact test used to determine differences in mortality at each day of the experiment. Sequencing metadata was initially analyzed using a Two-Way ANOVA to test for differences in ploidy and tissue. A One-Way ANOVA was then used to determine differences in sequencing metadata between tissues. qPCR validation of differentially expressed miRNA from sequencing were analyzed using a Two-Way ANOVA and if significant, a Tukey post-hoc test was run to determine which groups were statistically different. Finally, Fisher's exact test and Benjamini & Hochberg FDR correction was used to identify significantly impacted KEGG pathways by altered miRNA expression.

2.3 Results

2.3.1 Validation of Ploidy

Flow cytometry of erythrocytes from fish sampled at Day 0 and Day 3 was used to confirm ploidy of the two groups used in this trial. Sampled triploid fish had significantly larger erythrocytes compared to diploid fish (two-tailed t -test, $t_{30} = 4.390$, $p = 0.0001$). Additionally, DNA content (based on

propidium iodide fluorescence) was significantly higher in sampled triploid fish compared to sampled diploid fish (two-tailed t -test, $t_{30} = 3.391$, $p = 0.0020$).

2.3.2 Survival of Diploids and Triploids with live *Vibrio anguillarum* challenge

For this study, full-sibling Chinook salmon were assessed to determine whether increased ploidy impacted survival in response to an immune challenge (*Vibrio anguillarum*). Each fish received an IP injection of approximately 40 000 bacteria and mortality was monitored for 17 days. Overall, the diploid group had a survival of 45.45% while the triploid group had an overall survival of 34.60% (**Figure 2.1**). However, there was no difference in survival between the two groups over the entire infection (Mantel-Cox, $\chi^2 = 1.218$, $df = 1$, $p = 0.2697$) or between ploidies at each day after infection (Fisher's exact test, $p > 0.05$).

2.3.3 Sequencing metadata

Illumina high throughput small RNA sequencing was used to generate profiles of small RNA expression from the same fish across three separate tissues. Ploidy did not have an effect on any of the metadata parameters discussed (Two-Way ANOVA, $df = 1$, 50, $p > 0.05$), so all statistics were run to compare differences in sequencing metadata between tissues. Overall, an average of 14 million reads were generated and there was no difference between the tissues (**Figure 2.2A**; One-Way ANOVA, $F_{2,53} = 0.4417$, $p > 0.05$). Raw reads were trimmed using cutadapt to remove adapter sequences before filtering out low quality reads and reads larger than 35 bp or shorter than 18 bp. Hindgut samples had the highest number of reads after trimming, followed by spleen, and finally head kidney (One-Way ANOVA, $F_{2,53} = 116.7$, $p < 0.0001$) and trimmed reads for all three tissues had a mean length of 22 bp (**Figure 2.2B**). Bowtie2 aligned 94% of reads from hindgut and spleen to the Chinook salmon genome while 93% were aligned in head kidney (**Figure 2.2C**; One-Way ANOVA, $F_{2,53} = 6.040$, $p = 0.0043$). Hindgut (32%) and head kidney (35%) had significantly less percent of trimmed reads associated with miRNAs compared to spleen (48%; **Figure 2.2C**; One-Way ANOVA, $F_{2,53} = 33.56$, $p < 0.0001$). In total, between 225 and 248 miRNA genome coordinates had reads associated in each sample, with hindgut having significantly more miRNA genome coordinates with associated reads identified than head kidney and spleen (**Figure 2.2D**; One-Way ANOVA, $F_{2,53} = 42.96$, $p < 0.0001$). The pipeline used in this study (miARma-seq) was run without any errors.

2.3.4 Identification of tissue specific miRNA differences due to Ploidy and Infection

Read count files generated by miARma-seq for each tissue were then analyzed using DESeq2 to identify miRNA changes before and after infection. **Figures 2.3, 2.4, and 2.5** depict MA plots for hindgut, head kidney, and spleen respectively. Overall, comparisons between day 0 and day 3 (infection, **B & C**) had more miRNA altered than comparisons between ploidies (**A & D**). Outputs from DESeq2 can be found in supplemental materials for all three tissues. Each differentially expressed genome coordinate was then parsed to identify the mature miRNA it encodes for subsequent phenotypic analysis (**Tables 2.2-2.4**).

2.3.4.1 Hindgut differences in miRNA genome coordinates

Annotated miRNA genome coordinates were found to be differentially expressed in three out of four potential comparisons within the hindgut of diploid and triploid salmon. Overall, log₂ fold changes for all miRNA identified as differentially expressed were less than one (**Table 2.2**). Prior to infection (Day 0), four miRNA genome coordinates were upregulated in triploid fish (**Figure 2.3A**) which encoded two mature miRNA sequences (**Table 2.2**). In diploid fish 3 days after infection, 26 miRNA genome coordinates were upregulated, and 25 miRNA genome coordinates downregulated (**Figure 2.3B**). These genome coordinates encoded 18 and 16 mature miRNA respectively (**Table 2.2**). In the triploid group, infected fish had 31 miRNA genome coordinates upregulated while 24 miRNA genome coordinates were downregulated (**Figure 2.3C**) which each encoded 15 different mature miRNA (**Table 2.2**). However, at Day 3, no miRNA genome coordinates were differentially expressed between diploid and triploid fish (**Figure 2.3D**).

2.3.4.2 Head kidney differences in miRNA genome coordinates

Similar to hindgut, three out of four comparisons in head kidney tissue identified differentially expressed miRNA due to infection or changes in ploidy and log₂ fold changes varied between 0.2 and 3 (**Table 2.3**). Twelve miRNA genome coordinates were identified as upregulated and seven downregulated in triploid fish at Day 0 (**Figure 2.4A**). These coordinates encoded nine and four mature miRNA sequences respectively (**Table 2.3**). Additionally, two mature miRNA (ssa-miR-203a-3p and ssa-miR-1-3p) were identified as having high log₂ fold changes in this comparison (2.02 and 1.71 respectively). The diploid group had five genome coordinates upregulated and five downregulated after 3 days of infection (**Figure 2.4B**). Upregulated and downregulated genome coordinates each encoded three different mature miRNA (**Table 2.3**). The log₂ fold changes were 7.5

times greater in the downregulated miRNA (~3.1) when compared to the upregulated miRNA (~0.4). The triploid group had ten miRNA genome coordinates upregulated while only four were identified as downregulated after infection Day 3 (**Figure 2.4C**). Six mature miRNA were encoded by the upregulated genome coordinates and two mature miRNA were encoded by the downregulated genome coordinates, but all log2 fold changes were below 1 (**Table 2.3**). No miRNA genome coordinates were identified as differentially expressed between diploids and triploids at Day 3 of *Vibrio* infection (**Figure 2.4D**).

2.3.4.3 Spleen differences in miRNA genome coordinates

Annotated miRNA genome coordinates were identified as differentially expressed in all four comparisons for spleen tissue. Additionally, log2 fold changes varied between 0.2 and 2.0, with comparisons between ploidies having miRNA genome coordinates with higher log2 fold changes compared to infection comparisons (**Table 2.4**). Prior to infection, six miRNA genome coordinates were identified as upregulated in triploids and 18 miRNA genome coordinates downregulated (**Figure 2.5A**). Three mature miRNA were encoded by the upregulated genome coordinates and seven mature miRNA encoded by the downregulated genome coordinates (**Table 2.4**). Two downregulated miRNA (ssa-let-7e-5p and ssa-miR-1-3p) had log2 fold changes greater than 1 while all upregulated miRNA had lower log2 fold changes. Diploid fish had nine miRNA genome coordinates upregulated and 28 miRNA genome coordinates downregulated on Day 3 of the infection (**Figure 2.5B**). These genes encoded five mature miRNA and 11 mature miRNA, respectively (**Table 2.4**). One downregulated miRNA (ssa-miR-2188-3p) had a log2 fold change of 1.23, while the upregulated miRNA had log2 fold changes below 0.5 (**Table 2.4**). Triploid fish had 15 miRNA genome coordinates upregulated and 21 miRNA genome coordinates downregulated on Day 3 of the infection (**Figure 2.5C**). Upregulated genome coordinates were identified as encoding 11 mature miRNA while downregulated genome coordinates encoded 12 mature miRNA (**Table 2.4**). Two upregulated miRNA (ssa-miR-146d-5p and ssa-miR-146a-5p) were the only miRNA to have log2 fold changes greater than 1 in this comparison. In contrast to hindgut and head kidney, differentially expressed miRNA genome coordinates were identified between diploids and triploids at Day 3 of *Vibrio* infection. Thirteen miRNA genome coordinates were identified as upregulated, and 11 miRNA genome coordinates downregulated in the triploid fish (**Figure 2.5D**). Both upregulated and downregulated genome coordinates lists encoded seven different mature miRNA, with three

upregulated miRNA (ssa-miR-2188-3p, ssa-miR-144-5p, and ssa-miR-144-3p) having log2 fold changes that approached 2 (**Table 2.4**).

2.3.5 qPCR validation of DE miRNA

Sequencing results for differentially expressed miRNA were validated using qPCR to determine if differences were detectable and to increase the sample size for spleen and head kidney. Four miRNA were chosen based on their high normalized read count, differential expression in multiple comparisons, and high log2 fold changes. Hindgut (**Figure 2.6A**), head kidney (**Figure 2.6C**), and spleen (**Figure 2.6E**) levels of ssa-miR-1-3p were measured since this miRNA was differentially expressed between multiple comparisons and in all three tissues (**Tables 2.2-2.4**). Sequencing identified that hindgut expression increased at day 3 in both diploids and triploids (**Figure 2.6A**, **Table 2.2**), head kidney expression was significantly lower in triploids prior to infection but increased in triploids after three days (**Figure 2.6C**, **Table 2.3**), and spleen expression was significantly lower in triploids prior to infection but decreased to triploid levels in diploids after infection (**Figure 2.6E**, **Table 2.4**). Identified differences in hindgut and spleen were undetectable by qPCR; however, in the head kidney, day was found to have a significant effect on expression (Two-Way ANOVA, $F_{1,20} = 7.364$, $p = 0.0134$) with diploid day 0 having 4-fold higher expression of ssa-miR-1-3p compared to triploid day 3 (Tukey Post-hoc, $p = 0.0246$). Similarly, spleen expression of ssa-miR-146d-5p had no difference between groups in contrast to the sequencing identification of diploid elevated expression prior to infection (**Figure 2.6B**, **Table 2.4**; Two-Way ANOVA, $p > 0.05$). Spleen expression of ssa-miR-146d-5p was impacted by day (**Figure 2.6D**; Two-Way ANOVA, $F_{1,20} = 39.84$, $p < 0.0001$), with the diploid (2-fold) and triploid (3-fold) Day 3 fish having higher expression than the Day 0 fish (Tukey Post-Hoc, $p < 0.01$). However, splenic sequencing identified differences in expression between ploidies prior to infection and elevation in triploids after infection (**Figure 2.6D**, **Table 2.4**). Finally, spleen expression of ssa-miR-2188-3p (**Figure 2.6F**) had both a ploidy (Two-Way ANOVA, $F_{1,20} = 4.562$, $p = 0.0452$) and an interactive effect (Two-Way ANOVA, $F_{1,20} = 12.74$, $p = 0.0019$). Day 3 expression was 5-fold higher in triploid fish when compared to diploids (Tukey Post-Hoc, $p = 0.0033$). Sequencing differences between ploidies after infection were confirmed while differences between diploid fish before and after infection were not validated (**Figure 2.6F**, **Table 2.4**).

2.3.6 Analysis of miRNA targets using miRanda and KEGG

Genes predicted to be regulated by differentially expressed miRNA were analyzed to assess the phenotypic effects in each tissue. The miRanda algorithm was utilized with strict parameters to identify genes with greater probability of being regulated. **Table 2.5** outlines the pathways with significant enrichment in each comparison-associated gene list. Prior to infection (Comparison A), triploid hindguts and head kidneys are predicted to have altered signalling pathway expression; however, in spite of differences in miRNA abundance, spleen had no predicted KEGG pathway impacts. Gene lists associated with infection (Comparisons B & C) indicate that the diploid group is predicted to have more pathway impacts than triploids. Finally, triploids are predicted to have altered expression of signalling pathways in spleen prior to infection-associated mortality (Comparison D), especially in immune pathways such as TGF- β signalling and adipocytokine signalling.

2.4 Discussion

The objective of this study was to identify miRNA that are altered by ploidy and infection in Chinook salmon. MicroRNA from hindgut, head kidney, and spleen were sequenced to determine which tissue could be involved in poor triploid performance. Both diploids and triploids had miRNA altered after infection in all three tissues. Additionally, differences between ploidies were modest prior to infection in hindgut and head kidney. However, spleen differences were more dramatic between diploids and triploids, as observed with higher log₂ fold changes prior to *Vibrio*-associated mortalities. KEGG analysis of gene targets indicates that the miRNA identified as differentially expressed will impact immune regulation in both diploids and triploids.

2.4.1 Increased ploidy impacts miRNA expression in key immune tissues

The Day 0 sampling was utilized to identify miRNA that are altered strictly due to increased ploidy, rather than any applied stressor. Hindgut miRNA expression was similar, while a few miRNA with higher fold changes had lower expression in triploid head kidney and spleen. Diploid and triploid fish perform similarly unless conditions become stressful, so it is unsurprising that few differences were identified at this timepoint (Iunes et al., 2021a; Nuez-Ortín et al., 2017; O’Flynn, 1997). Salmonids have undergone two additional whole genome duplications, a teleost specific event occurring 320 million-years-ago and a salmonid-specific event occurring 88 million-years-ago (Macqueen and Johnston, 2014; Taylor et al., 2001). Maintenance of multiple gene copies is adaptive in these fishes,

allowing species-specific niche development over evolutionary timescales (Christensen and Davidson, 2017; Lien et al., 2016). Loci encoding miRNA are similarly maintained after genome duplications (Desvignes et al., 2021; Luo et al., 2018; Peterson et al., 2022). Therefore, artificially induced retention of extra chromosomes in salmonids is likely to have fewer negative effects compared to teleost families with diploids that have less gene copies. The KEGG analysis supports this conclusion since only three pathways are predicted to be impacted at Day 0, and only in hindgut and head kidney (**Table 2.5**). However, miRNA with higher fold changes at baseline environmental conditions in spleen and head kidney (ssa-miR-203a-3p, ssa-let-7e-5p, and ssa-miR-1-3p) should be investigated to identify whether these effects are family specific, and whether they drive phenotypic impacts on their respective gene targets.

The Day 3 timepoint was chosen for this experiment since IP injection of this *Vibrio* strain results in observable vibriosis symptoms and initial mortality events around day four (Semple et al., 2022). The fish utilized in this study were maintained in freshwater their entire lives and therefore had not been previously exposed to this saltwater-associated pathogen. Surprisingly, hindgut and head kidney had similar miRNA expression prior to infection-associated mortalities as indicated by **Figures 2.3D & 2.4D**. Transcriptional differences have been identified in triploid coho salmon head kidney and triploid goldfish hindgut; however, these differences are associated with protein coding genes after infection with *Piscirickettsia salmonis* or higher intestinal *Vibrio* presence, respectively (Cai and Wei, 2023; Correa et al., 2015). Although this could indicate that dysregulation of protein coding transcripts are the cause of poor triploid performance, the lack of miRNA differences identified in this study could be indicative of dysregulated epigenetic regulation. Expression of gene targets are related to the amount of cellular-associated miRNA, and therefore miRNA levels can act as a threshold to prevent excessive protein abundance (Stern-Ginossar et al., 2008). The dynamics of miRNA and their target mRNA are also tightly regulated in the immune response to prevent excessive immune-associated inflammation (Taganov et al., 2006). Therefore, protein expression of targets may be impacted by the lack of altered miRNA abundance in the triploids in this study, resulting in immune dysregulation. Exploring the relationship between miRNA and mRNA targets within the same tissue would elucidate whether normal epigenetic regulatory networks are impacted by increased ploidy.

Interestingly, the spleen of diploids and triploids had altered expression just prior to *Vibrio*-induced mortalities. The miRNA identified as having altered expression are predicted to target

immune pathways, implying that triploid fish have altered epigenetic regulation just prior to mortality. The teleost and mammalian spleen function similarly by sensing pathogen-associated molecular patterns in circulation and responding using resident cells such as T cells, B cells, and macrophages (Koppang et al., 2010, 2003). Spleen resident macrophages phagocytose pathogens early in infection and produce cytokines to recruit other immune cells, functioning as innate immune effectors (Uribe et al., 2011). Therefore, the altered miRNA expression identified could relate to a deficient innate immune function in triploid salmonids. Indeed, miRNA in fish have been identified as regulating toll-like receptor (TLR) expression, phagocytosis, and cytokine production (Nie et al., 2019; Wang et al., 2016). Additionally, triploid yellowtail tetra have reduced spleen expression of *il-1 β* and *tgfb β* , which supports this tissue as having altered innate immune function (Levy-Pereira et al., 2021). However, it's important to recognize that innate immune function in the spleen can recruit adaptive effectors to support pathogen clearance. In the spleen, B or T cells interact with antigen presenting cells (such as macrophages) to initiate adaptive immune responses by producing antibodies, removing infected cells, or activating cellular subsets for specific pathogens (Nakanishi et al., 2015). Therefore, the results presented here could also indicate that the regulation of early adaptive immune responses is altered in triploid fish prior to pathogen-associated mortality. It has been well established that miRNA are involved in regulating specificity for adaptive immune responses (Cobb et al., 2005; Mittelbrunn et al., 2011; Vigorito et al., 2007). Additionally, lymphoid precursor cell numbers in the spleen are significantly reduced in triploid fish (Lahnsteiner, 2020). Determining the role these miRNA have in regulating innate and adaptive immunity will provide clarity regarding whether immune cell development or immune cell function is inhibited in triploids.

2.4.2 Immune impacts of differentially expressed miRNA

Infection-induced differentially expressed miRNA identified in this study are predicted to regulate many pathways, including signalling pathways involved in immune function (**Table 2.5**). Clearly, diploids and triploids have different miRNA responses over 3-days of infection, although these differences had no impact on survival. Overall, this implicates that the altered miRNA response observed in triploids was sufficient, despite the predicted impact on pathways, to prevent reduced survival in this infection. However, the altered triploid miRNA response could be more detrimental for pathogens that can impact miRNA abundances in host tissues (e.g. *Edwardsiella tarda*; Li et al., 2023). Thus, the altered regulation identified could corroborate studies that have shown triploids being more susceptible to natural outbreaks from opportunistic pathogens (Ching et al., 2010).

Immune-associated gene expression is highly time dependent to allow for the return to homeostasis, and improper regulation (i.e. through miRNA dysregulation) is associated with chronic inflammation and autoimmunity (O'Connell et al., 2010; Perry et al., 2008; Taganov et al., 2006). However, the teleost immune system can be delayed or accelerated by environmental factors such as temperature (Abram et al., 2020; Bennoit and Craig, 2020). Altered miRNA dynamics in the absence of any difference in mortality between ploidies could be related to a delayed immune response. Indeed, triploid Atlantic salmon have demonstrated a delayed hyperferremic response, altered complement clearance following LPS stimulation, and delayed superoxide dismutase expression after H₂O₂ exposure (Chalmers et al., 2018; Langston et al., 2001). It is currently unclear whether the immune miRNA identified in the present study or immune gene expression are temporally shifted in triploid fish. Future studies that utilize multiple timepoints post-infection could provide clarity on immune gene dynamics in response to increased ploidy.

Despite the uncertainty regarding the impact of ploidy on immune function, the miRNA identified in this study have established roles in regulating immune responses. Hindgut, head kidney, and spleen all had altered expression of ssa-miR-1-3p (**Figure 2.6A, C, E**). Studies in teleosts indicate that this miRNA can be altered during smoltification, in response to hypoxia, and is implicated in cholesterol metabolism (Huang et al., 2017; Shwe et al., 2022, 2020; Zhu et al., 2018). Additionally, in humans miR-1-3p is involved in regulating immunological functions such as apoptosis and receptor mediated endocytosis (Coccia et al., 2020; Peng et al., 2017). Head kidney expression of ssa-let-7e-5p was elevated in diploid fish prior to infection (**Figure 2.6B**). Similar to miR-1-3p, let-7e-5p is altered during Atlantic salmon smoltification and is highly concentrated in rainbow trout ovarian fluid and plasma (Cardona et al., 2021; Shwe et al., 2022). Mammalian let-7e-5p has been shown to impact inflammatory signalling through regulating the TLR4 and JAK/STAT3 pathways (Androulidaki et al., 2009; Li et al., 2018). Expression of ssa-miR-146d was upregulated after infection in both ploidies as indicated by the qPCR results (**Figure 2.6D**). This is unsurprising due to the miR-146 family's well-established role in responding to immune stimulation. Mammalian and teleost models have confirmed that NF- κ B activation causes upregulation of miR-146a, which negatively regulates the MyD88-Traf6 signalling pathway to prevent excessive inflammation (Ordas et al., 2013; Perry et al., 2008; Taganov et al., 2006). Less than 10% of miRNA binding sites are conserved between humans and teleosts, validating the importance of the miR-146 family in regulating immunity (Xu et al., 2013).

The final miRNA that was measured was ssa-miR-2188-3p and was chosen because interactive effects were identified through sequencing. The qPCR results validated this interaction, where triploids had 4x higher expression compared to diploids after infection in spleen (**Figure 2.6F**). This vertebrate-specific miRNA is absent in mammals and previous work has identified that it responds to immune stimulation (Sha et al., 2014). Salmonid alphavirus infection in Atlantic salmon hearts, chlorpyrifos exposure in common carp head kidney, and Atlantic salmon monocyte to macrophage transition causes downregulation of miR-2188-3p (Andreassen et al., 2017; Liu et al., 2019; Smith et al., 2020). However, *Moritella viscosa* infection in olive flounder and polyI:C stimulation of Atlantic cod macrophages causes upregulation of the same miRNA (Eslamloo et al., 2018; Ramberg et al., 2022). These opposing results could be related to sampling time as studies within the first few hours of viral hemorrhagic septicemia virus infection or hypoxia exposure have shown that expression of the -5p version can be upregulated or downregulated rapidly (Najib et al., 2016; Sun et al., 2020). Further study is necessary to determine whether the ploidy differences observed here are due to delays in regular immune function, or complete dysregulation of miR-2188-3p's role in regulating immunity.

2.4.3 Study limitations & Future directions

Although this study has provided insight into miRNA dynamics after infection, there are more questions that need to be answered to confirm whether miRNA are involved in triploid immune dysfunction. First, this study only used one family to reduce genotypic impacts on epigenetic regulation. It has been established that family has an impact on performance when diploids and triploids are exposed to a pathogen (Weber et al., 2013). Therefore, future studies should validate that the miRNA identified as differentially expressed are shared between multiple families of Chinook salmon. Second, we chose to investigate two timepoints: 1) prior to infection to establish miRNA impacts due to increased ploidy and 2) after three days of infection to reduce injection-associated stress but identify differences prior to *Vibrio*-associated mortality. Infection in teleosts can induce changes in miRNA expression from hours to weeks after exposure (Andreassen et al., 2017; Gao et al., 2019). Investigating miRNA dynamics at more timepoints after *Vibrio* infection could identify more miRNA that are altered between ploidies as well as provide evidence for how ploidy impacts epigenetic regulation. Third, this study utilized known miRNA genome coordinates that were annotated through Ensembl to identify reads that encoded mature miRNAs. However, the Chinook genome annotation for miRNAs is incomplete since miR-155, a well-studied immune-associated

miRNA, was not identified in any comparison. Blasting the ssa-miR-155 sequence against Ensembl's most recent Chinook salmon genome assembly (GCA_002872995.1) identifies an unannotated region on chromosome 11. For the current study only genes that were annotated as miRNA were analyzed, so there are likely more differentially expressed genes in this study than reported here. Therefore, analyzing this dataset once the Chinook salmon genome has been annotated more thoroughly will provide new gene targets to explore. Lastly, the qPCR validation did not concur with the sequencing results. Successful validation of small RNA sequencing requires high expression of the miRNA of interest and higher fold changes (Yeri et al., 2018). The miRNA measured using qPCR all had high expression and log₂ fold changes >1 and therefore should have produced similar trends to the normalized read counts. This could be indicative of sequencing false positives since the head kidney and spleen sample size (n=4) was smaller than qPCR (n=6). Recent commentaries have discussed the issues with attempting to validate sequencing through qPCR due to the different chemistry that is involved in each technique (Coenye, 2021; Martyniuk, 2020; Sampathkumar et al., 2022). Therefore, results identified here should be investigated further to confirm whether the miRNA differences are biologically relevant under similar conditions.

2.4.4 Conclusion

This study is the first to investigate the miRNA changes associated with infection in diploid and triploid teleosts. Additionally, we have analyzed three separate immune tissues and identified that the spleen could be involved in the altered survival observed with changes in Chinook salmon triploids. Although the hindgut and head kidney had altered miRNAs after infection, no miRNAs were differentially expressed between diploids and triploids prior to *Vibrio*-associated mortality. The dramatic differences in miRNA expression after three days of infection in spleen could be indicative of dysregulation of immune-associated epigenetic regulation or a delayed response to infection. Predicted targets for the miRNA that were identified indicated that translation of genes involved in immune function were likely to be impacted. Future work will expand on the miRNA identified here to investigate how the transcriptional profile (including mRNAs) is altered between diploid and triploid Chinook salmon at more timepoints after infection.

Table 2.1: Primer information for qPCR validation

Primer efficiencies and r^2 for each gene target were determined using a pooled sample from hindgut, head kidney, and spleen. M scores for housekeeping genes were calculated using the CFX Maestro software (v 5.3.022.1030). Forward primers for miRNAs were based on their miRbase identity for *Salmo salar* and reverse primers are based on the proprietary Universal Reverse Primer (Agilent, 600037).

Target	Accession	Forward primer sequence	Reverse Primer sequence	Amplicon length (bp)	M Score	Efficiency	r^2	Reference
<i>18s rRNA</i>	KJ774722.1	GGCCGTTCTTAGTTGGTGGA	TTGCTCAATCTCGTGTGGCT	147	Hindgut: 0.861	97.8	0.995	-
					Head kidney: 0.615			
					Spleen: 0.450			
<i>ef1α</i>	FJ890356.1	ATGCCCTGTACTGGATTGC	TGGGGGCATCCTCAAGTTTC	105	Hindgut: 0.861	96.6	0.987	Thraya et al., 2019
					Head kidney: 0.615			
					Spleen: 0.450			
<i>ssa-miR-1-3p</i>	MIMAT0032472	TGGAATGTAAAGAAGTATGTAT	Universal Reverse Primer	-		93.1	0.989	-
<i>ssa-let-7e-5p</i>	MIMAT0032705	TGAGGTAGTAGATTGAATAGTT	Universal Reverse Primer	-		124.3	0.998	-
<i>ssa-miR-146d-5p</i>	MIMAT0032376	TGAGAACTGAATTCCAAGGGTT	Universal Reverse Primer	-		98.1	0.99	-
<i>ssa-miR-2188-3p</i>	MIMAT0032523	GCTGTGTGAGGTCAGACCTATC	Universal Reverse Primer	-		92.7	0.984	-

Table 2.2: Differentially expressed miRNA in hindgut tissue

Mature miRNAs were identified by blasting reads from *Oncorhynchus tshawytscha* with differentially expressed miRNA genome coordinates to the *Salmo salar* miRNA transcriptome in miRbase. The number of differentially expressed ensemble IDs associated with each mature miRNA are specified by “# IDs”. Log2 fold change (log2FC) was averaged between differentially expressed genome coordinates for each mature miRNA and a positive value indicates higher expression in the treatment group (second group for each comparison) and negative value indicates lower expression in the treatment group.

Comparison A: 2N - 3N Day 0			Comparison B: 2N Day 0 - Day 3			Comparison C: 3N Day 0 - Day 3		
Mature miRNA	# IDs	Average log2FC	Mature miRNA	# IDs	Average log2FC	Mature miRNA	# IDs	Average log2FC
ssa-miR-23a-3p	3	0.34	ssa-let-7e-5p	2	0.55	ssa-miR-194a-5p	1	-0.46
ssa-miR-133a-3p	1	0.28	ssa-miR-2188-3p	1	-0.87	ssa-miR-1-3p	3	0.72
			ssa-miR-194a-5p	1	-0.39	ssa-miR-7a-5p	9	0.33
			ssa-miR-458-3p	2	0.56	ssa-miR-30a-5p	3	-0.40
			ssa-miR-1-3p	3	0.59	ssa-miR-101a-3p	1	0.71
			ssa-miR-128-3p	3	-0.67	ssa-miR-2188-3p	1	-0.69
			ssa-miR-222a-5p	2	0.34	ssa-miR-146d-5p	1	0.67
			ssa-miR-101a-3p	1	0.61	ssa-miR-21b-5p	2	-0.49
			ssa-miR-148a-3p	1	0.40	ssa-miR-16a-5p	2	-0.38
			ssa-miR-29a-3p	1	0.56	ssa-miR-152-3p	2	0.51
			ssa-miR-194b-5p	1	-0.41	ssa-miR-730a-5p	1	0.55
			ssa-miR-196b-5p	1	-0.44	ssa-miR-10a-5p	1	0.45
			ssa-miR-29b-3p	2	0.58	ssa-miR-196a-5p	4	-0.49
			ssa-miR-10b-5p	1	0.45	ssa-miR-196b-5p	1	-0.43
			ssa-miR-24a-4-5p	1	-0.40	ssa-miR-9a-1-3p	1	0.39
			ssa-miR-455-5p	3	-0.41	ssa-miR-143-3p	2	0.41
			ssa-miR-135c-5p	1	-0.53	ssa-miR-15e-3p	1	0.53
			ssa-miR-218-5p	1	-0.45	ssa-let-7e-5p	2	0.34
			ssa-miR-214-3p	3	-0.30	ssa-miR-135a-5p	1	-0.48
			ssa-miR-142b-3p	2	-0.43	ssa-miR-194b-5p	1	-0.35
			ssa-miR-21b-5p	2	-0.38	ssa-miR-30e-5p	3	0.40
			ssa-miR-365-3p	1	0.49	ssa-miR-128-3p	3	-0.45
			ssa-miR-30a-5p	2	-0.24	ssa-miR-125b-5p	1	-0.44
			ssa-miR-10a-5p	1	0.37	ssa-miR-203a-3p	1	-0.40
			ssa-miR-499b-5p	1	-0.31	ssa-miR-148a-3p	1	0.29
			ssa-miR-143-3p	1	0.39	ssa-miR-21a-5p	1	-0.34
			ssa-miR-203a-3p	1	-0.41	ssa-miR-96-5p	1	0.23
			ssa-miR-29c-3p	1	0.46	ssa-miR-23a-3p	1	-0.33

ssa-miR-301b-5p	1	-0.46	ssa-miR-10b-5p	2	0.33
ssa-miR-15c-5p	1	0.35	ssa-miR-459-3p	1	-0.41
ssa-miR-152-3p	2	0.38			
ssa-miR-9a-1-3p	1	0.31			
ssa-miR-7a-5p	2	0.26			
ssa-miR-15e-3p	1	0.43			

Table 2.3: Differentially expressed miRNA in head kidney tissue

Mature miRNAs were identified by blasting reads from *Oncorhynchus tshawytscha* with differentially expressed miRNA genome coordinates to the *Salmo salar* miRNA transcriptome in miRbase. The number of differentially expressed ensemble IDs associated with each mature miRNA are specified by “# IDs”. Log2 fold change (log2FC) was averaged between differentially expressed genome coordinates for each mature miRNA and a positive value indicates higher expression in the treatment group (second group for each comparison) and negative value indicates lower expression in the treatment group.

Comparison A: 2N - 3N Day 0			Comparison B: 2N Day 0 - Day 3			Comparison C: 3N Day 0 - Day 3		
Mature miRNA	# IDs	Average log2FC	Mature miRNA	# IDs	Average log2FC	Mature miRNA	# IDs	Average log2FC
ssa-miR-203a-3p	1	-2.02	ssa-miR-203a-3p	1	-3.30	ssa-miR-146a-5p	2	0.71
ssa-miR-1-3p	3	-1.71	ssa-miR-205b-5p	3	-3.14	ssa-miR-29b-2-5p	1	0.63
ssa-miR-19a-3p	1	0.42	ssa-miR-216a-5p	1	-2.90	ssa-miR-1-3p	3	0.67
ssa-miR-142b-3p	2	0.35	ssa-miR-181a-5p	3	0.35	ssa-miR-26a-5p	3	-0.28
ssa-miR-22a-3p	1	-0.36	ssa-miR-146a-5p	1	0.48	ssa-miR-146d-5p	1	0.43
ssa-miR-142a-5p	1	0.35	ssa-miR-18b-3p	1	0.43	ssa-miR-152-3p	1	0.37
ssa-miR-18b-3p	1	0.41				ssa-miR-214-3p	2	0.39
ssa-miR-152-3p	2	-0.34				ssa-miR-25-3p	1	-0.35
ssa-miR-181a-5p	1	0.26						
ssa-miR-128-3p	3	0.30						
ssa-miR-301b-5p	1	0.32						
ssa-miR-210-5p	1	0.34						
ssa-let-7h-5p	1	0.27						

Table 2.4: Differentially expressed miRNA in spleen tissue

Mature miRNAs were identified by blasting reads from *Oncorhynchus tshawytscha* with differentially expressed miRNA genome coordinates to the *Salmo salar* miRNA transcriptome in miRbase. The number of differentially expressed ensemble IDs associated with each mature miRNA are specified by “# IDs”. Log2 fold change (log2FC) was averaged between differentially expressed genome coordinates for each mature miRNA and a positive value indicates higher expression in the treatment group (second group for each comparison) and negative value indicates lower expression in the treatment group.

Comparison A: 2N - 3N Day 0			Comparison B: 2N Day 0 - Day 3			Comparison C: 3N Day 0 - Day 3			Comparison D: 2N - 3N Day 3		
Mature miRNA	# IDs	Average log2FC	Mature miRNA	# IDs	Average log2FC	Mature miRNA	# IDs	Average log2FC	Mature miRNA	# IDs	Average log2FC
ssa-let-7e-5p	2	-1.11	ssa-miR-2188-3p	1	-1.23	ssa-miR-146d-5p	1	1.03	ssa-miR-2188-3p	1	1.98
ssa-miR-1-3p	3	-1.33	ssa-let-7e-5p	2	-0.84	ssa-miR-146a-5p	2	1.10	ssa-miR-144-5p	1	1.90
ssa-miR-7132a-5p	1	-0.81	ssa-let-7a-5p	7	-0.57	ssa-miR-21b-5p	2	0.74	ssa-miR-144-3p	1	1.81
ssa-miR-16a-5p	2	-0.80	ssa-miR-1-3p	3	-0.73	ssa-miR-150-5p	1	-0.74	ssa-miR-150-5p	1	-0.53
ssa-miR-146d-5p	1	-0.48	ssa-miR-9a-3-3p	1	-0.68	ssa-miR-92b-3p	2	-0.76	ssa-miR-724-5p	2	-0.82
ssa-miR-199a-3p	4	0.31	ssa-miR-144-5p	1	-0.77	ssa-miR-15b-3p	1	-0.66	ssa-miR-7a-5p	7	0.46
ssa-let-7a-5p	7	-0.35	ssa-miR-23a-3p	2	0.47	ssa-miR-144-5p	2	0.52	ssa-miR-222b-5p	1	0.49
ssa-let-7g-5p	2	-0.39	ssa-miR-144-3p	1	-0.74	ssa-let-7b-5p	2	-0.57	ssa-miR-190a-5p	1	-0.82
ssa-miR-458-3p	1	0.40	ssa-miR-7a-5p	7	-0.51	ssa-miR-106a-3p	1	-0.53	ssa-miR-204-5p	3	-0.50
ssa-miR-30e-3p	1	0.27	ssa-miR-29b-2-5p	1	0.56	ssa-miR-107-3p	1	0.55	ssa-miR-456-3p	2	-0.39
			ssa-miR-16a-5p	2	-0.61	ssa-miR-222a-3p	2	0.50	ssa-miR-101a-3p	1	0.47
			ssa-let-7g-5p	2	-0.53	ssa-miR-29b-2-5p	1	0.55	ssa-miR-10a-5p	1	-0.31
			ssa-miR-199a-3p	4	0.37	ssa-miR-455-5p	2	-0.44	ssa-miR-15e-3p	1	0.48
			ssa-miR-15a-5p	1	-0.52	ssa-miR-129-5p	3	-0.54	ssa-miR-15b-3p	1	-0.41
			ssa-miR-23b-3p	1	0.40	ssa-miR-456-3p	2	-0.40			
			ssa-miR-204-5p	1	0.45	ssa-miR-10b-5p	2	-0.36			
						ssa-miR-27c-3p	2	-0.37			

	ssa-miR-21a-5p	1	0.38	
	ssa-miR-128-3p	2	-0.46	
	ssa-miR-222b-5p	1	0.43	
	ssa-miR-15e-3p	1	0.52	
	ssa-miR-23b-3p	1	0.42	
	ssa-miR-10d-5p	1	-0.34	

Table 2.5: Predicted KEGG pathway impacts

Significant phenotypic impacts on KEGG pathways based on predicted targets for differentially expressed miRNA in each tissue and comparison. Gene targets predicted to be impacted by miRNA changes in each tissue were identified using miRanda (version 3.3a) and gene lists for each comparison analyzed using KOBAS (version 3.0.3). Pathways listed are predicted to be significantly regulated in hindgut (grey), head kidney (pink), and spleen (green).

Comparison A: 2N - 3N Day 0		
Pathway	ID	p-Value
Insulin signaling pathway	sasa04910	0.01826
Wnt signaling pathway	sasa04310	0.01984
Neuroactive ligand-receptor interaction	sasa04080	0.04109
Comparison B: 2N Day 0 - Day 3		
Pathway	ID	p-Value
Neuroactive ligand-receptor interaction	sasa04080	0.00165
TGF-beta signaling pathway	sasa04350	0.00417
MAPK signaling pathway	sasa04010	0.01472
FoxO signaling pathway	sasa04068	0.01472
Glycerophospholipid metabolism	sasa00564	0.04290
Hedgehog signaling pathway	sasa04340	0.04099
FoxO signaling pathway	sasa04068	0.04099
AGE-RAGE signaling pathway in diabetic complications	sasa04933	0.04099
Neuroactive ligand-receptor interaction	sasa04080	0.04099
FoxO signaling pathway	sasa04068	0.03177
TGF-beta signaling pathway	sasa04350	0.03177
Adipocytokine signaling pathway	sasa04920	0.03177
Insulin signaling pathway	sasa04910	0.04637
Mannose type O-glycan biosynthesis	sasa00515	0.04637
Nitrogen metabolism	sasa00910	0.04637
MAPK signaling pathway	sasa04010	0.04637
Glycosaminoglycan biosynthesis - heparan sulfate / heparin	sasa00534	0.04637
Glycosphingolipid biosynthesis - lacto and neolacto series	sasa00601	0.04637
Wnt signaling pathway	sasa04310	0.04637
Neuroactive ligand-receptor interaction	sasa04080	0.04637
mTOR signaling pathway	sasa04150	0.04637
Comparison C: 3N Day 0 - Day 3		
Pathway	ID	p-Value
TGF-beta signaling pathway	sasa04350	0.00354
Neuroactive ligand-receptor interaction	sasa04080	0.00616
Neuroactive ligand-receptor interaction	sasa04080	0.00082
mTOR signaling pathway	sasa04150	0.01640
Comparison D: 2N - 3N Day 3		
Pathway	ID	p-Value
TGF-beta signaling pathway	sasa04350	0.00364
mTOR signaling pathway	sasa04150	0.00947
Adipocytokine signaling pathway	sasa04920	0.04910
Wnt signaling pathway	sasa04310	0.04910
Insulin signaling pathway	sasa04910	0.04910

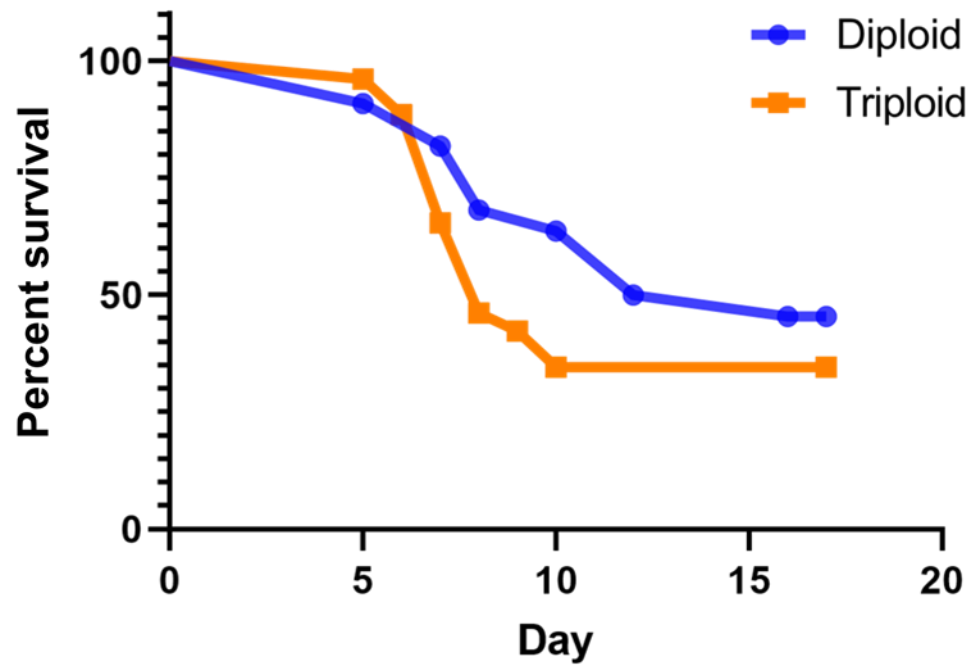


Figure 2.1: Survival curve for full-sibling diploid and triploid *Oncorhynchus tshawytscha*
 Nine-month-old chinook salmon (diploid n = 27, triploid n = 31) infected with an intra-peritoneal injection of *Vibrio anguillarum* were monitored for 2 weeks in 200 L barrels. Calculation for mortality accounted for fish sampled at the Day 3 timepoint. There was no difference in survival between the two ploidies (Mantel-Cox, $\chi^2 = 1.218$, df = 1, $p = 0.2697$).

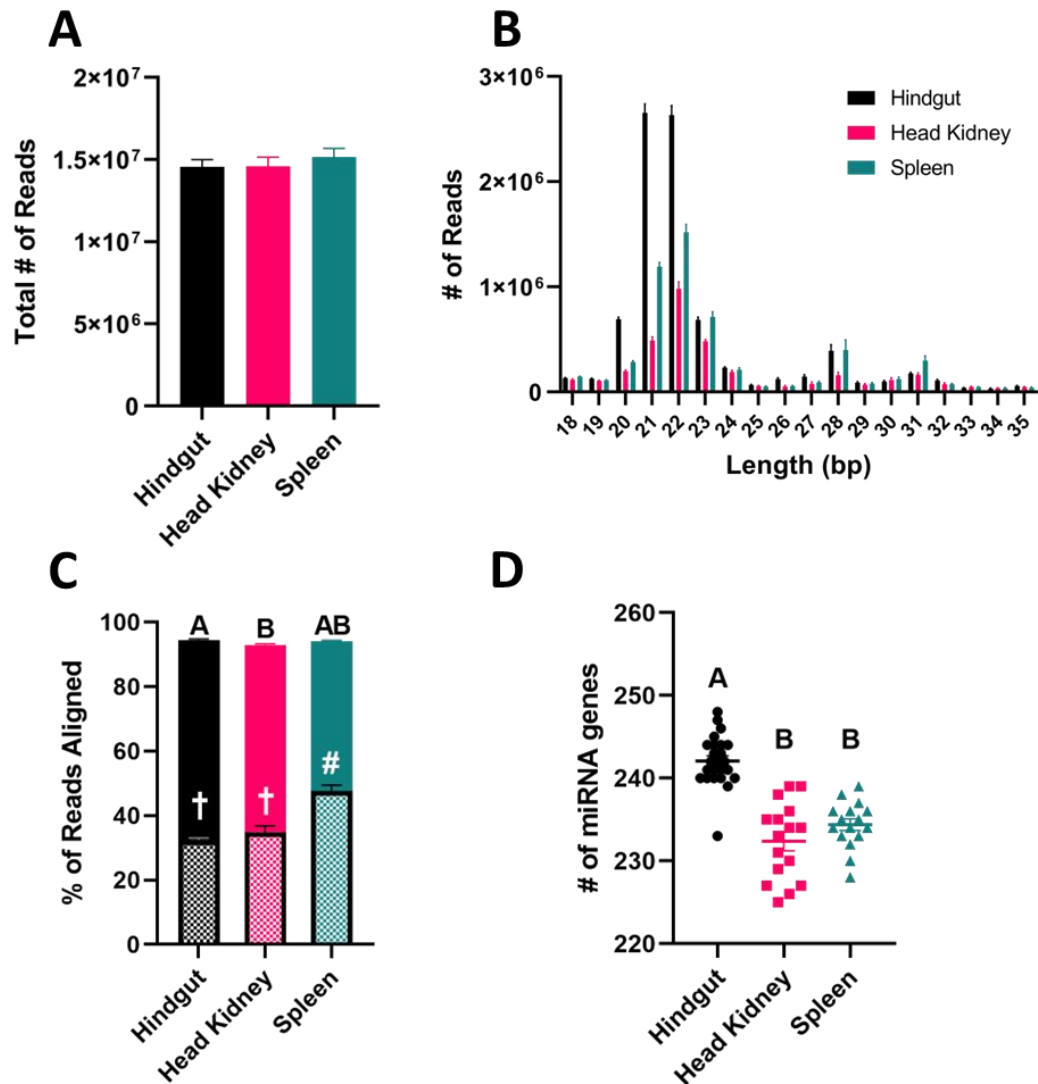


Figure 2.2: Sequencing metadata for small RNA sequencing across three tissues

Reads for hindgut (black, $n = 24$), head kidney (pink, $n = 16$) and spleen (teal, $n=16$) were generated using Illumina Novaseq 6000. All values are presented as mean $\pm$ SEM and groups that do not share a letter or symbol are statistically different from each other (One-Way ANOVA, Tukey post-hoc, $p < 0.05$). **A)** Total reads generated. **B)** Read length distribution after trimming based on length and quality. Average read length was 22 bp and reads remaining after trimming was highest in the hindgut, followed by spleen, and finally head kidney (One-Way ANOVA, Tukey post-hoc, $p < 0.0001$). **C)** Percentage of trimmed reads aligned to the Chinook salmon genome. Total percent of trimmed reads is indicated in solid bars while hash-marked bars indicate the percent of total trimmed reads associated with miRNA genome coordinates. **D)** Total number of miRNA genome coordinates identified with aligned reads.

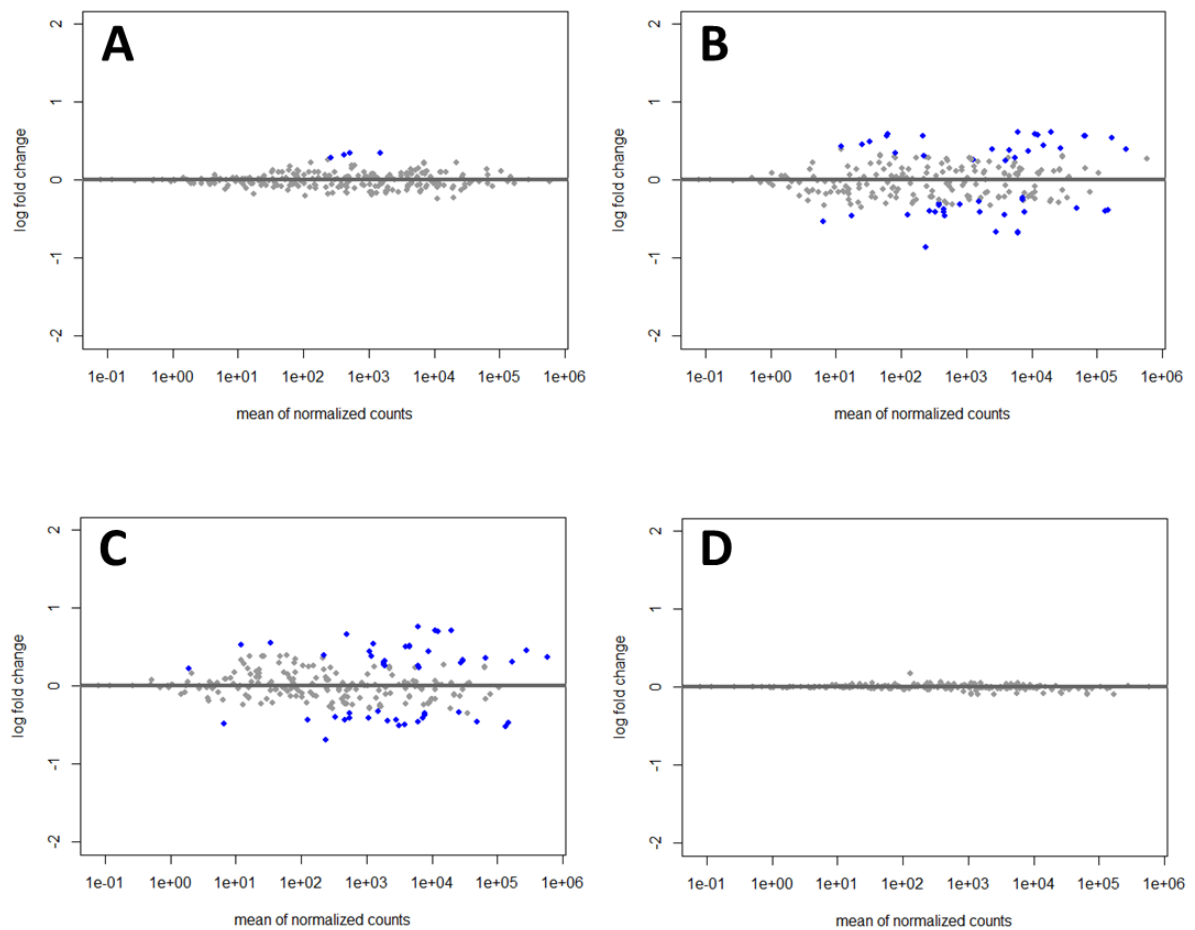


Figure 2.3: Hindgut differential expression results for miRNA genome coordinates between ploidies or infection with *Vibrio anguillarum*

MA plots depict the shrunk (using the *ashr* R package) log fold changes against normalized counts for miRNA genome coordinates identified in hindgut tissue ($n = 6$ per group). Each point represents a single miRNA genome coordinate, and points in blue indicate a significantly higher (above 0) or lower (below 0) expression in the treatment group based on an adjusted p value of 0.05. **A)**

Comparison between diploids and triploids prior to infection (Day 0). **B)** Comparison between diploids prior to infection and after three days of infection. **C)** Comparison between triploids prior to infection and after three days of infection. **D)** Comparison between diploids and triploids after three days of infection.

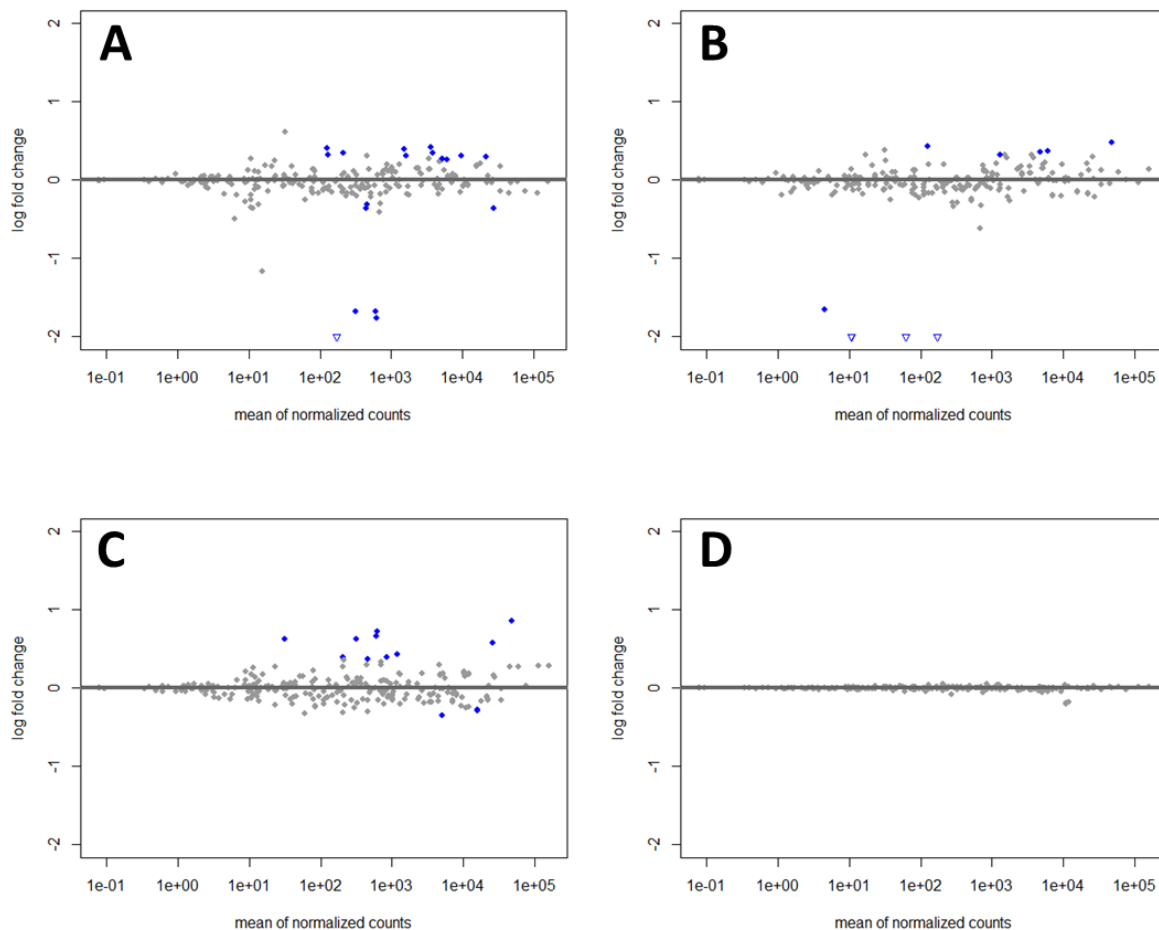


Figure 2.4: Head kidney differential expression results for miRNA genome coordinates between ploidies or infection with *Vibrio anguillarum*

MA plots depict the shrunk (using the *ashr* R package) log fold changes against normalized counts for miRNA genome coordinates identified in hindgut tissue ($n = 4$ per group). Each point represents a single miRNA genome coordinate, and points in blue indicate a significantly higher (above 0) or lower (below 0) expression in the treatment group based on an adjusted p value of 0.05. Inverted triangles represent miRNA with log2 fold changes less than -2. **A)** Comparison between diploids and triploids prior to infection (Day 0). **B)** Comparison between diploids prior to infection and after three days of infection. **C)** Comparison between triploids prior to infection and after three days of infection. **D)** Comparison between diploids and triploids after three days of infection.

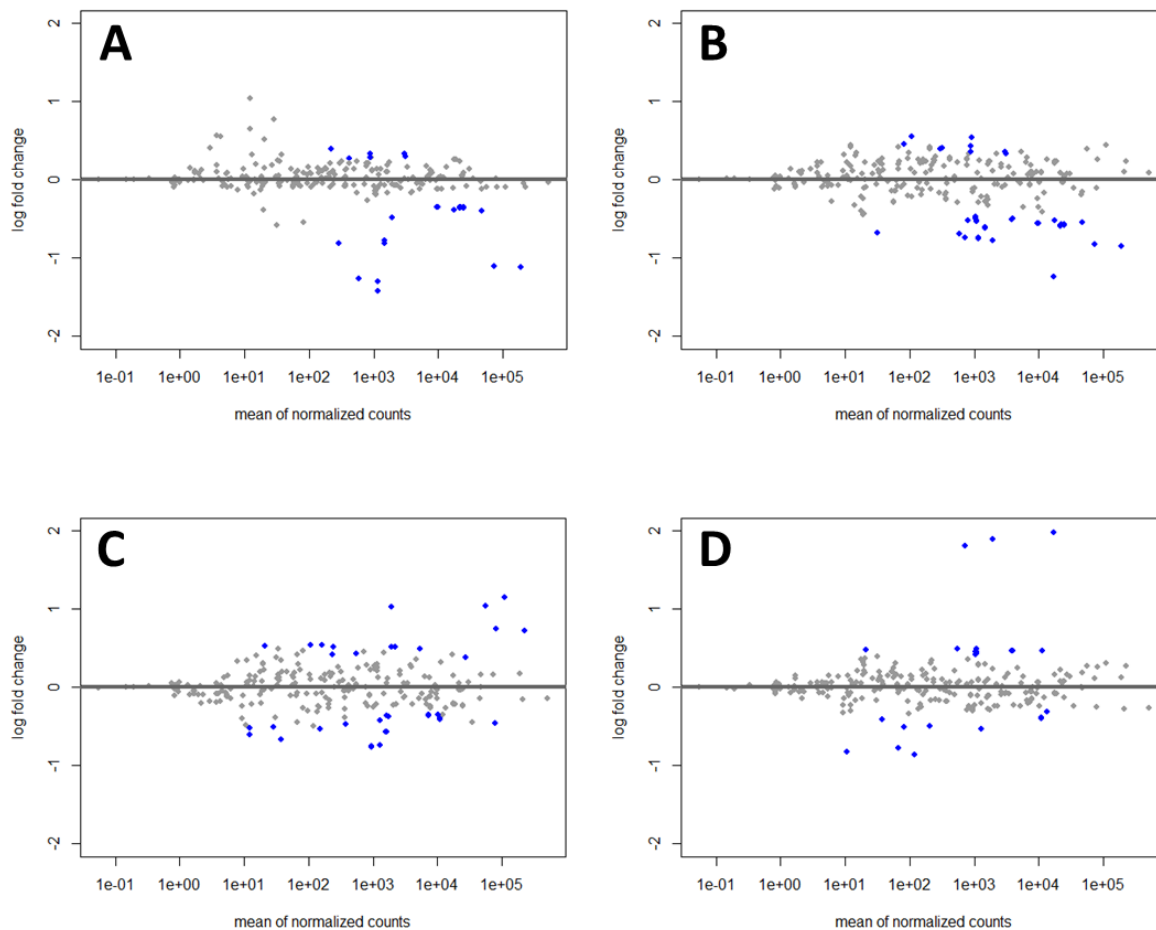


Figure 2.5: Spleen differential expression results for miRNA genome coordinates between ploidies or infection with *Vibrio anguillarum*

MA plots depict the shrunk (using the *ashr* R package) log fold changes against normalized counts for miRNA genome coordinates identified in hindgut tissue ($n = 4$ per group). Each point represents a single miRNA genome coordinate, and points in blue indicate a significantly higher (above 0) or lower (below 0) expression in the treatment group based on an adjusted p value of 0.05. **A)** Comparison between diploids and triploids prior to infection (Day 0). **B)** Comparison between diploids prior to infection and after three days of infection. **C)** Comparison between triploids prior to infection and after three days of infection. **D)** Comparison between diploids and triploids after three days of infection.

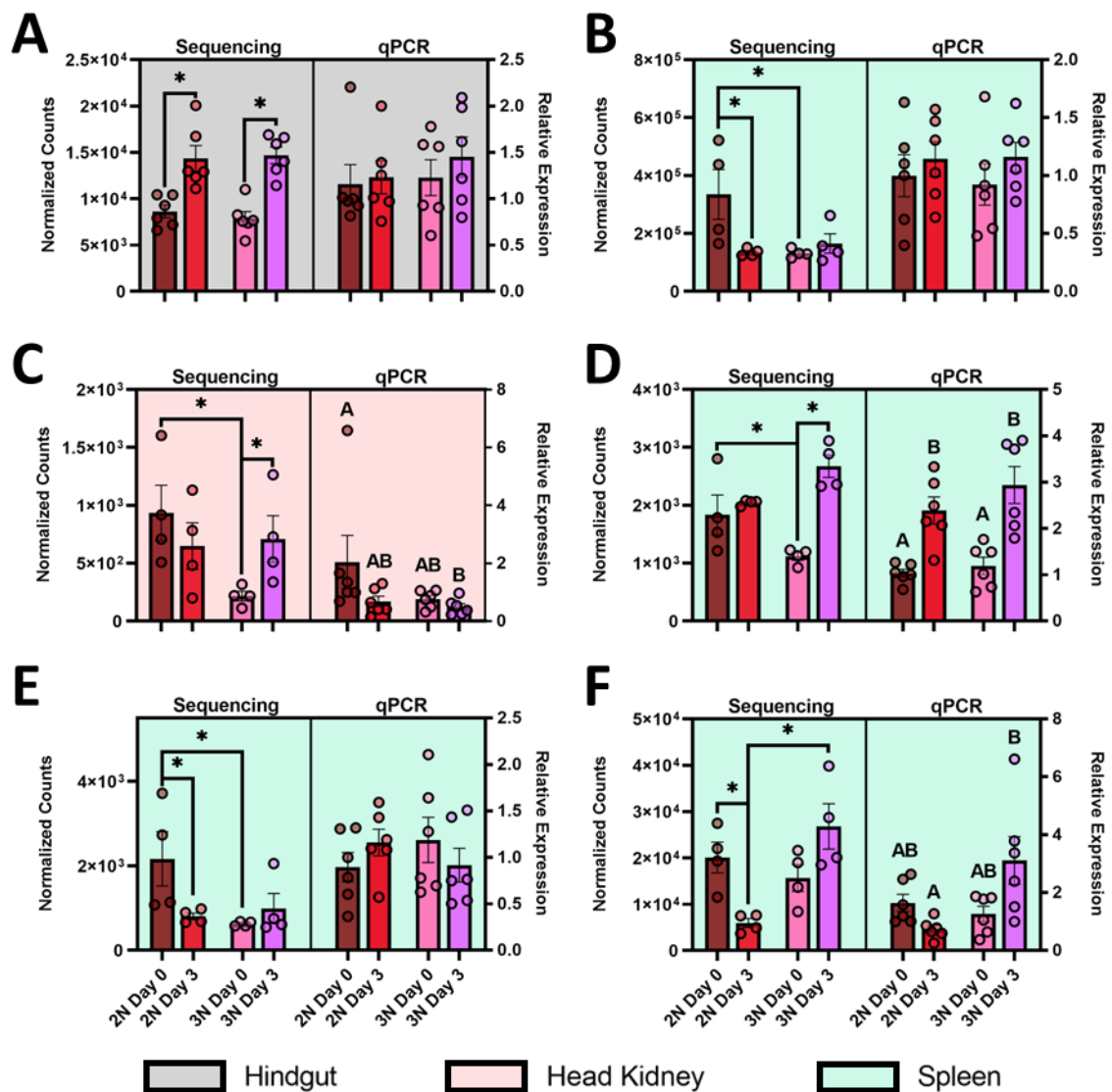


Figure 2.6: qPCR validation of miRNA identified as differentially expressed

DESeq2 was used to identify differentially expressed miRNA and generate normalized counts (mean $\pm$ SEM, left side of each graph) for four miRNA in hindgut (grey, $n = 6$), head kidney (pink, $n = 4$) or spleen (green, $n = 4$). A star indicates a significant difference based on an adjusted p value of 0.05. Differentially expressed miRNA were measured using qPCR (mean $\pm$ SEM, $n = 6$ for all tissues, right side of each graph) to confirm the identified impacts of ploidy or *Vibrio anguillarum* infection. Bars that do not share a common letter indicate that they are statistically different from each other (Two-Way ANOVA, Tukey post-hoc $p < 0.05$). **A)** Hindgut expression of ssa-miR-1-3p. **B)** Spleen expression of ssa-let-7e-5p. **C)** Head kidney expression of ssa-miR-1-3p. **D)** Spleen expression of ssa-miR-146d-5p. **E)** Spleen expression of ssa-miR-1-3p. **F)** Spleen expression of ssa-miR-2188-3p.

2.5 Acknowledgements

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Chapter 3

Recovery from acute thermal exposure impacts immune gene expression in diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha*)

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3.1 Introduction

Organisms experience constant environmental perturbations and have to respond to maintain homeostasis in spite of changes to biotic and abiotic factors. The immune system is particularly important for organism health since it is involved in combating infection using controlled cellular pathways. In teleost fishes, water-borne pathogens are prevalent and therefore they have a robust innate immune system to prevent infection establishment (Mauduit et al., 2022; Rombout et al., 2005; Wang et al., 2023a). In general, this system is broken down into the innate immune system and the adaptive immune system. The innate immune system is made up of physical barriers (e.g. epithelial lining), humoral mediators (e.g. general antibodies and the complement system), and cellular activators (e.g. phagocytic antigen presenting cells) (reviewed by Uribe et al., 2011). The teleost adaptive immune system is less effective compared to the innate system due to reduced immunological memory compared to mammals (Bromage et al., 2004; reviewed by Magor, 2015; Vigliano et al., 2006). The adaptive system provides specificity to immune function by detecting specific pathogen-associated peptides in cell surface receptors called major histocompatibility complex proteins (MHC; class I for intracellular peptides and class II for extracellular peptides; Blum et al., 2013; Chen et al., 2010). These tightly controlled networks are required to maintain homeostasis, and therefore understanding how they are impacted by changes in the environment is vital to understand how climate change will impact organism health.

The teleost immune system not only has to contend with constant assault from pathogens, but also abiotic changes in the environment. As climate change progresses, aquatic organisms will have to contend with increases in global mean temperatures in combination with increased incidence of acute thermal events (Intergovernmental Panel On Climate Change (IPCC), 2023). Fish are ectothermic poikilotherms, which means that their body temperature fluctuates with the external environment (Fry, 1947; Schulte et al., 2011). Acute changes in water temperature impacts immune gene expression in rainbow trout (*Oncorhynchus mykiss*) gills and spleen, indicating that rapid changes in temperature can govern immune function (Guo et al., 2023). Additionally, temperature plays a key role in the timing of immune function, with low temperatures delaying immune activity while elevated temperatures can accelerate them (Abram et al., 2019; Bennoit and Craig, 2020; Bly and Clem, 1991). This change in timing can be detrimental to maintaining survival, especially considering that pathogen dynamics are also impacted by changes in temperature. The abundance of aquatic bacteria and parasites increase at elevated temperatures (Ewing et al., 1986; Lages et al.,

2019; Stocking et al., 2006; Vezzulli et al., 2016). Additionally, the virulence of pathogens can increase under elevated temperatures, resulting in worse outcomes for fish living in these conditions (Lages et al., 2019). Investigating how immune expression changes after acute temperature fluctuations could provide evidence for whether these changes are predictive of biotic assaults.

These drastic environmental changes will put wild fish populations at greater risk; therefore, finding ways to reduce further anthropogenic pressure will help to maintain their health and well-being. Aquaculture, while an important worldwide food source, directly impacts wild fishes through escapees (Atalah & Sanchez-Jerez, 2020). These escapees can directly interact with wild fishes, competing for resources or impacting genetic diversity should they successfully breed (Fleming et al., 2000; Lura & Sægrov, 1991; Skaala et al., 2006). One solution to reduce these impacts is to farm triploid fish since they are physiologically sterile and, in salmon, are less likely to impact the freshwater migration of wild fishes (reviewed by Benfey, 1999; Glover et al., 2016). However, triploids have reduced survival under elevated temperatures and worse performance in response to infection (Aunsmo et al., 2022; Ching et al., 2010; Hyndman et al., 2003). These issues are thought to be due to changes in gene copy numbers, and under stressful conditions can result in altered gene expression which is associated with reduced performance (Ching et al., 2010). Therefore, understanding the transcriptional impacts at baseline conditions and in response to stress will provide evidence for how farmed fish are expected to perform as climate change progresses.

This study was conducted to assess how recovery from acute thermal stress impacts immune gene expression. Both diploid and triploid Chinook salmon (*Oncorhynchus tshawytscha*) were exposed to a critical thermal maximum (CT_{max}) trial and sampled after 1 hour of recovery at ambient water conditions. The teleost gill is important for respiratory function and contains immune cells, including the interbranchial lymphoid tissue (Aas et al., 2017; Haugarvoll et al., 2008; Koppang et al., 2010; reviewed by Wilson and Laurent, 2002). Therefore, it was collected to assess how acute changes in temperature could impact the organism's ability to sense/respond to pathogens. The ventricle of the heart was also collected because it responds to acute increases in temperature and is directly involved in governing thermal tolerance limits (reviewed by Ern et al., 2023; Sandblom et al., 2016). Transcript abundance of genes involved in heat shock (*hsp70* & *hsp90aa1*), recognizing pathogens (pattern recognition receptors, PRRs; *tlr2* & *nlrp3*), endogenous peptide presentation (MHC class I; *tapasin*, *mhc1a*, & *b2m*), exogenous peptide presentation (MHC class II; *mhc2a* & *mhc2b*), or intercellular cell signaling (*il1b*, *il8*, & *tgfb*) were measured to understand whether

reduced triploid performance is associated with altered expression. It is hypothesized that reduced triploid performance is due to altered expression after recovery from thermal stress.

3.2 Methods

3.2.1 Study organisms

Chinook salmon embryos were produced in October 2019. Embryos destined to remain diploid were exposed to hatchery water two minutes post-fertilization before being transferred directly to flow through incubation stacks (Heath Tecna). At 30 minutes post-fertilization, the rest of the embryos were exposed to high pressure (5 minutes at 10 000 psi) to induce retention of the second polar body (Johnson et al., 2004). Embryos were then moved to parallel flow through stacks and cultured under similar conditions until 9-months of age (July 2020). Diploid fish were immunized (Vibrogen 2, ELANCO Canada Ltd., Mississauga, Ontario, Canada) and transferred to open sea net pens, while triploid fish were maintained in land-based freshwater troughs due to reduced infrastructure at the aquaculture facility. Diploids were kept in salt water until 28-months of age (February 2022), when they were transferred to the same outdoor freshwater artificial stream system as triploids. Water quality parameters (temperature and dissolved oxygen) for this flow through system were monitored continuously. This artificial stream system is supplied through groundwater input and fluctuates with seasonal conditions; however, the temperature (6.5°C to 14.2°C) and dissolved oxygen (7.8 mg/L to 11.9 mg/L) remained within seasonal tolerable parameters for all fish.

In August 2022, diploid and triploid fish were transferred from the outdoor system to two separate flow-through freshwater hatchery troughs (2000 L, 10-12°C, dissolved oxygen 7.8-8.5 mg/L, 12h light:12h dark). Every day, fish were fed to satiety in the morning and afternoon using commercial fish feed (Taplow Feeds, Chilliwack, British Columbia, Canada) except when fish were fasted 24-hours before sampling. Fish were allowed to acclimate for 12 days, and all animal care followed the Canadian Council for Animal Care guidelines and had previously been approved by the University of Waterloo's Animal Care Committee (AUPP# 43212; 43415).

3.2.2 Acute thermal stress

For this trial, fish were collected in batches of five and either sampled directly from the trough (control) or sampled after exposure to an acute thermal maximum (CT_{max}) trial. For the CT_{max} trial, fish were moved to a 200 L recirculating bath and allowed to acclimate at ambient water conditions ($\sim 12^{\circ}\text{C}$) for one hour. After acclimation, water temperature was increased at a constant rate of 0.23°C per minute using a 240V Laing Infinity Electric Heater (Laing Thermotech, San Diego, California, USA). Fish were monitored during this acute stress until they reached their CT_{max} point (loss of equilibrium, as described by Becker and Genoway, 1979), at which time they were moved from the thermal bath to individual aerated recovery plastic tote tanks. Data for CT_{max} of fish utilized in this study can be found in Chapter 4 (Heat control group). All individuals were allowed to recover at ambient water conditions ($\sim 12^{\circ}\text{C}$) for one hour to allow for transcriptional changes in response to thermal challenge.

After one hour of recovery, fish were euthanized with diluted clove oil (0.4 mL/L of water; Sigma-Aldrich, C8392) to sample for tissues of interest. Two tissues were measured in this study to assess the immunological effects of acute thermal challenge. Ventricles were perfused with phosphate buffered saline to remove lumen-associated cellular material and allow for assessment of tissue-associated responses, before being bisected and stored in RNAlater. The lamellae at the tip of the first two gill arches on the right side of each individual fish was excised and stored in RNAlater. Both tissues were subsequently flash frozen in a portable dry shipper (-200°C ; VWR, 55709-232). Eight fish (four diploids, four triploids) were identified as having signs of disease at sampling (sores, thin blood, skin lesions) while two fish (one diploid, one triploid) were not sufficiently recovered one hour after CT_{max} . Therefore, these individuals were removed from further analysis (Control 2N $n = 9$, 3N $n = 7$; Thermal Stress Recovered 2N $n = 8$, 3N $n = 8$).

3.2.3 Gene expression analysis

The impact of acute thermal stress on immune gene expression was assessed in the ventricle and gill. Two diploid ventricles samples were lost in transport, one for the control group and one for the thermal stress recovered group. Therefore, $n = 8$ and 7 for these groups, respectively. Total RNA was extracted from both tissues using Qiagen's miRNeasy mini kits (Qiagen, 217004). Approximately 50 mg of tissue was homogenized using a bead mill homogenizer (Fisher Scientific, 15340163) and manufacturer's protocol followed without deviation. Additionally, to remove any DNA

contamination, all samples were treated with Qiagen's On-Column DNase Digestion (Qiagen, 76254) and, after elution, RNA was stored at -80°C until analysis. Eluted RNA concentrations were quantified spectrophotometrically using a Take3 plate and a Synergy H1 microplate reader (Agilent BioTek, SH1MSN). A total of 250 ng of RNA was reverse transcribed into cDNA for each sample using the qScript cDNA Supermix (Quantabio, 95048), following manufacturer's protocol without deviation. All samples were diluted 1:10 using molecular grade water (Sigma, W4502) and stored at -20°C until analysis.

All qPCR reactions for this chapter were run in triplicate using a LightCycler 480 II (Roche, 05015278001). Each 10 µL reaction contained 2.5 µL of diluted cDNA, 2.5 µL of 2 µM primer mix (containing forward and reverse primer, final concentration in reaction of 0.5 µM), and 5 µL of Wisent Advanced qPCR Master Mix (Wisent, 800-435 QL). Reactions were initiated by incubating at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 5 seconds), annealing (60°C or reported temperature in **Table 3.1** for 30 seconds), and extension (72°C for 8 seconds). Finally, to confirm that only one amplicon was generated, a melt curve (incremental increase from 65°C to 97°C, 5 seconds per °C) was analyzed and only wells containing one peak accepted. Each run contained a non-template control and if amplification was identified in these wells, the entire plate was repeated. Primer efficiency (**Table 3.1**) was calculated using a 7-point dilution series (utilizing pooled samples for each tissue) based on previously published techniques (Pfaffl, 2001). Reference gene stability was determined using geNorm (Vandesompele et al., 2002) through RefFinder (Xie et al., 2023). Based on MIQE guidelines (Bustin et al., 2009), *elongation factor 1α (ef1α)* and *β actin* were identified as stable in gill and *mRNA-decapping enzyme 1α (dcp1α)* and *β actin* were identified as stable in ventricle. Therefore, two reference genes were used for each tissue to calculate relative transcript abundance (Livak & Schmittgen, 2001) and all samples were normalized to the diploid control group.

3.2.4 Statistical analysis

All graphs depict the individual relative abundance values, with the mean ± standard error of the mean (SEM) for each group represented by the horizontal lines. Graphpad Prism (v10.2.1) was used to analyze differences between treatment groups and ploidies with Two-Way Analysis of Variance (ANOVA). All models also contained an interaction term. Normality (Shapiro-Wilk) and heteroscedasticity (Spearman's) of residuals were validated for all models and if residuals did not

pass these assumptions, relative abundance values were transformed to be able to perform parametric analyses. For all tests, alpha was set to the standard 0.05. Due to the number of comparisons, false discovery rate corrections (FDR) were conducted for Two-Way ANOVA *p*-values using the two-stage step-up method (Benjamini et al., 2006). Genes were grouped based on their function for this correction, labelled as heat shock protein (*hsp70* & *hsp90aa1*), pattern recognition receptors (*tlr2* & *nlrp3*), major histocompatibility complex I (*tapasin*, *mhc1a*, & *b2m*), major histocompatibility complex II (*mhc2a* & *mhc2b*), or cytokines (*il1b*, *il8*, & *tgfb*). After FDR correction, if a significant main effect was identified by the Two-Way ANOVA, Tukey's Post-Hoc were conducted to identify differences between groups.

3.3 Results

Relative transcript abundance of heat shock and immune genes was assessed in the gill (**Figure 3.1**) and ventricle (**Figure 3.2**) to identify whether ploidy or acute thermal stress recovery impacted their expression. Each figure depicts the relative abundance of genes involved in heat shock responses (**A** & **B**), pathogen sensing (**C** & **D**), endogenous peptide presentation to immune cells (**E**, **F**, & **G**), exogenous peptide presentation to immune cells (**H** & **I**), or intercellular communication (**J**, **K**, & **L**).

3.3.1 Gill heat shock and immune gene expression

Heat shock gene relative expression was assessed to confirm that acute thermal stress recovered fish were mounting a heat shock response at the gill. Expression of *hsp70* (**Figure 3.1A**) was found to have a treatment effect (Two-Way ANOVA, $F_{1,28} = 587.8$, $p < 0.0001$), where thermal stress recovered fish (irrespective of ploidy) had ~500x higher abundance relative to control groups (Tukey's Post-Hoc, $p \leq 0.05$). Similarly, expression of *hsp90aa1* (**Figure 3.1B**) had a treatment (Two-Way ANOVA, $F_{1,27} = 167.1$, $p < 0.0001$) and interaction (Two-Way ANOVA, $F_{1,27} = 4.910$, $p = 0.049$), with treatment groups having significantly elevated transcript abundance (Tukey's Post-Hoc, $p \leq 0.05$). In spite of the interaction, there was no difference between ploidies within each treatment (Tukey's Post-Hoc, $p > 0.05$).

Genes involved in extracellular (*tlr2*) and intracellular (*nlrp3*) pathogen sensing were assessed to identify differences associated with ploidy and/or acute thermal stress recovery. Gill expression of *tlr2* (**Figure 3.1C**) had both a ploidy (Two-Way ANOVA, $F_{1,28} = 16.71$, $p = 0.0009$)

and treatment effect (Two-Way ANOVA, $F_{1,28} = 6.285$, $p = 0.0192$). Triploid fish had ~50% as many transcripts compared to diploid control fish (Tukey's Post-Hoc, $p \leq 0.05$). However, the diploid thermal stress-recovered group had similar expression relative to all other groups (Tukey's Post-Hoc, $p > 0.05$). Gill expression of *nlrp3* (**Figure 3.1D**) had a ploidy effect (Two-Way ANOVA, $F_{1,28} = 11.12$, $p = 0.0038$). Triploid fish had significantly higher expression relative to acute thermal stress recovered diploid fish (Tukey's Post-Hoc, $p \leq 0.05$). Similar to *tlr2*, this relationship was not identified between triploids and the diploid control group (Tukey's Post-Hoc, $p > 0.05$).

Genes involved in the MHC class I pathway, both loading peptides (*tapasin*) and the structural component of the cell surface protein (*mhc1a* & *b2m*), were assessed to identify whether gill expression was impacted. Expression of *tapasin* (**Figure 3.1E**) was not significantly impacted by ploidy (Two-Way ANOVA, $F_{1,28} = 6.508$, $p = 0.0693$), treatment (Two-Way ANOVA, $F_{1,28} = 0.6150$, $p = 0.5274$) or their interaction (Two-Way ANOVA, $F_{1,28} = 0.089$, $p = 0.7164$). Similarly, *mhc1a* (**Figure 3.1F**) abundance was not impacted by ploidy (Two-Way ANOVA, $F_{1,28} = 2.289$, $p = 0.297$), treatment (Two-Way ANOVA, $F_{1,28} = 0.8025$, $p = 0.5274$), or their interaction (Two-Way ANOVA, $F_{1,28} = 0.6643$, $p = 0.6975$). However, *b2m* (**Figure 3.1G**) relative abundance was identified as having a ploidy effect (Two-Way ANOVA, $F_{1,28} = 38.29$, $p < 0.0001$). Acute thermal stress recovered triploid fish had significantly higher *b2m* expression relative to both diploid groups (Tukey's Post-Hoc, $p \leq 0.05$). Additionally, triploid controls had higher expression relative to diploid controls (Tukey's Post-Hoc, $p < 0.0001$).

Genes involved in formation of the MHC class II protein (*mhc2a* & *mhc2b*) were assessed to investigate whether ploidy and/or recovery from thermal stress impacted gill exogenous peptide presentation. Relative transcript abundance of *mhc2a* (**Figure 3.1H**) had a ploidy effect (Two-Way ANOVA, $F_{1,28} = 12.47$, $p = 0.0079$), where triploid fish had significantly elevated abundance relative to diploid thermal stress recovered fish (Tukey's Post-Hoc, $p \leq 0.05$). This difference was not observed between diploid controls and any other group (Tukey's Post-Hoc, $p > 0.05$). Additionally, abundance of *mhc2b* (**Figure 3.1I**) was not significantly impacted by ploidy (Two-Way ANOVA, $F_{1,28} = 1.647$, $p = 0.2755$), treatment (Two-Way ANOVA, $F_{1,28} = 0.5686$, $p = 0.48$), or their interaction (Two-Way ANOVA, $F_{1,28} = 0.1262$, $p = 0.6345$).

Finally, relative expression of cytokines (*il1b*, *il8*, and *tgfb*) was assessed to identify whether immune cell signalling was impacted in the gill for this study. Gill abundance of *il1b* (**Figure 3.1J**)

was identified as having both a ploidy (Two-Way ANOVA, $F_{1,28} = 19.84$, $p < 0.0001$), and treatment effect (Two-Way ANOVA, $F_{1,28} = 140.9$, $p < 0.0001$). At baseline, there was no difference between diploid and triploid fish (Tukey's Post-Hoc, $p = 0.0897$). However, both ploidies had significantly elevated abundance after recovery from thermal stress (Tukey's Post-Hoc, $p \leq 0.05$). Additionally, triploid expression at this timepoint was significantly higher than diploids (Tukey's Post-Hoc, $p = 0.0033$). Abundance of *il8* (**Figure 3.1K**) was also significantly impacted in this study, with ploidy effects (Two-Way ANOVA, $F_{1,28} = 26.61$, $p < 0.0001$), treatment effects (Two-Way ANOVA, $F_{1,28} = 36.10$, $p < 0.0001$), and interaction (Two-Way ANOVA, $F_{1,28} = 7.765$, $p = 0.005$). Triploid fish recovered from acute thermal stress had significantly higher abundance relative to all other groups (Tukey's Post-Hoc, $p \leq 0.05$). Relative transcript abundance of *tgfb* (**Figure 3.1L**) had a significant ploidy effect (Two-Way ANOVA, $F_{1,28} = 9.072$, $p = 0.0035$), where triploid fish recovered from acute thermal stress had higher expression relative to diploid control fish (Tukey's Post-Hoc, $p = 0.041$).

3.3.2 Ventricle heat shock and immune gene expression

Ventricle heat shock gene (*hsp70* & *hsp90aa1*) expression was assessed to confirm that the heart responded molecularly to the thermal stress. Relative transcript abundance of *hsp70* (**Figure 3.2A**) had a treatment effect (Two-Way ANOVA, $F_{1,26} = 1289$, $p < 0.0001$), where acute thermal stress recovered fish had ~400x the number of transcripts compared to control fish (Tukey's Post-Hoc, $p \leq 0.05$). Ventricle-associated *hsp90aa1* (**Figure 3.2B**) abundance had both a ploidy (Two-Way ANOVA, $F_{1,26} = 6.972$, $p = 0.0145$) and treatment effect (Two-Way ANOVA, $F_{1,26} = 238.1$, $p < 0.0001$). Similar to *hsp70*, thermally recovered fish had ~10x greater relative abundance compared to non-heat stressed individuals (Tukey's Post-Hoc, $p \leq 0.05$). However, triploid control fish had ~2x greater relative abundance of *hsp90aa1* compared to diploid fish (Tukey's Post-Hoc, $p = 0.0249$).

Abundance of PRRs (*tlr2* & *nlrp3*) were assessed to investigate changes in the ventricle's ability to sense pathogen presence. Abundance of *tlr2* (**Figure 3.2C**) did not change in response to ploidy (Two-Way ANOVA, $F_{1,26} = 2.439$, $p = 0.137$), treatment (Two-Way ANOVA, $F_{1,26} = 0.1361$, $p = 0.500$), or their interaction (Two-Way ANOVA, $F_{1,26} = 0.3587$, $p = 0.4657$). However, relative transcript abundance for *nlrp3* (**Figure 3.2D**) was identified as having both a ploidy (Two-Way ANOVA, $F_{1,26} = 50.39$, $p < 0.0001$) and treatment effect (Two-Way ANOVA, $F_{1,26} = 9.281$, $p = 0.0111$). Triploid control fish had significantly lower abundance relative to both diploid groups (Tukey's Post-Hoc, $p \leq 0.05$) and the acute thermal stress recovered fish had even less expression,

relative to both the diploid groups (Tukey's Post-Hoc, $p \leq 0.05$) and the triploid controls (Tukey's Post-Hoc, $p = 0.0093$).

MHC class I expression was assessed in the ventricle to identify whether genes involved in endogenous antigen presentation were impacted. Ventricle abundance of *tapasin* (**Figure 3.2E**) was identified as having a ploidy effect (Two-Way ANOVA, $F_{1,26} = 19.43$, $p = 0.00168$). Triploid fish all had significantly less *tapasin* abundance relative to diploid control fish (Tukey's Post-Hoc, $p \leq 0.05$). Additionally, triploid acute thermal stress recovered individuals had significantly lower abundance relative to diploids exposed to the same stress (Tukey's Post-Hoc, $p = 0.02$). Abundance of *mhc1a* (**Figure 3.2F**) was not impacted by ploidy (Two-Way ANOVA, $F_{1,26} = 1.163$, $p = 0.407$), treatment (Two-Way ANOVA, $F_{1,26} = 1.888$, $p = 0.407$), or their interaction (Two-Way ANOVA, $F_{1,26} = 0.491$, $p = 0.5875$). Similarly, ventricular abundance of *b2m* (**Figure 3.2G**) had no ploidy effect (Two-Way ANOVA, $F_{1,26} = 0.04883$, $p = 0.868$), treatment effect (Two-Way ANOVA, $F_{1,26} = 1.242$, $p = 0.407$), or interaction (Two-Way ANOVA, $F_{1,26} = 1.483$, $p = 0.407$).

MHC class II expression (*mhc2a* & *mhc2b*) was assessed in the ventricle to identify whether exogenous peptide presentation was impacted by ploidy or thermal stress. Similar to the structural genes for MHC class I, ventricle abundance of *mhc2a* (**Figure 3.2H**) was not impacted ploidy (Two-Way ANOVA, $F_{1,26} = 0.1728$, $p = 0.715$), treatment (Two-Way ANOVA, $F_{1,26} = 6.932$, $p = 0.0889$), or their interaction (Two-Way ANOVA, $F_{1,26} = 0.4778$, $p = 0.7151$). The same response was identified for ventricular *mhc2b* (**Figure 3.2I**), with a lack of any ploidy effects (Two-Way ANOVA, $F_{1,26} = 0.3769$, $p = 0.715$), treatment effects (Two-Way ANOVA, $F_{1,26} = 0.4411$, $p = 0.715$), or their interaction (Two-Way ANOVA, $F_{1,26} = 0.3076$, $p = 0.7151$).

Finally, ventricular expression of cytokines (*il1b*, *il8*, & *tgfb*) were assessed to identify whether immune signalling was impacted by the groups in this tissue. Relative transcript abundance of ventricle *il1b* (**Figure 3.2J**) had a treatment effect (Two-Way ANOVA, $F_{1,26} = 156.2$, $p < 0.0001$). Acute thermal stress recovery was associated with a 10-15x higher abundance relative to control groups (Tukey's Post-Hoc, $p \leq 0.05$), irrespective of ploidy. Ventricle abundance of *il8* (**Figure 3.2K**) was not impacted by ploidy (Two-Way ANOVA, $F_{1,26} = 2.342$, $p = 0.290$), treatment (Two-Way ANOVA, $F_{1,26} = 3.403$, $p = 0.214$), or their interaction (Two-Way ANOVA, $F_{1,26} = 0.360$, $p = 0.6648$). Similarly, no impact of ploidy (Two-Way ANOVA, $F_{1,26} = 0.5866$, $p = 0.631$), treatment (Two-Way

ANOVA, $F_{1,26} = 0.7885$, $p = 0.631$), or interaction (Two-Way ANOVA, $F_{1,26} = 0.00004$, $p = 0.9287$) was found for relative transcript abundance of ventricular *tgfb* (**Figure 3.2L**).

3.4 Discussion

The objective of this study was to characterize the immunological implications of triploidy, while also assessing how each ploidy would respond after recovery from acute thermal stress. Overall, both tissues had specific genes that responded to increased ploidy but there was no clear trend in specific immune functions altered in triploids. Gill had more differentially expressed transcripts, as indicated by altered abundance of PRRs (*tlr2* & *nlrp3*) and cytokines (*il1b*, *il8*, & *tgfb*). Ventricles had few immune impacts, except for *nlrp3* and *tapasin* which had lower abundance in triploid fishes and decreased after recovery from thermal stress. In both tissues, *il1b* abundance had similar response to heat shock proteins, indicating that the transcript relative abundance of this inflammatory cytokine responds to thermal stress.

Clear heat shock response was mounted in both tissues as indicated by significant upregulation of *hsp70* and *hsp90aa1*. A 1-hour recovery post-CT_{max} was chosen because it has been validated that the molecular heat shock response is upregulated at this timepoint (Fangue et al., 2011; Weber et al., 2023). Therefore, any changes in immune transcript abundance are likely associated with heat shock responses since Hsp70 and Hsp90 are upregulated during the teleost heat shock response (reviewed by Roberts et al., 2010). These protein chaperones act to refold misfolded proteins or target them for degradation (Hartl et al., 2011; Kiang, 1998). However, Hsp90 has constitutive functions in protein folding and signal transduction, and therefore has high cellular abundance even under non-stress conditions (Taipale et al., 2010). In this study, *hsp90aa1* had higher abundance in triploids relative to diploids, which could be due to these alternative roles rather than increased triploid ventricular protein denaturation in the absence of stress.

Expression of PRRs involved in sensing external (*tlr2*) and internal (*nlrp3*) had altered abundance in the gill and ventricle, respectively. Toll-like receptor 2 (*tlr2*) is a cell surface PRR and is involved in sensing bacterial pathogen associated molecular patterns (PAMPs) by dimerizing with Tlr1 or Tlr6, depending on the PAMP (Aliprantis et al., 1999; Takeda, 2004). However, although *tlr2* expression in fish is modulated by infection, rainbow trout Tlr-2 is unable to stimulate Nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) transcription factor activity after PAMP exposure

(Basu et al., 2012; Brietzke et al., 2016; S. Guo et al., 2024; Wu et al., 2018). Although the function of this PRR is unknown in salmonids, it has immunomodulatory capacity and the reduced expression in triploid gills is indicative of altered pathogen sensitivity in these fish. NOD-like receptor pyrin domain 3 (Nlrp3) is a subunit of the inflammasome, which is transcribed after immune stimulation (via NF- κ B; Bauernfeind et al., 2009). The inflammasome is then activated by many different signals, including (but not limited to) bacterial RNA (Gupta et al., 2014), reactive oxygen species (Zhou et al., 2011), and altered ion (K^+ , Na^+ , Cl^+ , & Ca^{2+}) concentrations (Muñoz-Planillo et al., 2013; Schorn et al., 2011; Tang et al., 2017; Weber & Schilling, 2014). The relationship between Nlrp3 and K^+ is particularly interesting for the current study since reduced cellular $[K^+]$ is associated with inflammasome activation (Muñoz-Planillo et al., 2013). Elevated temperature is associated with altered cardiac intracellular $[K^+]$ due to myocyte ion leakage (Haverinen & Vornanen, 2020). Na^+ influx-associated cell swelling reduces $[K^+]$, resulting in inflammasome activation (Schorn et al., 2011); therefore, reduced triploid cardiac *nlrp3* could be a compensatory mechanism to prevent excessive inflammation. Triploids have larger cells (Benfey, 1999), and therefore the changes in intracellular $[K^+]$ could be more impactful in these individuals. Additionally, acute stressors increase plasma $[K^+]$ due to ion leakage from skeletal muscle, which is associated with acidosis (Ellis et al., 2013; Nielsen & Lykkeboe, 1992; Thomas et al., 1987; Wells et al., 1986). Although plasma $[K^+]$ has been found to be similar between ploidies after stress (Ellis et al., 2013; Lahnsteiner, 2022; Preston et al., 2017), the reduced *nlrp3* in triploids after thermal stress recovery could be to further mitigate any inflammasome activation due to hyperkalemia during the CT_{max} trial. However, this does not explain why diploids did not have a similar reduced expression and does not explain the elevated expression in triploid gills. This altered triploid gill expression could be due to gene dosage effects in triploids (see below), or increased abundance of immune cells in triploid gills. Further study on the electrochemical implications of increased ploidy and how this interacts with the immune system could provide clarity on reduced triploid performance.

In this study, expression of both endogenous (MHC class I) and exogenous (MHC class II) did not clearly indicate whether these pathways are impacted by triploidy or exposure to thermal stress. Specific gene changes provide interesting insight into the impacts of these treatments at the tissue level. Relative abundance of *tapasin* was lower in triploid ventricles relative to diploid control fish. Tapasin is a component of the peptide loading complex for MHC class I and, in rainbow trout, connects the transporter associated with antigen processing (TAP) and the MHC class I protein (Sever

et al., 2018). Therefore, this data could implicate reduced peptide loading of MHC class I in triploid salmon hearts. Expression of *tapasin* in rainbow trout has been shown to be impacted by low temperature *in vitro* (Abram et al., 2019), but no studies have looked at how high temperature could influence this gene. Thermally recovered diploids did not have the same difference in expression, which could indicate some thermal regulation of this gene. Expression of *mhc1a* did not change in either tissue, in spite of other studies that have shown that abundance changes in response to ploidy (Ching et al., 2010) and exposure to thermal stress (Guo et al., 2023). Sampling at different timepoints could be the source of this discrepancy, as Guo et al., 2023 sampled directly at CT_{max} in contrast to allowing for recovery. Elevated *b2m* in triploid gills have been previously documented, indicating that this could be a common expression profile associated with these fishes (Ching et al., 2010). The ploidy difference is most clear between both triploid groups and the control diploids, which could indicate some thermal regulation of this gene, in corroboration with other studies (Perez-Casanova et al., 2008; Rodrigues et al., 1998). However, the biological implications of this dysregulation are unclear since, in addition to forming the MHC class I surface receptor, B2m is also involved in antibody transport and iron regulation (Feder et al., 1998; Praetor & Hunziker, 2002; Schaible et al., 2002). The only significant change in MHC class II occurred in the gill, where triploids had higher abundance of *mhc2a* relative to thermally recovered diploids. Similar to *nlrp3* expression in the same tissue, this difference might be more related to gene dosage impacts that thermal stress exacerbates, rather than altered MHC class II abundance. Although expression of MHC class II decreases in response to cold stress, high temperatures have not been identified as impacting genes in this pathway (Dittmar et al., 2014; Nath et al., 2006; Raida & Buchmann, 2007). Overall, it appears that ploidy impacts only specific MHC gene expression (rather than expression of the entire complex), and that thermal exposure has some impact on these altered genes.

Interestingly, cytokine expression was altered significantly in the gills, and in the ventricle *il1b* abundance validated that this gene is upregulated after thermal stress. Upregulation of *il1b* transcripts after thermal stress is consistent with *in vitro* and *in vivo* studies in many different fish species (Chen et al., 2014; Guo et al., 2023; Khosravi-Katuli et al., 2021; Perez-Casanova et al., 2008; Zhang et al., 2015). Stress-induced upregulation of *il1b* has been identified in fish and is thought to be driven in part by cortisol stimulation (Fast et al., 2008). The fish exposed to thermal stress had elevated plasma cortisol (Rogozynski et al., 2024) which could be driving the increase after thermal stress recovery. Additionally, it is speculated that increases in Hsp70 upregulates *il1b* expression,

since exposing grass carp (*Ctenopharyngodon idella*) head kidney leukocytes to recombinant Hsp70 resulted in increased expression of *il1b* and elevated release of the cytokine (Zhang et al., 2015). Although it is expected that thermal stress would increase expression of this transcript, it is important to note that triploid abundance of *il1b* was higher after recovery from heat stress compared to the recovered diploids. Gill expression of *il1b* has been found to be similar between diploid and triploid Chinook salmon after *Vibrio* infection and lower in triploid yellow tail tetra (*Astyanax altiparanae*) without infection (Ching et al., 2010; Levy-Pereira et al., 2021). However, H₂O₂ exposure resulted in time-dependent differences in gill *il1b* expression between diploid and triploid Atlantic salmon (*Salmo salar*; Chalmers et al., 2018). Therefore, a more realistic explanation for this ploidy difference after recovery from thermal stress is due to altered timing in the expression of this transcript in triploids after exposure to stress. A similar response was identified in gill expression of *il8*, where only triploid fish had elevated abundance after recovery from thermal stress. Il-8 (also called Cxcl8) is a pro-inflammatory chemokine that can be produced by most cells and is involved in recruiting neutrophils to infection sites (Leonard & Yoshimura, 1990). Studies in fish corroborate that mRNA expression of this chemokine responds to heat stress (Esam et al., 2022; Lee et al., 2023; Liang et al., 2022). The gill difference between ploidies after recovery from thermal stress provides further evidence for altered expression timing dynamics in triploids, since the only group with significantly elevated *il8* abundance was thermal stress recovered triploids. The final gene investigated was *tgfb*, which is an anti-inflammatory cytokine that can be induced by proinflammatory cytokines such as Il-1 β (Yang et al., 2014). The only statistical difference identified was in the gill, between diploid control fish and triploid thermal stress recovered fish, in spite of upregulation of *il1b* in both tissues. Although *tgfb* expression did not follow the same trend as *il1b*, there is some evidence for it being thermally modulated in fish. Simulated heat waves in three-spine stickleback (*Gasterosteus aculeatus*) and Chinese tongue sole (*Cynoglossus semilaevis*) have been shown to upregulate *tgfb* in head kidney and brain, respectively (Dittmar et al., 2014; Y. Wang, Su, et al., 2023). The lack of anti-inflammatory signalling occurring in conjunction with pro-inflammatory signalling could implicate dysregulated immune function, but measurements of protein levels for Il-1 β would be necessary to confirm whether the transcriptional impacts are functionally relevant.

Overall, thermal stress appears to result in immunomodulatory impacts in the gill and ventricle at the transcriptional level. However, it is important to note that this study did not go beyond relative mRNA abundance, so interpretation of the results should be carefully considered. When an

extra copy of DNA is added (e.g. triploidy in salmon), gene expression of protein-coding transcripts can change due to changes in abundance of regulatory genes (reviewed by Birchler et al., 2001). Three potential results will occur in organisms with increased gene copies: positive relative abundance changes (gene dosage effect), decreased relative abundance changes (inverse dosage effect), or no difference in relative abundance (dosage compensation). Throughout this entire dataset all three expression impacts are present, which can complicate interpretation of the phenotypic effects. Therefore, investigating the protein abundance of these genes is vital to understand how immune function is altered in triploids and after thermal stress. Additionally, making sure to account for how changes in cell number (Small et al., 2024; Van De Pol et al., 2020) and increases in plasma membrane area to maintain surface area to volume ratio (Benfey, 1999) could impact function of these genes will provide insight into the physiological implications of increased ploidy.

Although this study provides some clarity on the altered performance of triploids in response to thermal stress, there are study limitations that need to be addressed. Firstly, only one timepoint after thermal stress was accounted for. Immune genes are dynamic and expressed in a time-dependent manner to allow for pathogen clearance before the return to homeostasis (Soto-Dávila et al., 2024). Across all genes measured, triploid differences were rarely associated with both diploid groups which indicates that expression of these genes would be altered if sampling occurred at CT_{max} , or even longer after thermal stress. Therefore, future studies should utilize multiple timepoints to get a clearer explanation regarding how immune function is impacted after thermal stress in triploids. Secondly, diploids and triploids in this study had different rearing conditions. Diploids were immunized and kept in saltwater net pens for 1.5 years while triploids were maintained in fresh water throughout their lives. Diploid and triploid Atlantic salmon have similar responses to vaccination, although there are ploidy differences in specific immune parameters (e.g. macrophage respiratory burst) in spite of vaccination status (Chalmers et al., 2016). Vaccination has been shown to impact expression of MHC transcripts across tissues (Koppang et al., 1998a; 1998b); however, this response required re-vaccination to observe long term impacts. Overall, vaccination-associated differences might not be as important due to poor immunological memory in fish as this trial was conducted two years after immunization (Bromage et al., 2004; reviewed by Magor, 2015; Vigliano et al., 2006). However, a bigger issue is the extensive time diploids spent in salt water. Seawater has high pathogen loads so fish are under greater immunological assault during saltwater residency (Mauduit et al., 2022). Although six months in freshwater should be enough time for any differences in immune expression

to be reduced, this increased exposure to pathogens could impact how gene expression responds to thermal stress. Smoltification (during transfer from freshwater to saltwater) and parr-reversion (during transfer back to freshwater) could also be impacting the expression profiles observed. Diploid and triploid rainbow trout have similar immune responses to saltwater transfer (Taylor et al., 2007), and some evidence exists for freshwater residency in Chinook salmon resulting in similar hormonal changes to smoltification (Cowley et al., 1994). In another rainbow trout study, saltwater acclimation and vaccination resulted in differential immune transcript expression after exposure to a chase stressor (Khansari et al., 2024). Therefore, it cannot be disregarded that these rearing condition differences resulted in the ploidy effects identified. Future work should validate whether these profiles exist in similarly cultured salmon. Thirdly, the immune implications in this study are hard to interpret without a co-occurring infection. The immune system is highly regulated to prevent excessive cell damage in the absence of pathogens, so any changes in expression that occur might not have functional repercussions. Utilizing a pathogen after exposure to acute thermal stress would provide clarity on whether triploids have altered performance in response to these multiple stressors.

In conclusion, this study identified that recovery from an acute thermal challenge impacted immune gene expression in both diploid and triploid Chinook salmon. The gill appeared to have more ploidy differences compared to the ventricle, and these differences could result in triploid salmon having reduced capacity to sense pathogens in the environment. This study also validated that thermal stress modulates specific immune genes that could be involved in improving fish response to opportunistic pathogens that are more prevalent at elevated temperature. Future work should further investigate these changes at the protein level to validate phenotypic impacts from increased ploidy and utilize a pathogen challenge to confirm whether altered expression is protective or detrimental.

Table 3.1: Primer information for gene expression analysis

M scores were calculated using RefFinder (Xie et al., 2023) for each tissue that the associated reference gene was used. Primer efficiency and r^2 for each gene was calculated based on a 7-point standard dilution series (Pfaffl, 2001). A “-” for M score was used if that gene was not utilized as a reference gene for that tissue. Genes without a reference were designed for the present study.

Target	Accession	Forward primer sequence	Reverse Primer sequence	Amplicon length (bp)	Gill				Ventricle				Reference
					M Score	Annealing Temp	Efficiency (%)	r^2	M Score	Annealing Temp	Efficiency (%)	r^2	
<i>dcp1a</i>	XM_024375248	CGTCGTAGTTCGACCATAAA	CCACCCACAGAATCGAGAAA	-	-	60°C	94.4	0.999	0.661	60°C	91.5	0.999	Giroux <i>et al.</i> , 2019
<i>ef1α</i>	XM_024396038.2	CGCACAGTAACACCGAACTAATTAAGC	GCCTCCGCACTTGATAGATCAGATG	131	0.557	60°C	100.2	0.999	-				Thraya <i>et al.</i> , 2019
<i>β actin</i>	XM_042316123.1	TGGACTTTGAGCAGGAGATGG	AGGAAGGAGGGCTGGAAGAG	139	0.557	60°C	99.3	0.999	0.541	60°C	93.3	0.991	Ma <i>et al.</i> , 2019
<i>hsp70</i>	XM_042307454.1	GAGACAGGCCACTAAGGACG	GACATGCCTTTGTCCATGCC	108		60°C	95.3	0.998		60°C	100.5	0.994	
<i>hsp90aa1</i>	XM_042300925.1	ATGATCGGGCAGTTCCGGTG	AGTGTCACCTTTGACAGTGAA	141		65°C	90.2	0.995		65°C	105	0.997	von Biela <i>et al.</i> , 2020
<i>tlr2</i>	XM_042298531.1	GCCATAGTTCACCTTCATGGAGGTT	TGGAACGTTGCGTCCTCTAC	74		60°C	101.6	0.999		60°C	101.8	0.985	
<i>nlrp3</i>	XM_024386851.2	TGGAGCCATATTCATCGCCA	TACCTTTGCTCCGACTCTC	97		60°C	93.8	0.994		60°C	92.6	0.994	
<i>tapasin</i>	XM_024441639.2	AGGGACGGAGGCTCTACAATC	GCTGAAGTGCGCCACACA	101		60°C	99.5	0.998		60°C	108	0.999	
<i>mhc1a</i>	XM_024441638.2	ATGCTGTTCTTGCCGTCTTTCT	AAAGTCCGTCCCTCAGTGTCTCT	123		60°C	100.7	0.995		60°C	95.3	0.999	
<i>b2m</i>	L47354.1	CAGACCTGGCCTTGAACAG	AAGCCCCAAGTTTCAGCTCT	228		55°C	108	0.998		55°C	104.6	0.994	
<i>mhc2a</i>	XM_024408463.2	CCTTACTGAGCCCCTGACAC	GCAACTCCCATTAGCCCCAG	108		60°C	95.1	0.996		60°C	103	0.999	
<i>mhc2b</i>	XM_042296278.1	AAGGTCCTCAGCTGGGTCAA	GCTCAACTGTCTTGCCAGTATGG	99		60°C	97.5	0.997		60°C	99.6	0.999	
<i>il1b</i>	XM_024418276.2	ACCGAGTTCAAGGACAAGGA	CATTCATCAGGACCCAGCAC	181		60°C	98.4	0.991		60°C	104.3	0.999	
<i>il8</i>	XM_024415648.2	ATTGAGACGGAAAGCAGACG	CGCTGACATCCAGACAAATCT	136		60°C	108.2	0.988		60°C	100.1	0.995	Wang <i>et al.</i> , 2020
<i>tgfb</i>	XM_024397891.2	TGTGGGGAGACAACACAAGG	AAACCAGCGCCATCAAAAAGG	164		60°C	102.9	0.99		60°C	100.4	0.999	

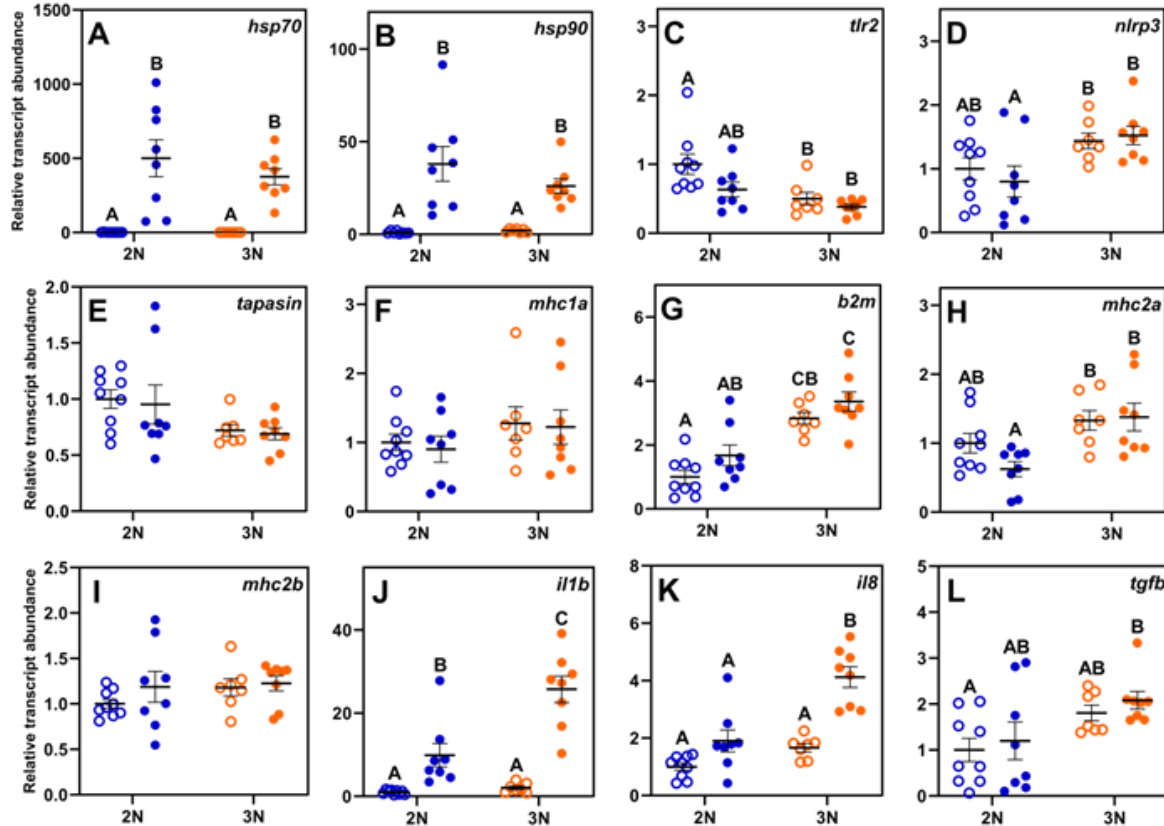


Figure 3.1: Gill relative transcript abundance in control (open circle) and acute thermal stress recovered (filled-in circle) diploid and triploid Chinook salmon

Control fish (2N n = 9, 3N n = 7) were sampled directly from stocking troughs while heat fish (2N n = 8, 3N n = 8) were sampled after one hour of recovery from CT_{max} . All expression was normalized to diploid control fish. Genes measured are associated with heat shock responses (A & B), pathogen sensing (C & D), intracellular peptide presentation to immune cells (E, F, & G), extracellular peptide presentation to immune cells (H & I), or intracellular communication (J, K, & L). Horizontal lines for each group indicate mean ($\pm$ SEM) and groups that do not share a common letter are statistically different from each other (Two-Way ANOVA, Tukey's post-hoc, $p \leq 0.05$).

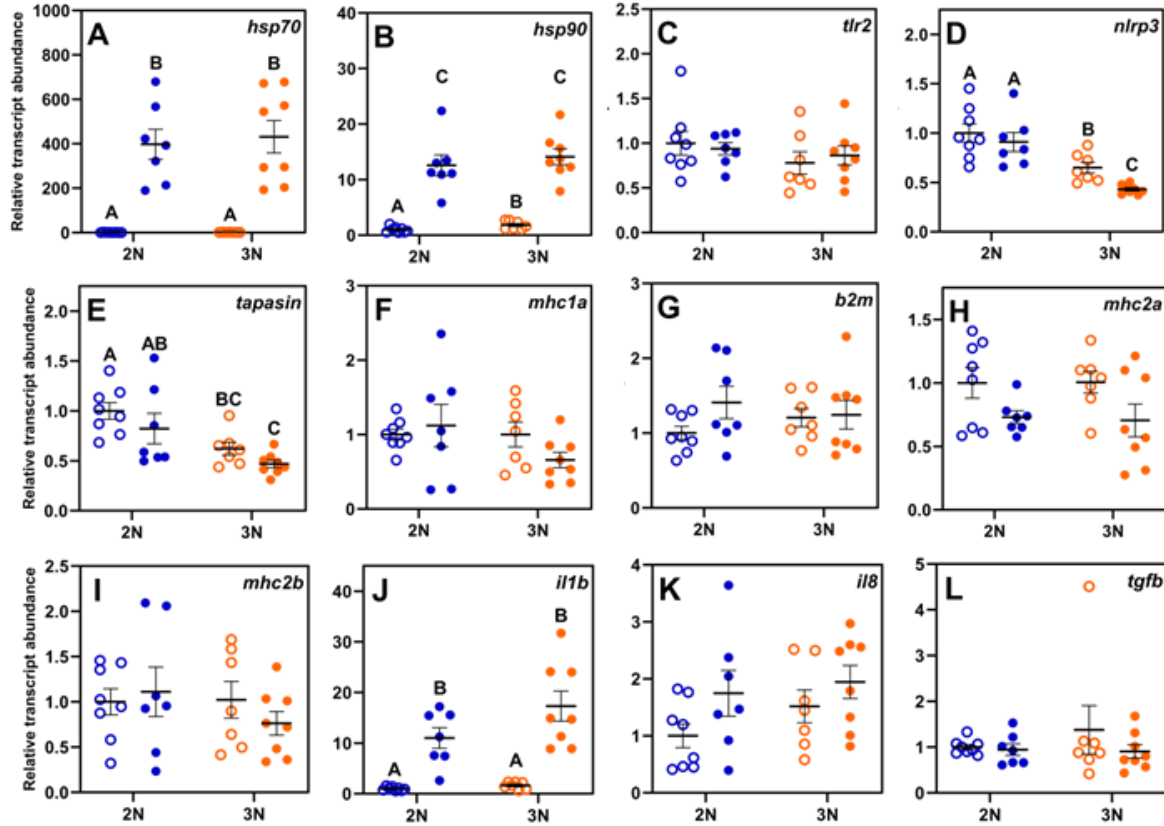


Figure 3.2: Ventricle relative transcript abundance in control (open circle) and acute thermal stress recovered (filled-in circle) diploid and triploid Chinook salmon

Control fish (2N n = 8, 3N n = 7) were sampled directly from stocking troughs while heat fish (2N n = 7, 3N n = 8) were sampled after one hour of recovery from CT_{max} . All expression was normalized to diploid control fish. Genes measured are associated with heat shock responses (A & B), pathogen sensing (C & D), intracellular peptide presentation to immune cells (E, F, & G), extracellular peptide presentation to immune cells (H & I), or intracellular communication (J, K, & L). Horizontal lines for each group indicate mean ($\pm$ SEM) and groups that do not share a common letter are statistically different from each other (Two-Way ANOVA, Tukey's post-hoc, $p \leq 0.05$).

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Chapter 4

Reduced thermal performance of triploid Chinook salmon (*Oncorhynchus tshawytscha*) is associated with cardiac arrhythmia and altered cardiorespiratory gene expression in the ventricle

4.1 Introduction

Climate change-associated anthropogenic disturbances will exacerbate aquatic stressors and sea-based aquaculture will have to adapt to these issues. To this end, changes in abiotic indices (i.e temperature, dissolved oxygen) may directly impact the physiology of finfish stocks while more frequent extreme weather events will increase infrastructure damage (reviewed by Reid et al., 2019). These disturbances may also increase the risk of escapees, which are of particular concern due to their potential to interact and breed with wild populations (Jensen et al., 2010; Lura and Sægrov, 1991; Skaala et al., 2006). Alterations in genetic diversity directly impact traits that are important for survival such as thermal tolerance, which can increase pressure on already at-risk endemic species (Debes et al., 2021). Farming of triploid (autopolyploid) fishes offers a sustainable solution to this problem since these fish are physiologically sterile and less likely to migrate into spawning habitats should they escape (Benfey, 1999; Glover et al., 2016). Triploid fishes have three chromosome copies due to retention of the second polar body after fertilization, which prevents chromosomal alignment during gametogenesis (reviewed by Piferrer et al., 2009). Although some autopolyploids can produce viable gametes (e.g. genus *Acipenser*; Gille et al., 2015; Havelka et al., 2014; Pšenička et al., 2011), female salmonid triploids are more successfully sterile and their widespread farming would reduce pressure on at-risk ecosystems.

The primary challenges in culturing triploid salmon stem from their reduced performance under stressful conditions. Triploid salmonids exhibit higher mortality in response to osmotic and pathogen stressors, which are regularly experienced in aquaculture during the production cycle (Ching et al., 2010; Jhingan et al., 2003; Johnson et al., 2019). Additionally, triploid fishes are more susceptible to the effects of climate change due to their reduced tolerance of elevated temperature. Triploid salmonids have lower thermal optima relative to diploids, as indicated by their reduced metabolic scope at elevated temperatures (Altimiras et al., 2002; Atkins and Benfey, 2008). Triploid brook trout (*Salvelinus fontinalis*) and Atlantic salmon (*Salmo salar*) have altered plasma and muscle metabolite (lactate, glucose) levels when exposed to elevated temperatures, which indicates altered utilization of anaerobic pathways (Hyndman et al., 2003; Sambras et al., 2018). To this end, chronic exposure to high temperatures results in higher mortalities in triploid rainbow (*Oncorhynchus mykiss*) and brook trout in comparison to diploids (Hyndman et al., 2003; Ojolick et al., 1995). Moreover, triploid brook trout and Atlantic salmon have lower critical thermal maximum (CT_{max}) and exhibit

reduced survival compared to diploids when oxygen levels become hypoxic (Ellis et al., 2013; Hansen et al., 2015).

Acute increases in temperature raise aerobic demands in ectothermic poikilotherms, increasing pressure on the cardiovascular system to deliver oxygen to tissues. Heart rate increases proportionally to temperature until reaching maximum heart rate, at which point cardiac function starts to diminish (Farrell et al., 1996; Gamperl et al., 2022; Heath and Hughes, 1973). At these thermal limits, the salmonid heart is unable to acquire enough oxygen for its own aerobic metabolism since it primarily receives deoxygenated venous return blood supply (Eliason et al., 2013; A.P Farrell, 2002). Athletic fishes such as salmon lack extensive coronary circulation throughout the heart and have adapted to increase oxygen diffusion into the myocardium from this oxygen-poor supply. Plasma accessible carbonic anhydrase (Ca4) is present in the luminal cells of atrium and spongy myocardium, which reduces blood pH in cardiac lumen (Alderman et al., 2016). This facilitates offloading of excess O₂ bound to pH-sensitive hemoglobin, which diffuses into the spongy myocardium in response to high tissue myoglobin content (Bailey and Driedzic, 1986; Rummer et al., 2013; Rummer and Brauner, 2015). This oxygen delivery system is of particular interest since triploids have been identified as having reduced arterial oxygen content during swimming and reduced hypoxia tolerance (Bernier et al., 2004; Jensen and Benfey, 2022; Samba et al., 2017; Scott et al., 2015).

In recent years, studies have shown that triploid rainbow trout hearts become arrhythmic at lower temperatures relative to diploids (Verhille et al., 2013). However, no studies have investigated whether the oxygen diffusion system is hindered in triploid fishes, but previous work has identified that regulation of gene expression in response to stress is impacted by ploidy (Cadonic et al., 2023; Ching et al., 2010). Triploid Chinook salmon (*Oncorhynchus tshawytscha*) have altered microRNA (miRNA) expression before and after pathogen stress, which is associated with reduced immune function (Cadonic et al., 2023). MicroRNAs regulate gene expression by binding to the 3' untranslated region (UTR) of specific genes and either targeting the transcript for degradation or reducing translational efficiency (Lee et al., 1993; Wightman et al., 1993; Zeng et al., 2003). Therefore, it is possible that differential expression and/or gene dosage of miRNAs in triploid salmon could contribute to their altered cardiac function. Indeed, miRNAs respond to thermal stress in teleosts, have been identified as regulating heat stress-associated gene expression, and regulate cardiac responses to low oxygen (Chen et al., 2022; Liu et al., 2022; Vasadia et al., 2019). However,

whether reduced thermal performance in triploids is associated with altered miRNA expression remains unclear.

The objective of the present study is to characterize cardiac performance of diploid and triploid Chinook salmon in response to an acute temperature challenge. It is hypothesized that the reduced thermal performance of triploid salmonids is due to earlier onset of cardiac arrhythmia which is associated with altered cardiorespiratory gene expression. To test this hypothesis, diploid and triploid salmon were exposed to a CT_{max} trial to assess the impact of increased ploidy on thermal tolerance. Cardiac performance was monitored prior to, during, and after the trial using implantable heart rate loggers to assess whether ploidy impacts heart function in response to acute changes in temperature. Plasma metabolites (cortisol, lactate, and glucose) were utilized as stress markers and measured in all treatment groups to assess whether ploidy impacted stress responses to surgery and/or recovery from CT_{max}. Finally, heart atrium and ventricle expression of heat shock proteins (Hsps; *hsp90aa1* and *hsp70*) and cardiorespiratory genes (*myoglobin*, *carbonic anhydrase 4*, and *ca4*-targeting *miR-24a-5p*) was assessed after recovery from CT_{max} to identify whether altered expression could explain reduced triploid performance.

4.2 Methods

4.2.1 Experimental animals

Cardiac impacts of acute temperature exposure were assessed using 2.5-year-old female diploid (590 ± 28.86 g) and triploid (252.45 ± 11.49 g) Chinook salmon. Triploidy was induced using high pressure (10 000 psi) just after fertilization, following previously published protocols (Johnson et al., 2004). Diploid fish were immunized (Vibrogen 2, ELANCO Canada ltd, Mississauga, Ontario, Canada) and moved to an open-sea net pen at 10 months of age while, due to reduced saltwater infrastructure, triploid fish were maintained in freshwater throughout their lives. Diploids were moved back to freshwater six months prior to this experiment and cultured in the same freshwater outdoor artificial spawning channel system as triploid fish. Dissolved oxygen ($7.8\text{--}11.2$ mg L⁻¹) and temperature ($6.5\text{--}14.2^\circ\text{C}$) readings were monitored consistently and remained within tolerable seasonal physiological parameters for Chinook salmon over the six months before this experiment. Approximately 50 fish of each ploidy were transferred to aerated 2500 L hatchery troughs and

allowed to acclimate for four days prior to experimental handling. Water quality parameters (temperature, pH, conductivity, and dissolved oxygen) and mortalities were assessed daily. Fish were fed to satiety twice daily using commercial fish feed (Taplow Feeds, British Columbia, Canada) except when fish were fasted 24-hours before handling or sampling. All animal usage and experimental techniques followed the Canadian Council for Animal Care guidelines and had previously been approved by the University of Waterloo's Animal Care Committee (AUPP# 43212; 43415).

4.2.2 Surgical implantation of Star-Oddi HRT loggers

Star-Oddi micro heart rate and temperature loggers (micro-HRT, Star-Oddi, Garðabær, Iceland) were surgically implanted in fish to assess heart rate impacts during and after recovery from thermal stress. Loggers were programmed to take electrocardiogram (ECG) and temperature measurements every minute at a recording frequency of 100 Hz. Readings were delayed by four days since this has been found to be sufficient for heart rate to return to resting levels after surgery (Føre et al., 2021). Once loggers were programmed, individual fish were anesthetized using sodium bicarbonate buffered tricaine methanesulphonate (150 mg L⁻¹ MS-222, Sigma-Aldrich, E10521) until they lost equilibrium. Fish were then moved to a surgical table and held ventral side up in a damp foam bed, while anesthetic was continuously passed across the gills using a pump submerged in a tricaine-dosed water bath. A 2 cm incision was created between the pectoral fins, and the logger inserted towards the pericardium with electrodes contacting the ventral body wall. The logger was then anchored to the ventral wall using a stitch at the anterior side of the incision, before closing the incision using 1-2 sutures as per manufacturer instructions. Fish were then held in recovery troughs until they were able to swim freely and continuously monitored for surgical-associated mortality. The entire surgical procedure, from sedation to recovery in the trough, lasted less than ten minutes for all individuals. Fifteen fish for each ploidy underwent this protocol with only one surgical-associated mortality in the diploid fish. To identify any metabolic impacts on stress markers for recovery from surgery, 10 fish for each ploidy underwent the same procedure (anesthesia, surgery, recovery for four days, no logger inserted) and sampled using protocols described below.

4.2.3 Critical Thermal Maximum protocol

Critical thermal maximum (CT_{max}) was assessed in batches of five for each ploidy using a 200 L recirculating bath. Fish were moved from their respective troughs at the same time each day and

allowed to acclimate in the bath at ambient water temperatures for one hour. Although longer acclimation would have been ideal, one hour was chosen due to data constraints of heart loggers. Additionally, this acclimation time has been shown to be sufficient for CT_{max} trials (Turko et al., 2020). Temperature and dissolved oxygen content was assessed throughout the experiment using submerged probes (Vernier, Beaverton, Oregon, USA) and remained constant during this acclimation. After acclimation, the water temperature was increased by a 240V Laing Infinity Electric Heater (Laing Thermotech, San Diego, California, USA) at a constant rate of 0.23°C per minute. Throughout the experiment, water was oxygenated using an air pump and oxygen concentration never decreased to below 8.0 mg L⁻¹. Trials were continued until individuals reached their thermal maximum as indicated by loss of equilibrium, when fish were transferred to oxygenated plastic tote baths (~12°C) to recover for one hour.

After recovery, fish were euthanized in diluted clove oil (0.4 mL L⁻¹ of water; Sigma-Aldrich, C8392) to sample tissues of interest. Blood was collected from the posterior caudal vein using heparinized syringes and centrifuged for 5 minutes at 2500 x g. After centrifugation, plasma was flash frozen in a portable dry shipper (-200°C; VWR, 55709-232). Heart tissue (ventricle and atrium) was excised and perfused with sterile phosphate buffered saline to remove excess blood within the cardiac lumen. The ventricle and atrium from each individual were then bisected and stored in RNAlater before flash freezing.

11 fish (seven diploids, four triploids) were identified as having signs of disease at sampling (sores, thin blood, skin lesions) while three fish (two diploid, one triploid) were not sufficiently recovered one hour after CT_{max} . Therefore, these individuals were removed from further analysis. Four treatment groups were sampled for this experiment for each ploidy: directly from stocking trough without acute temperature exposure (control; 2N n = 9, 3N n = 7); surgically recovered for four days without acute temperature exposure (surgical control; 2N n = 10, 3N n = 10); only exposed to acute temperature increase and recovery (heat control; 2N n = 8, 3N n = 8); and surgically implanted with HRT loggers, recovered for four days, exposed to the acute temperature increase, and allowed to recover (logger; 2N n = 10, 3N n = 15).

4.2.4 Heart rate and temperature measurements

Measurements of heart rate (f_H) and temperature indices throughout the study were analyzed using data collected by the implanted HRT loggers. Vernier-logger recorded water temperature and

implanted HRT logger temperature increased at the same rate, irrespective of ploidy ($0.23^{\circ}\text{C min}^{-1}$; two-tailed t -test, $t_{23} = 1.543$, $p = 0.1366$); however, due to differences in mass between the diploid and triploid fish, the difference in temperature between water and internal body temperature was greater in diploid fish (**Figure C.1**). CT_{max} and thermal tolerance can vary with size (Di Santo and Lobel, 2017; Gomez Isaza et al., 2019), so the heat control fish predicted internal body temperature for CT_{max} was calculated by plotting the log weight against the difference in temperature for the logger implanted fish (**Figure C.2**).

Figure 4.1 depicts the different measurements taken throughout the experiment. Logger recordings of six-second ECG measurements were taken once a minute, starting at 9:00am until one hour recovery post- CT_{max} (approximately 1:00pm). ECG readings were analyzed using Star-Oddi's HRT analyzer (v1.1.0) to determine changes in f_{H} (in beats per minute; BPM) throughout the experiment. Resting f_{H} was determined by averaging the readings during the first hour when fish were undisturbed in the surgical recovery trough. Handling-associated f_{H} was similarly averaged based on the second hour of the experiment, when fish were acclimating in the recirculating temperature bath at ambient water temperature. Maximum f_{H} was identified as the highest f_{H} measurement and occurred in all fish prior to CT_{max} , while minimum f_{H} was identified as the lowest f_{H} measurement over the entire four-hour recording period which occurred during recovery from CT_{max} . The f_{H} associated with the ECG at one-hour post- CT_{max} was recorded as the recovery f_{H} . Differences in heart rates for each individual fish were calculated to quantify the f_{H} plasticity during the experiment, and included resting to max f_{H} scope, resting to handling f_{H} scope, handling to max f_{H} scope, resting to recovery f_{H} scope, and maximum to minimum f_{H} scope. Factorial scope was calculated to identify the overall magnitude of f_{H} plasticity from resting to max f_{H} (Prystay et al., 2017). Temperature measurements from loggers associated with important f_{H} metrics were also assessed over the same period. Arrhenius breakpoint temperature (T_{ab}) was calculated using the *segmented* R package (v1.6-4; Muggeo, 2008). The temperature associated with the first incidence of ECG recorded arrhythmias (as described by Anttila et al., 2013) was recorded as the T_{arr} . The difference in temperature from the CT_{max} trial initial start temperature to T_{arr} ($|T_{\text{arr}}|$) and CT_{max} ($|\text{CT}_{\text{max}}|$) was calculated to determine the temperature range each ploidy could tolerate before arrhythmia and loss of equilibrium, respectively. Finally, the temperature associated with maximum f_{H} was compared to CT_{max} for each individual to identify ploidy differences for when maximum cardiac rate was reached.

4.2.5 Plasma metabolite analysis

Plasma cortisol was measured using the DetectX® Cortisol Immunoassay Kit (Arbor Assays, KAPDB270). Diluted samples (100x) were run in triplicate. Assays were run as per manufacturer instructions and final plasma concentration determined by comparing transmittance at 450nm to a 7-point standard curve on a Synergy H1 Hybrid plate reader (BioTek instruments, Winooski, Vermont, USA). Two plasma samples in the heat control diploid group were not included due to sample loss during transport.

Plasma lactate was measured using previously published enzymatic assays (Borowiec et al., 2018). Samples were acidified using an equal parts dilution in 8% PClO_4 and briefly centrifuged at 4°C to remove precipitate. All samples were run in duplicate and diluted to fall within the 6-point standard curve. Each well contained 170 mM glycine, 140 mM hydrazine sulphate, 2.0 mM β -Nicotinamide adenine dinucleotide (β -NAD⁺; Sigma, N0632), and 5U lactate dehydrogenase (Sigma, L2500). Plates were incubated at 37°C for 60 mins and final absorbance read at 340nm on a SpectraMax 190 Microplate Reader. Two plasma samples in the heat control diploid group were not included due to sample loss during transport.

Plasma glucose was measured using the Glucose Colorimetric/Fluorometric Assay kit (Sigma, MAK263). Samples were diluted 100x and deproteinated at 4°C using 10 kDa MWCO spin columns (Pall, OD010C34). All samples were run in duplicate. Plasma concentration was determined by comparing the absorbance at 570nm to a 6-point standard curve on a Synergy H1 Hybrid plate reader (BioTek instruments, Winooski, Vermont, USA). Four plasma samples in the triploid surgical control group were not included due to insufficient reagents and two plasma samples in the heat control diploid group were not included due to sample loss during transport.

4.2.6 Gene expression analysis

Cardiac transcription of genes associated with responding to heat and cardiovascular oxygen diffusion were measured in atrium and ventricle separately. Additionally, potential miRNA regulators for these genes were predicted using miRanda (version v3.3a; Enright et al., 2003) with previously established strict cutoffs (alignment score > 140, free energy score < -20 kcal mol⁻¹) to reduce potential false positive predictions (Kostyniuk et al., 2019). MiRanda identified that *miR-24a-5p* is predicted to bind to the 3' UTR of carbonic anhydrase 4 (XM_024442862.2), with an alignment score of 143 and a minimum free energy of -258.33 kcal mol⁻¹ (**Figure C.4**). Therefore, *miR-24a-5p* was measured in

both cardiac tissues to identify whether ploidy impacts epigenetic regulators of genes associated with cardiac oxygen diffusion.

Total RNA (including miRNAs) from control and heat control tissues (ventricle n = 7-8, atrium n = 7-9) was extracted using Qiagen's miRNeasy mini kits (Qiagen, 217004). Differences in sample size between tissues and the sample sizes for groups described above are due to transport-associated loss of samples. Tissues were homogenized using a bead mill homogenizer (Fisher Scientific, 15340163) and all samples were treated to remove DNA contamination using Qiagen's On-Column DNase Digestion protocol (Qiagen, 76254). Total RNA was quantified using a Qubit fluorometric assay (Invitrogen, Q10211) and cDNA generated using the Agilent miRNA 1st-strand cDNA Synthesis kit (Agilent, 600036). For both tissues, a total of 1000 ng of RNA was polyadenylated prior to following the reverse transcription protocol without deviation. After reverse transcription, each cDNA sample was diluted to 15x and stored at -20°C. Additionally, a pooled RNA sample for each tissue was reverse transcribed to generate a 7-point standard curve for calculating primer efficiency calculation as per Minimal Information for Publication of Quantitative Real-Time PCR Experiments guidelines (MIQE; Bustin et al., 2009). All qPCR reactions were run using a CFX96 Touch Real-Time PCR Detection System (Biorad, Mississauga, Ontario, Canada). Each 10 µL PCR reaction contained 3 µL of diluted cDNA, 0.8 µL of 3.125 µM forward primer, 0.8 µL of 3.125 µM reverse primer or miRNA Universal Reverse Primer (Agilent, 600037), 0.4 µL of molecular grade H₂O (Sigma, W4502) and 5 µL of SsoAdvanced SYBR Green Master Mix (Biorad, 1725274). The protocol for each qPCR run followed the manufacturer guidelines for the SYBR Green master mix, with polymerase activation initially (95°C for 30 seconds), followed by 40 repeated cycles of denaturation (95°C for 10 seconds) and annealing/extension (60°C for 15 seconds). Annealing and extension for *carbonic anhydrase 4 (ca4)* was run at 55°C for atrium and 59°C for ventricle samples to have MIQE acceptable primer efficiencies (**Table 4.1**). Finally, runs finished with a melt curve (incremental increase by 0.5°C from 65°C to 95°C) to confirm single amplicon size throughout the experiment. All samples were run in triplicate and each run contained a non-template control to detect potential contamination. Relative gene expression was calculated using CFX Maestro 2.3 (Biorad, v5.3.022.1030) using *18s ribosomal RNA* and *elongation factor 1a (ef1a)* as stable (GeNorm M score cutoff < 1.0) housekeeping genes. Normalized expression ($\Delta\Delta C_q$) was generated for all groups relative to diploid controls. Primer sequences, amplicon lengths, primer efficiencies, and housekeeping gene M scores and r^2 values are presented in **Table 4.1**.

4.2.7 Statistical analysis

All figures depict individual values, with mean ($\pm$ standard error of the mean; SEM) represented unless otherwise stated. For all statistical tests, a p value cutoff of 0.05 was used to denote significance. CT_{max} , logger-recorded heart rate and temperature indices, and correlations between cardiorespiratory gene relative abundance were analyzed in R (v4.3.0). Plasma metabolites (cortisol, lactate, and glucose) were analyzed in Graphpad Prism (v9.5.0). CT_{max} did not pass normality; therefore, a permutation Two-Way ANOVA ($n = 50\,000$ iterations) was utilized to identify differences between ploidies in each treatment group (*Imperm* v2.1.0, Manly, 2006). Logger recorded indices for heart rate and temperature metrics were analyzed using a two-tailed t -test. Plasma metabolites were analyzed using a Two-Way ANOVA to identify differences between ploidies and/or groups, and if models detected significance for metabolites, specific group differences were identified using a Tukey post-hoc test. All gene expression data was analyzed in Graphpad Prism (v9.5.0) and if residuals for models were not normally distributed, data was transformed to run parametric analyses. Two-Way ANOVAs were used to determine differences between ploidy and temperature treatment and if models detected significance, specific group differences were identified using a Tukey post-hoc test. Finally, since cardiorespiratory transcript abundance was not normally distributed, Spearman's correlations were conducted between cardiorespiratory genes (*miR-24a-5p*, *ca4*, *mb*) to assess how expression changes related across all ploidies, treatment groups, and tissues.

4.3 Results

4.3.1 Thermal tolerance metrics

For this study, CT_{max} was utilized to identify whether ploidy impacted thermal tolerance. Diploids ($26.15 \pm 0.19^{\circ}\text{C}$) and triploids ($25.97 \pm 0.19^{\circ}\text{C}$) had similar CT_{max} temperatures and treatment group (heat control or logger fish) had no impact on CT_{max} (Permutation two-way ANOVA, 50 000 iterations, $n = 8-15$, $p > 0.05$; **Figure 4.2**).

4.3.2 Logger-recorded responses to handling and temperature

During the CT_{max} trial, heart rate for both ploidies increased linearly until maximum heart rate was reached, at which point it decreased prior to reaching CT_{max} (**Figure C.3**). Logger implanted fish were assessed for different heart rate and temperature responses throughout the experiment to

determine whether triploids have reduced cardiac performance (**Table 4.2**). Resting heart rate ($f_{H \text{ resting}}$), maximum heart rate ($f_{H \text{ max}}$), minimum heart rate ($f_{H \text{ min}}$), and recovery-associated heart rate ($f_{H \text{ recovery}}$) were all not significantly different between ploidies (two-tailed t -test, $df = 23$, $p > 0.05$). However, the heart rate associated with handling was ~8 BPM higher in triploid fish (two-tailed t -test, $t_{23} = -2.597$, $p = 0.016$). Similar to most heart rate metrics, all heart rate scope indices were similar between ploidies (two-tailed t -test, $df = 23$, $p > 0.05$). Most logger-recorded temperature measurements were also similar between ploidies, except for the temperature associated with the first incidence of cardiac arrhythmia (T_{arr}) which was ~1.5°C lower in triploid fish (two-tailed t -test, $t_{23} = 2.385$, $p = 0.025$) and the difference between CT_{max} trial start temperature and T_{arr} ($|T_{arr}|$), which was 1.6°C lower in triploid fish (two-tailed t -test, $t_{23} = 2.382$, $p = 0.026$).

4.3.3 Plasma metabolite analysis

Plasma cortisol, lactate, and glucose were measured to assess the impact of surgical implantation and/or acute thermal stress on circulating stress markers. Circulating cortisol (**Figure 4.3A**) had both a treatment (Two-Way ANOVA, $F_{3,67} = 30.49$, $p < 0.0001$) and ploidy effect (Two-Way ANOVA, $F_{3,67} = 10.39$, $p = 0.0020$). CT_{max} -recovered groups (heat control and logger) had significantly elevated plasma cortisol relative to non-heat-exposed groups (Tukey post-hoc, $p \leq 0.05$), except for diploid surgical controls, which had similar levels to diploid heat controls and triploid logger groups (Tukey post-hoc, $p > 0.05$).

Treatment had a significant impact on plasma lactate levels (**Figure 4.3B**; Two-Way ANOVA, $F_{3,67} = 139.9$, $p < 0.0001$), where CT_{max} -recovered groups had significantly elevated lactate compared to both control groups (Tukey post-hoc, $p \leq 0.05$). Additionally, triploid CT_{max} -recovered logger-implanted fish had significantly elevated plasma lactate levels compared to heat control triploid fish after recovery from CT_{max} (Tukey post-hoc, $p = 0.04$).

Circulating glucose had a significant treatment effect (**Figure 4.3C**; Two-Way ANOVA, $F_{3,63} = 30.25$, $p < 0.0001$). Surgically manipulated groups (surgical control and logger) had significantly elevated plasma glucose when compared to control groups, regardless of ploidy (Tukey post-hoc, $p \leq 0.05$). Additionally, heat control groups of both ploidies had similar circulating glucose after recovery from CT_{max} compared to the control groups (Tukey post-hoc, $p > 0.05$).

4.3.4 Atrial and ventricular gene expression

Atrium and ventricular expression of genes associated with heat shock responses (*hsp90aa1* & *hsp70*) and cardiorespiratory function (*ca4*, *mb*, & *ca4*-targeting *miR-24a-5p*) were measured to assess ploidy and temperature associated changes in non-surgical fish.

Atrium expression of *hsp90aa1* (**Figure 4.4A**) and *hsp70* (**Figure 4.4B**) increased in CT_{max}-recovered fish relative to controls, irrespective of ploidy (Two-Way ANOVA, Tukey post-hoc, $p \leq 0.05$, **Table C.3**). A similar relationship was observed for *miR-24a-5p* (**Figure 4.4C**), where CT_{max}-recovered diploids and triploids had ~2-fold higher expression in atrium relative to control fish (Two-Way ANOVA, Tukey post-hoc, $p \leq 0.05$). Carbonic anhydrase 4 (*ca4*; **Figure 4.4D**) did not change in response to ploidy or treatment (Two-Way ANOVA, $p \geq 0.05$). However, ploidy (Two-Way ANOVA, $F_{1,27} = 10.89$, $p = 0.0027$) and treatment (Two-Way ANOVA, $F_{1,27} = 4.941$, $p = 0.035$) effects were identified for myoglobin (*mb*; **Figure 4.4E**), where CT_{max}-recovered triploid fish had significantly higher expression relative to diploid fish (Two-Way ANOVA, Tukey post-hoc, $p \leq 0.05$).

Similar to atrium, ventricle expression of *hsp90aa1* (**Figure 4.5A**) and *hsp70* (**Figure 4.5B**) increased in CT_{max} recovered fish (Two-Way ANOVA, Tukey post-hoc, $p \leq 0.05$, **Table C.4**). However, a ploidy effect was identified for *hsp90aa1* (Two-Way ANOVA, $F_{1,26} = 5.944$, $p = 0.022$), where control triploid fish had ~2-fold higher expression relative to control diploids (Tukey post-hoc, $p = 0.029$). Similarly, ploidy effects were identified for all three cardiorespiratory genes. Triploid fish had significantly higher ventricle *miR-24a-5p* expression relative to diploid fish (**Figure 4.5C**; Two-Way ANOVA, $F_{1,26} = 8.576$, $p = 0.007$). Conversely, *ca4* expression was significantly lower in triploid ventricles relative to diploid CT_{max}-recovered fish (**Figure 4.5D**; Tukey post-hoc, $p \leq 0.05$). Ventricular expression of *mb* also was impacted by ploidy (**Figure 4.5E**; Two-Way ANOVA, $F_{1,26} = 39.34$, $p < 0.0001$), where triploid fish had ~2-fold greater expression relative to diploid fish (Tukey post-hoc, $p \leq 0.05$).

Spearman's correlations were conducted between *miR-24a-5p*, *ca4*, and *mb* to assess whether cardiorespiratory genes have related changes in expression (**Figure 4.6**). There was no significant relationship between abundance of *miR-24a-5p* and its' predicted target *ca4* (**Figure 4.6A**; Spearman's correlation, $\rho = -0.163$, $p = 0.2169$). However, cardiac abundance of *ca4* and *mb* were negatively correlated (**Figure 4.6B**; Spearman's correlation, $\rho = -0.45$, $p < 0.001$), while abundance

of *mb* and *miR-24a-5p* had a positive relationship (**Figure 4.6C**; Spearman's correlation, $\rho = 0.514$, $p < 0.0001$).

4.4 Discussion

The objective of this study was to assess the cardiac performance of triploid Chinook salmon in comparison to diploids, before, during, and after recovery from a CT_{max} trial. Overall, triploids were less resilient to elevated temperature as indicated by their lower magnitude of thermal change before arrhythmias developed and lower arrhythmic temperatures. Plasma metabolite results indicate that thermal challenge caused increased stress and anaerobic respiration; however, surgical manipulation resulted in elevated metabolic markers of stress. Finally, gene expression results indicate that both ploidies have similar heat shock responses in atrium and ventricle at 1-hour post CT_{max} . Cardiorespiratory gene expression (*ssa-miR-24a-5p*, *ca4*, & *mb*) was temperature responsive in the atrium, while differences between ploidies were identified for these genes in the ventricle.

Triploid hearts became arrhythmic at lower temperatures compared to diploid hearts, which agrees with previous findings in rainbow trout (Verhille et al., 2013). In that study, trout were anesthetized, and maximum heart rate induced using atropine sulfate and isoproterenol before being exposed to an incremental thermal challenge. This study validates that free swimming triploids also have earlier onset of cardiac arrhythmia in response to acute increases temperature. The physiological mechanisms that govern when arrhythmias first occur during CT_{max} trials are unclear, although they appear to be species-specific. For example, in rainbow trout, hypoxic swimming causes arrhythmias to develop only when coronary blood supply is ligated (Steffensen and Farrell, 1998). Similarly, thermal stress-associated cardiac arrhythmias in Chinook salmon have been shown to be associated with reduced venous oxygenation and myocardial acidosis (Clark et al., 2008). Oxygen availability likely contributes significantly to cardiac collapse at high temperatures in salmonids, and triploid Chinook salmon have reduced arterial oxygen content during swimming compared to diploids (Bernier et al., 2004). Although this could explain the earlier arrhythmia onset, other mechanisms of cardiac failure could contribute as well. In coronary circulation-lacking opaleye (*Girella nigricans*) oxygen availability is not the sole cause of temperature induced cardiac failure (Schwieterman et al., 2023). These authors speculated that cardiac upper thermal limits could be driven by membrane depolarization or reactive oxygen species (ROS) buildup, that both occur at thermal extremes (Iftikar

and Hickey, 2013; Vornanen et al., 2014). Whether ploidy disrupts electrical conductance or ROS accumulation is currently unknown and should be investigated to understand triploid cardiac collapse at extreme temperatures.

Triploid fishes have reduced survival at chronically elevated temperatures (Hyndman et al., 2003; Ojolick et al., 1995), while they perform similarly to diploids when water temperature increases acutely (Benfey et al., 1997; Bowden et al., 2018). This is corroborated in the current study where CT_{max} was not impacted by ploidy. However, alternative thermal tolerance endpoints prior to CT_{max} are being proposed as more ecologically relevant. The agitation point has been utilized to indicate the temperature when fish would be seeking cooler waters to optimize metabolic function (Breau et al., 2011). Triploid salmonids have lower thermal optimum based on metabolic rate studies (Altimiras et al., 2002; Atkins and Benfey, 2008). Agitation is a behavioural measurement and triploid fishes have altered behaviour when compared to diploids as indicated by their reduced aggression and poor foraging performance (Carter et al., 1994; Garner et al., 2008). Unfortunately, for this study, determining individual fish agitation was not feasible and the capability for recording trials to achieve a blind assessment of agitation was not possible. Investigating this endpoint during future triploid CT_{max} trials would be more physiologically relevant to aquaculture-based fishes that are unable to find thermal refuge in open-sea net pens or land-based troughs.

Molecular regulation of cardiac genes important for tissue oxygenation could be involved in the reduced cardiovascular function at high temperatures. Altered epigenetic regulation is associated with the poor immune response in triploid Chinook salmon (Cadonic et al., 2023). The strong correlation between *ssa-miR-24a-5p* and *myoglobin* implicates expression of this miRNA is associated with oxygenation of the myocardium. Although this same relationship was not identified between miR-24a-5p's target *ca4*, miRNA can act to reduce protein abundance without impacting mRNA expression (Wightman et al., 1993). Ventricular expression of cardiorespiratory genes indicates altered oxygen dynamics in triploids since plasma accessible carbonic anhydrase and myocyte-associated myoglobin act to enhance oxygenation of the spongy myocardium (Alderman et al., 2016; Bailey and Driedzic, 1986). Expression of these two genes was negatively correlated, indicating compensatory expression in triploids to maintain cardiac oxygenation. Triploids have larger cells to contain more DNA, and therefore cells have lower surface area to volume ratio (Benfey, 1999). Altered plasma-accessible *ca4* is likely only an issue under stressful conditions, such as those posed by acute increases in temperature, which puts more pressure on the cardiovascular

system (Alderman et al., 2016). However, it has been established that triploids have reduced circulating oxygen content relative to diploids (Bernier et al., 2004; Graham et al., 1985). This reduced blood oxygen content could explain the elevated cardiac myoglobin expression since myoglobin content increases in response to low partial pressures of oxygen (Ewart and Driedzic, 1987; Fraser et al., 2006; reviewed by Hoppeler and Vogt, 2001). Another explanation for the elevated myoglobin abundance relates to the evolutionary history of salmon. Interestingly, Chinook salmon have only one myoglobin gene copy despite undergoing multiple whole genome duplications during evolution (Helfenrath et al., 2021; Macqueen and Johnston, 2014; Taylor et al., 2001). Higher myoglobin gene copy numbers are associated with thermally variable environments and since salmon prefer cooler, more thermally stable waters, maintenance of multiple gene copies offers no evolutionary advantage (Armstrong et al., 2016; Helfenrath et al., 2021). Furthermore, loss of cardiac myoglobin has occurred throughout the teleost lineage, even in fishes with high oxygen demands (e.g. *Gasterosteus aculeatus*; Macqueen et al., 2014). Increases in the number of gene loci due to triploidization could have resulted in positive dosage effects for myoglobin compared to genes with multiple copies that salmonids have evolved to regulate. The interactions between miR-24a-5p, carbonic anhydrase 4, and myoglobin in the teleost heart requires further study to understand cardiac oxygenation, and further study into how this network is impacted in triploids at the cellular level.

Another key finding of this study is the impact of surgical recovery on both ploidies. It has been established that ceramic coated Star-Oddi tags have comparably less immunological impacts relative to other surgically implanted tags, especially over the timelines in the present study (Heath et al., 2023). Additionally, previous work has identified that four days of recovery from logger implantation is sufficient for heart rate to return to resting conditions (Føre et al., 2021). The ECG recordings during the first hour indicate that this recovery period was sufficient for heart rate to return to resting conditions since resting heart rates of Chinook salmon are reported to be 40-60 bpm (Clark et al., 2008; Twardek et al., 2021). However, plasma results indicate that surgical manipulation resulted in metabolic impacts after this four-day recovery period. Triploids that underwent surgery had elevated plasma lactate after recovery from CT_{max}, and groups that underwent any surgery had elevated circulating glucose relative to individuals that had not undergone sedation and four-day recovery. Triploid fishes have elevated lactate levels after thermal stress and have reduced capacity to clear lactate from circulation relative to diploids (Hyndman et al., 2003; Sambraus et al., 2018). Similarly, salmonids are described as “glucose intolerant” since they are less efficient at clearing

circulating glucose compared to other organisms (Forbes et al., 2019; Legate et al., 2001; Mazur et al., 1992). Four days of chronic social stress in rainbow trout is associated with elevated plasma glucose, likely driven by cortisol's role in liberating carbohydrate stores (Culbert and Gilmour, 2016). The elevation of lactate and glucose in surgical groups indicates increased anaerobic respiration and lasting impacts of cortisol-mediated gluconeogenesis. Future studies that investigate cardiac thermal tolerance in triploids should allow for sufficient recovery from surgery (six days minimum based on Føre et al., 2021) to reduce potential multiple stressor impacts

Although this study offers explanations for the reduced thermal performance of triploid fishes, rearing differences between ploidies likely confounded the responses observed. Diploids were maintained under similar conditions to triploids for six months prior to the CT_{max} trial, which should be sufficient time to acclimate them to the temperature conditions triploids were maintained in since thermal acclimation takes 4-5 weeks (Johansen et al., 2021; Schulte et al., 2011). However, smoltification is associated with extensive alterations in endocrine and physiological parameters to prepare for tolerating the osmotic challenge associated with marine development (Nilsen et al., 2008). Although some traits that made them tolerant of saltwater were lost during parr-reversion (return from saltwater to freshwater), there could be lasting impacts that made diploids respond differently to this experiment. The triploids utilized in this study were maintained in freshwater their entire lives which is similar to the residency phenotype that occurs in salmonids such as rainbow and brook trout. Freshwater residency in salmon is thought to occur in some species due to genetics, food availability, and water temperature (Brannon et al., 2004; Ferguson et al., 2019; Nevoux et al., 2019; Sloat and Reeves, 2014; Wysujack et al., 2009). The literature on freshwater residency compared to parr-reversion is limited, although freshwater residency in Chinook salmon causes hormonal changes similar to smoltification (Cowley et al., 1994). Saltwater acclimation in brook trout does initially decrease CT_{max} compared to freshwater acclimated trout, but this discrepancy is lost after 16 days in saltwater (Shaughnessy and McCormick, 2018). In a natural context, parr-reversion in Pacific salmon is associated with spawning migration, and as such the cardiac changes associated with this stressful event have been investigated. Populations of Sockeye salmon (*Oncorhynchus nerka*) with different migration distances have differences in cardiac transcriptional responses to swimming and warming (Anttila et al., 2013). Additionally, migrating salmon develop extensive arteriosclerosis in their coronary circulation, which can be detrimental to their survival since obstruction of coronary circulation reduces aerobic scope and CT_{max} (reviewed by A. P. Farrell, 2002; Farrell et al., 1990; Van

Wert et al., 2024). Although migration-associated stress is likely more severe than the freshwater facility the diploids were transferred to, arteriosclerosis is thought to be initially caused by the increased growth rate in marine environments (Saunders et al., 1992). Therefore, the higher mass in the diploid groups could impact how they responded during recovery from thermal stress due to altered metabolic stores and cardiac function. Yet, unlike mammals, heart rate does not scale with mass in Chinook salmon (Clark and Farrell, 2011); therefore, the altered mass might not have impacted the heart rate metrics measured. Correcting CT_{max} for the internal body temperature was utilized to mitigate body-size associated impacts on thermal tolerance (reviewed by Ern et al., 2023; Messmer et al., 2017) since, presumably, thermal tolerance is governed by the temperature that tissues are at rather than the external water temperature. However, due to these rearing differences, it is impossible to determine whether responses identified are associated with ploidy or their altered life history. Finally, this study did not include controls that accounted for fish handling. It is known that even short-term handling stress (minutes) can alter plasma metabolite concentration and tissue abundance of mRNAs and miRNAs for hours following the stressor (Cadonic et al., 2020; Ikert et al., 2021a, 2021b). Fish in the Control group were utilized to assess the metabolite and molecular impacts of only ploidy rather than any stressor, while the Surgical Control group was utilized to identify any surgically associated stress markers prior to any thermal stress. Although the severity of the acute thermal stress likely had a greater impact on the data collected, handling likely contributed to the results for the two CT_{max} exposed groups.

In conclusion, this study supports the idea that triploid salmon are less resilient to acute increases in temperature due to earlier onset of cardiac arrhythmia. Triploids were also less resilient to surgical and handling stress as indicated by their elevated plasma lactate and elevated handling-associated heart rate. Finally, we have identified that triploid ventricles have altered cardiorespiratory expression that could relate to their reduced thermal performance. Recent work has commented on the role of electrical conductance, rather than oxygen limitation, as contributing to thermal tolerance and cardiac arrhythmia (reviewed by Ern et al., 2023; Jutfelt et al., 2019; Schwieterman et al., 2023; Vornanen et al., 2014). Future studies should investigate neural signal transduction in triploids to elucidate whether reduced cardiac function is related to electrical excitation in these fishes. Altered cardiorespiratory function could be due to dysregulated epigenetic regulators (miRNA) or inherent genetic impacts of increased ploidy. Future studies should validate these impacts at the cellular level

and in other salmonid species to confirm whether altered cardiorespiratory function is common to autopolyploid fishes and whether it is associated with reduced cardiac oxygenation.

Table 4.1: Primer information for gene expression analysis

M scores, primer efficiencies, and r^2 values were calculated using CFX Maestro software (Biorad, v5.3.022.1030). Primers without a reference (-) were designed for the current study. Reverse primer for *miR-24a-5p* is based on Agilent's proprietary Universal Reverse Primer (Agilent, 600037).

Target	Accession	Forward primer sequence	Reverse Primer sequence	Amplicon length (bp)	Atrium				Ventricle				Reference
					M Score	Annealing Temp	Efficiency (%)	r ²	M Score	Annealing Temp	Efficiency (%)	r ²	
<i>18s rRNA</i>	KJ774722.1	GGCCGTTCTTAGTTGGTGGA	TTGCTCAATCTCGTGTGGCT	147	0.472	60°C	95.0	0.989	0.418	60°C	94.3	0.988	Cadonic et al., 2023
<i>ef1α</i>	FJ890356.1	ATGCCCTGTACTGGATTGC	TGGGGGCATCCTCAAGTTTC	105	0.472	60°C	100.0	0.990	0.418	60°C	104.8	0.994	Thraya et al., 2019
<i>hsp90aa1</i>	XM_042300925.1	ATGATCGGGCAGTTCGGTG	AGTGTCAACTTTGACAGTGAA	141		60°C	99.2	0.984		60°C	100.7	0.989	von Biela et al., 2020
<i>hsp70</i>	XM_042307454.1	GAGACAGGCCACTAAGGACG	GACATGCCTTTGTCCATGCC	108		60°C	99.4	0.992		60°C	94.8	0.992	Rogozynski et al., 2024
<i>ca4</i>	XM_024432671.2	GATTGGTGGAGACCTGGAGA	GTCTTTCAGGGCATCTTCCA	172		55°C	110.0	0.992		59°C	97.8	0.993	Alderman et al., 2016
<i>mb</i>	XM_024439779.2	CGCTGTCACCTACAAGGAGC	AGTCACAAGCAAACGAGCGT	73		60°C	97.0	0.993		60°C	100.2	0.996	-
<i>miR-24a-5p</i>	MIMAT0032556	TGCCTACTGAACTGGTATCAGT	Universal Reverse Primer	-		60°C	101.2	0.996		60°C	98.5	0.994	-

Table 4.2: Heart rate and temperature indices derived from heart logger data over entire CT_{max} trial

Data was collected from diploids (n = 10) and triploids (n = 15) implanted with heart rate loggers (Star-Oddi) and factors that are bold for each measurement indicate a difference between ploidies (Two-tailed T-test, $p < 0.05$). Descriptions of how factors were calculated can be found above and all values are mean $\pm$ SEM. Heart rate values (f_H ; beats per minute) were determined through electrocardiogram analysis and temperature values (T) are in $^{\circ}\text{C}$.

	Diploid	Triploid
f_H resting	47.45 $\pm$ 1.87	47.22 $\pm$ 2.70
f_H handling	63.70 $\pm$ 1.56	71.33 $\pm$ 2.15
f_H peak	129.10 $\pm$ 4.18	128.07 $\pm$ 2.76
f_H min	30.60 $\pm$ 2.97	33.67 $\pm$ 1.76
f_H recovery	50.89 $\pm$ 5.36	55.33 $\pm$ 3.85
Resting-Peak scope	81.65 $\pm$ 4.44	80.84 $\pm$ 3.97
Resting-Handling scope	16.26 $\pm$ 2.01	24.11 $\pm$ 3.47
Handling-Peak scope	65.40 $\pm$ 4.36	56.74 $\pm$ 2.82
Resting-Recovery scope	-3.79 $\pm$ 5.11	-8.11 $\pm$ 4.00
Peak-Min scope	98.50 $\pm$ 4.54	94.40 $\pm$ 3.75
Factorial scope	2.76 $\pm$ 0.13	2.85 $\pm$ 0.18
T_{ab}	23.00 $\pm$ 0.60	21.92 $\pm$ 0.27
T_{arr}	24.17 $\pm$ 0.54	22.75 $\pm$ 0.33
$T_{peak\ fH}$	23.59 $\pm$ 0.63	22.29 $\pm$ 0.33
T_{arr}	11.32 $\pm$ 0.59	9.68 $\pm$ 0.40
$ CT_{max} $	13.55 $\pm$ 0.31	12.72 $\pm$ 0.34
$T_{peak-CTmax\ scope}$	2.82 $\pm$ 0.55	3.41 $\pm$ 0.30

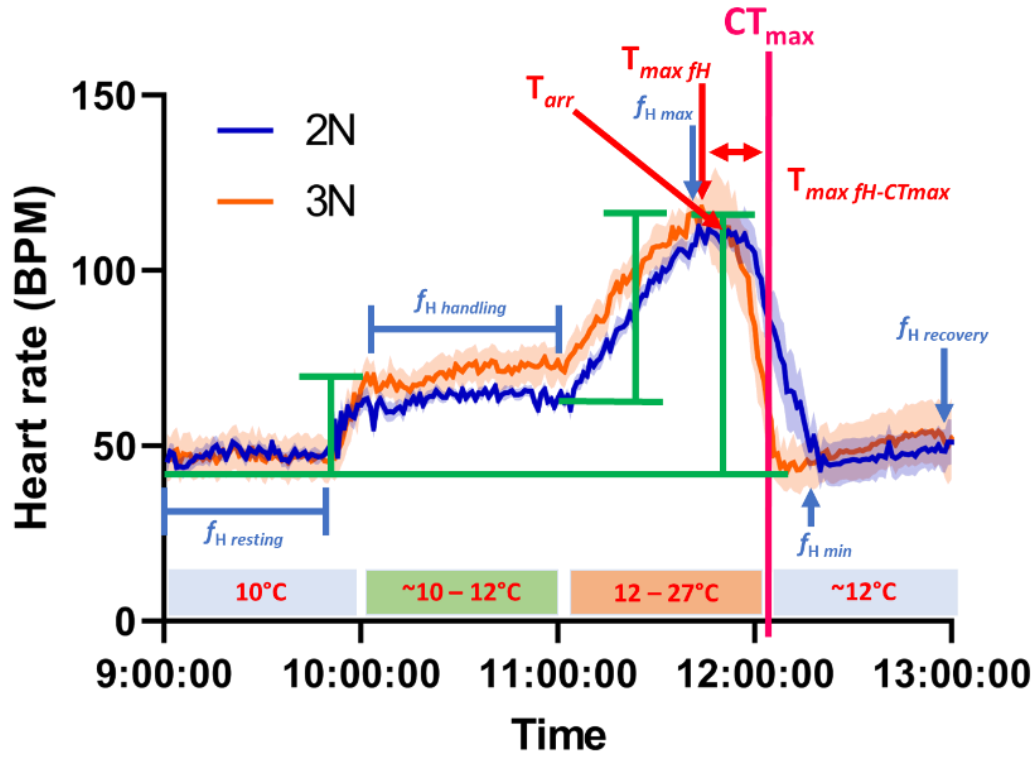


Figure 4.1: Heart rate and temperature indices measured throughout CT_{max} experiment

Diploid (blue, $n = 10$) and triploid (orange, $n = 15$) heart rates (mean $\pm$ SEM) during multiple stages of experiment to assess cardiac responses to different stressors. Fish were left undisturbed for the first hour in stocking troughs (10°C), after which they were moved to the recirculating temperature bath and allowed to acclimate for one hour at ambient water temperatures ($10\text{--}12^{\circ}\text{C}$). After this acclimation, water temperature was increased at a rate of $0.23^{\circ}\text{C minute}^{-1}$ until fish lost equilibrium (CT_{max}). Fish were then allowed to recover at ambient water temperature ($\sim 12^{\circ}\text{C}$) for a subsequent hour before sampling tissues for further analysis. Heart rate indices are labelled in light blue, temperature indices in red, and differences between heart rate indices labelled in green.

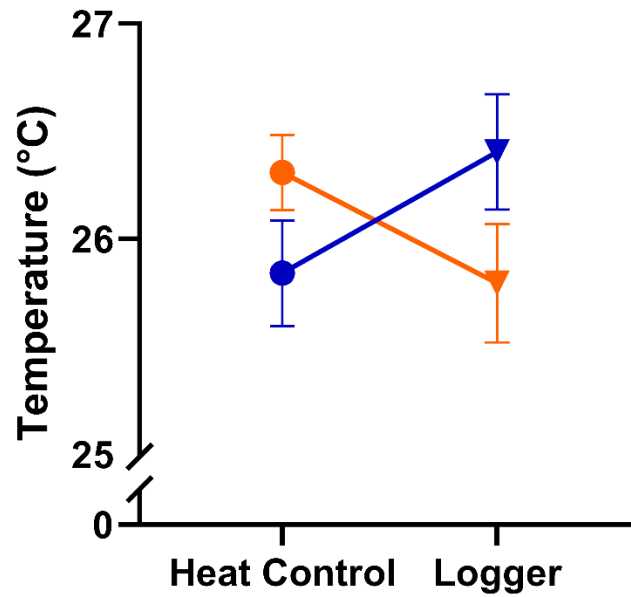


Figure 4.2: CT_{max} for diploid and triploid Chinook salmon

Diploid (blue) and triploid (orange) Chinook salmon CT_{max} . Heat control (circle; 2N n = 8, 3N n = 8) fish were only exposed to a CT_{max} challenge while Logger (triangle; 2N n = 10, 3N n = 15) fish were surgically implanted with Star-Oddi HRT loggers and allowed to recover for four days before CT_{max} challenge. Individual points represent mean ($\pm$ SEM) CT_{max} for that group.

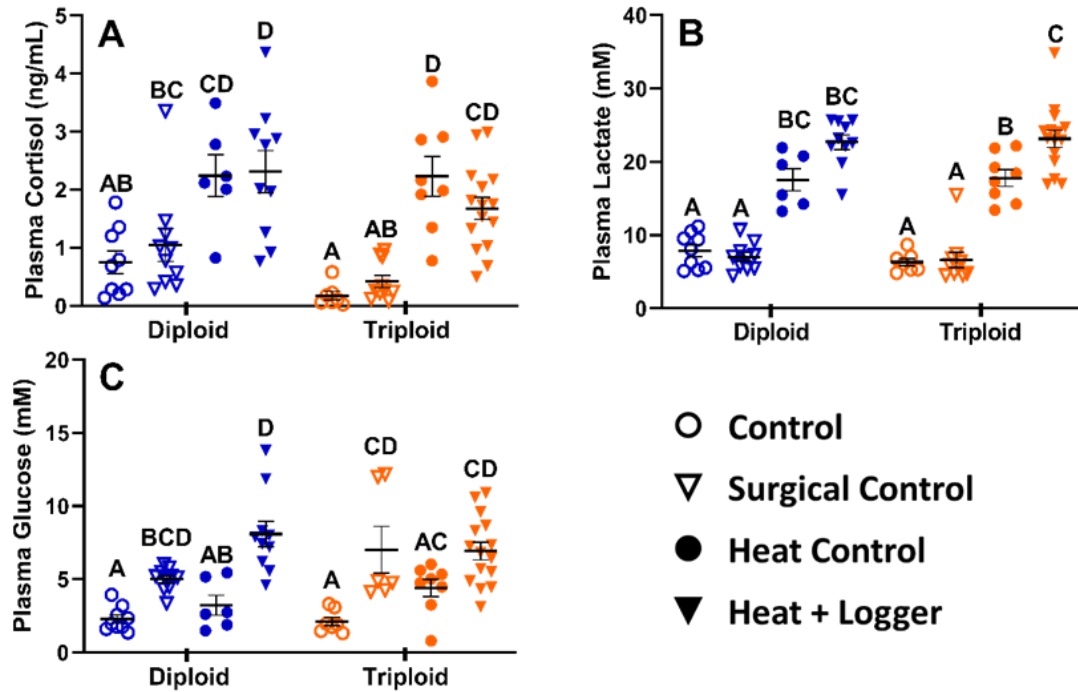


Figure 4.3: Plasma cortisol (A), lactate (B), and glucose (C) concentration of diploid and triploid Chinook salmon before (Control/Surgical control) or after (Heat control/Logger) a CT_{max} trial

Control fish (2N n = 9, 3N n = 7) were sampled directly from their trough, while surgical control fish (2N n = 10, 3N n = 6-10) were sampled four days after surgery. Heat control (2N n = 6, 3N n = 8) and Heat Logger fish (2N n = 10, 3N n = 15) were sampled after one hour of recovery from CT_{max} . Groups that do not share a common letter are statistically different from each other (Two-Way ANOVA, Tukey post-hoc, $p \leq 0.05$).

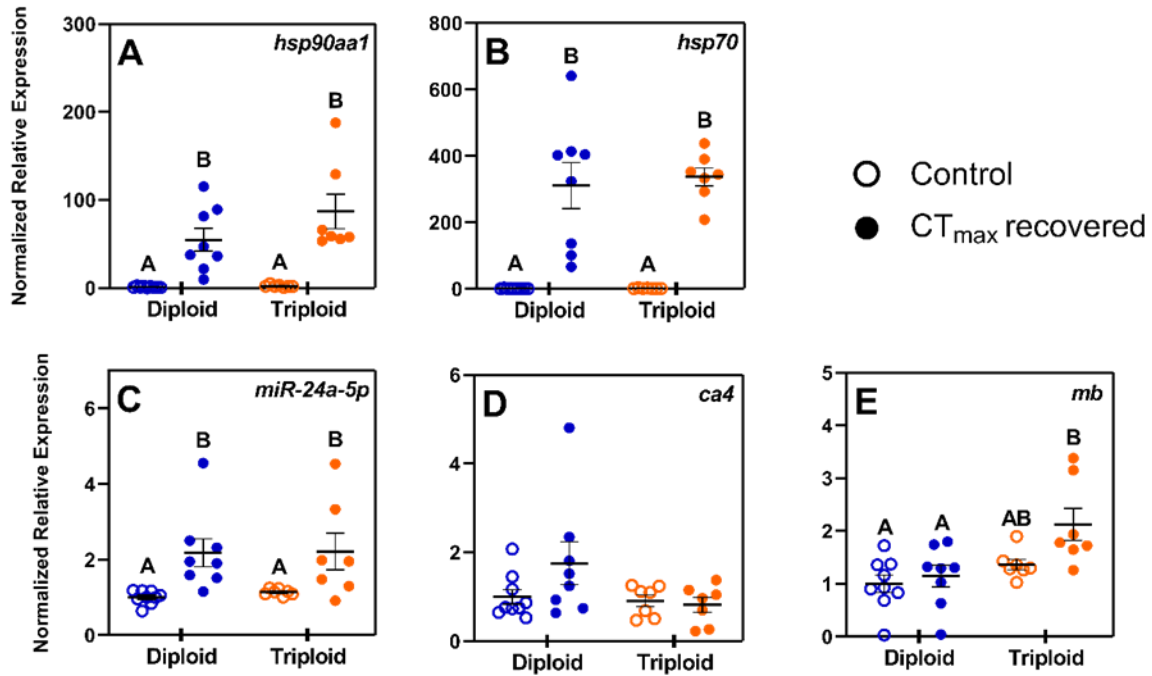


Figure 4.4: Atrium gene expression in control (open circle) and CT_{max} recovered (filled-in circle) diploid and triploid Chinook salmon

Control fish (2N n = 9, 3N n = 7) were sampled directly from stocking troughs while heat fish (2N n = 8, 3N n = 7) were sampled after one hour of recovery from CT_{max}. All expression was normalized to diploid control fish. Horizontal lines for each group indicate mean (± SEM) and groups that do not share a common letter are statistically different from each other (Two-Way ANOVA, Tukey post-hoc, $p \leq 0.05$).

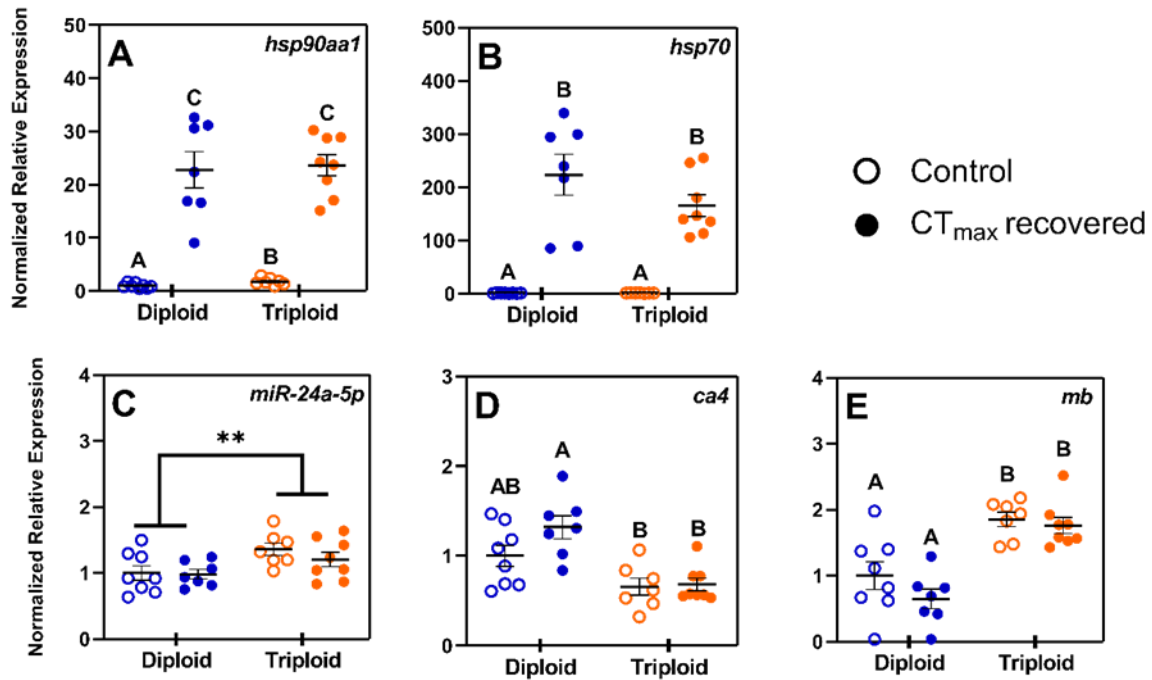


Figure 4.5: Ventricle gene expression in control (open circle) and CT_{max} recovered (filled-in circle) diploid and triploid Chinook salmon

Control fish (2N $n = 8$, 3N $n = 7$) were sampled directly from stocking troughs while heat fish (2N $n = 7$, 3N $n = 8$) were sampled after one hour of recovery from CT_{max}. All expression was normalized to diploid control fish. Horizontal lines for each group indicate mean ($\pm$ SEM) and groups that do not share a common letter or are denoted by asterisks are statistically different from each other (Two-Way ANOVA, Tukey post-hoc, $p \leq 0.05$).

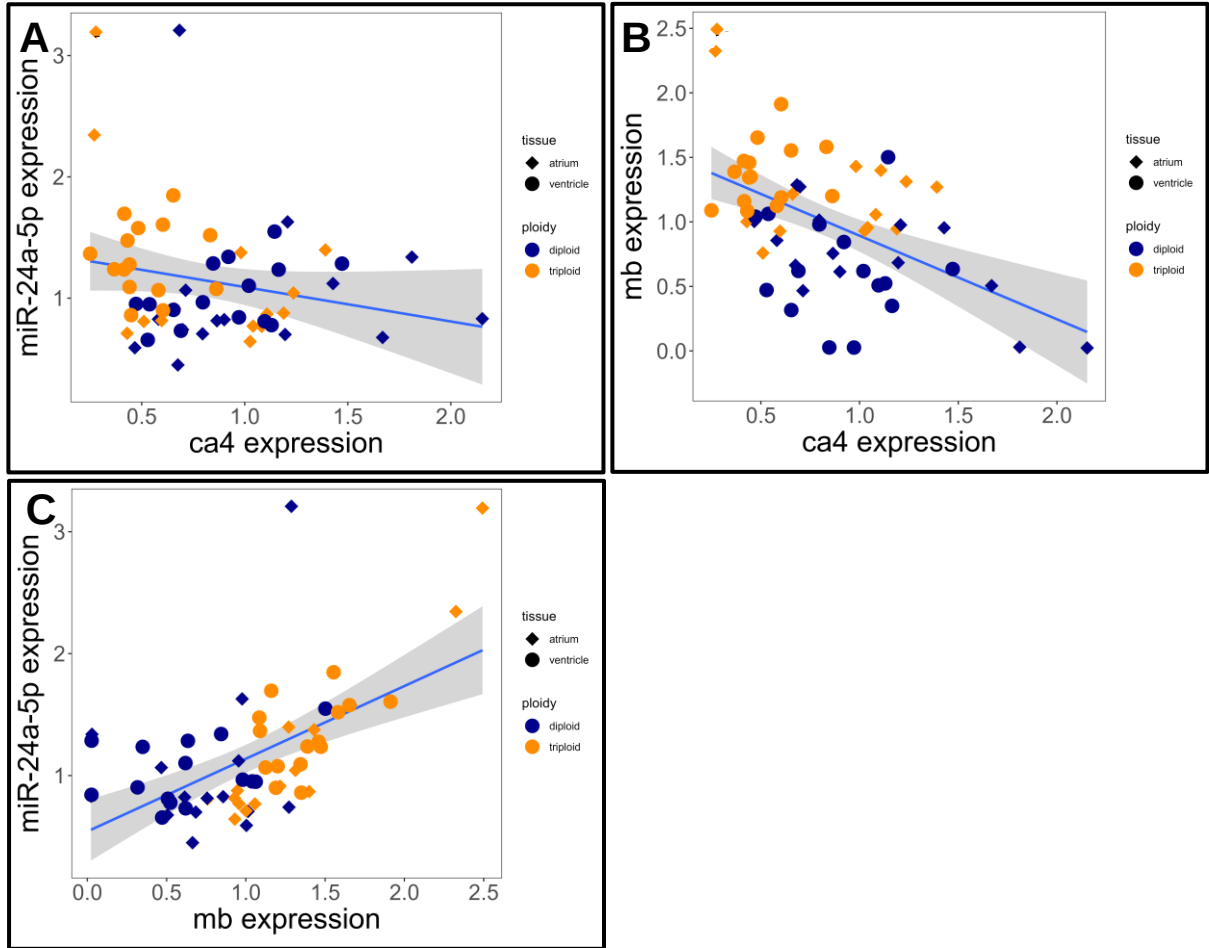


Figure 4.6: Spearman's correlations between cardiac expression of cardiorespiratory genes (*ca4*, *mb*, & *miR-24a-5p*)

Expression of genes in atrium (diamond) or ventricle (circle) of diploid (blue) and triploid (orange) Chinook salmon. Each panel represents Spearman's correlation between the three genes (95% confidence interval in grey). The relationship in panel A is not significant (Spearman's correlation, $\rho = -0.163$, $p = 0.2169$), while the relationship in panels B (Spearman's correlation, $\rho = -0.45$, $p < 0.001$) and C (Spearman's correlation, $\rho = 0.514$, $p < 0.0001$) are significant.

4.5 Acknowledgements

I would like to acknowledge and thank Yellow Island Aquaculture (Dr. Ann Heath, Elliot Haugen, and the rest of the YIAL team) for their help maintaining the health and wellbeing of the fish studied. Additionally, I would like to thank Maureen Latimer, Earl Heath, & Krista Deane for their help with tissue sampling. I would like to thank Dr. Britney Firth for her help with CT_{max} study design. I would like to thank Dr. Gail Schwieterman & Dr. Anthony Farrell for their advice in analyzing the heart rate data. Finally, I would like to thank my coauthors for the study (Noah Rogozynski & Dr. Manuel Soto-Dávila) for their help with the glucose and cortisol data collection.

Chapter 5

General Conclusions

5.1 Overall thesis findings

Climate change is only going to worsen as humanity continues to rely on fossil fuels for energy. Yet these anthropogenic-induced disturbances will not only exacerbate negative impacts on human health, but also on the ecosystems around us. Utilizing more environmentally friendly practices in all aspects of society will reduce pressures on our biosphere. Pacific salmonids are particularly susceptible to these environmental perturbations, so farming of triploids on the west coast would improve wild population health as climate change progresses. However, before widespread implementation of triploid aquaculture, determining the reasons for their poor stress tolerance is necessary since their reduced performance would decrease profitability for the aquaculture industry. This thesis aimed to investigate the molecular dysregulation associated with increased ploidy in Chinook salmon (*Oncorhynchus tshawytscha*) before and after two different stressors (pathogen and heat). The data reported (summarized in **Figure 5.1**) addresses gaps in knowledge showing both genetic and epigenetic dysregulation in these fish, offering explanations for their reduced performance, and providing targets for breeding programs and/or therapeutic development.

Chapter 2 investigated the molecular impacts (miRNA) of increased ploidy before and after infection with *Vibrio anguillarum*. Small RNA sequencing identified that in the absence of stress, increased ploidy had few epigenetic impacts across the immune tissues (hindgut, head kidney, and spleen) investigated. After infection, miRNA abundance was altered in all three tissues which could be supporting immune function as indicated by KEGG pathway analysis of predicted targets. However, the KEGG results predict that diploids have greater impact on pathways that are involved in immune function relative to triploids across all three tissues. Additionally, just prior to infection-associated mortality, hindgut and head kidney miRNA expression was similar between diploids and triploids. Only in the spleen were differences identified at Day 3 and indicated that molecular regulation of immune function was altered in triploid fishes prior to *Vibrio*-associated mortality.

Chapter 3 continued investigating immune impacts of increased ploidy but focused on how relative abundance of immune genes (mRNA) were altered during recovery from acute thermal stress. Overall, there was no clear pattern of ploidy-induced dysregulation of MHC class I, MHC class II, or cytokine expression. However, gill expression of *tlr2* and ventricle expression of *nlrp3* were lower in triploid fish, implicating potential alteration of pathogen sensing in triploids. Tissue specific alterations after thermal stress provided some insight into immune dynamics during a cellular heat-

shock response. In particular, cytokine expression in the gill was thermally responsive, especially for pro-inflammatory (*il-1 β* and *il-8*) cytokines. Variability of thermal stress-recovered fish implicated that many of these immune genes are thermally responsive in some capacity, but due to variability after thermal recovery this response could be impacted in a time-dependent manner.

Finally, Chapter 4 returned to investigating miRNAs, but focused on the cardiovascular impacts of increased ploidy in response to thermal stress. Cardiac performance was quantified using implantable heart rate and temperature loggers to determine whether ploidy impacted heart function. Triploids were less resilient to acute thermal stress as indicated by their earlier incidence of cardiac arrhythmia. Quantification of cardiovascular genes in the heart implicated that oxygen diffusion dynamics are altered in the ventricle, which could relate to the reduced thermal performance in triploids. Additionally, miRNA could be involved in cardiorespiratory function since *miR-24a-5p* abundance was altered in the ventricle and its expression was positively correlated with *myoglobin*.

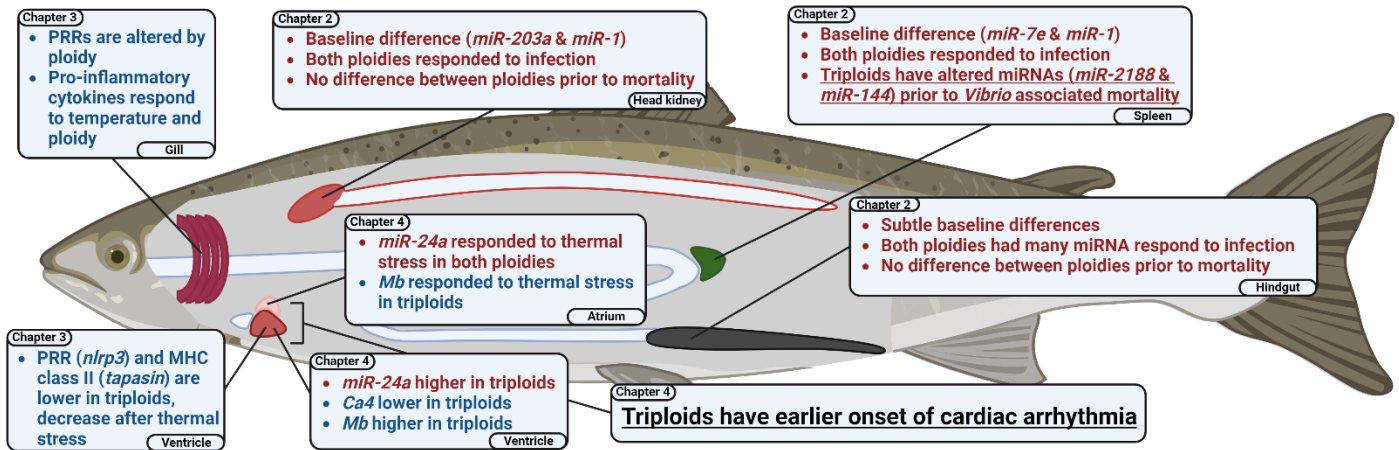


Figure 5.1: Thesis results representing tissue-specific impacts of increased ploidy in Chinook salmon

Diagram representing overall tissue impacts on triploid fish identified in this thesis. Points in red represent microRNA impacts, points in blue represent mRNA, and points in black represent physiological differences. Figure generated in BioRender.

5.2 Increased ploidy impacts gene expression

Few ploidy differences were identified prior to stress across all three studies, which corroborates previous work that shows triploids have similar performance to diploids in the absence of stress (Hyndman et al., 2003; Nuez-Ortín et al., 2017; Van De Pol et al., 2020). Although Chapter 3 did not have any phenotypic measurements to indicate that triploids had reduced performance, the gene expression changes implicate altered immune stimulation in response to acute high temperatures. Chapter 2 showed that ploidy impacted abundance of epigenetic regulators, and therefore likely had an impact on regulation of immune and metabolic function; however, this was not associated with altered *Vibrio*-associated mortality. The results from Chapter 4 provide an explanation for the reduced thermal performance of triploids being related to altered regulation of the oxygen-diffusion system in the heart. Previous work had identified that triploid Chinook salmon have reduced circulating oxygen content during swimming (Bernier et al., 2004) and triploid rainbow trout hearts fail at lower temperatures (Verhille et al., 2013). This molecular dysregulation adds to this story and provides a potential explanation for cardiac deficiency in triploids.

Importantly, these altered abundances were not identified in all tissues. Most molecular studies tend to focus on one tissue and then extrapolate conclusions about the overall performance of the organism. For example, Ching et al. (2010) investigated how mRNA expression changed after infection with the same bacteria used in Chapter 2. Utilizing microarray data from only the head kidney, they found that there was altered abundance of protein-coding immune genes within the first day of infection. Although my results are later in the immune response, utilizing the hindgut, head kidney, and spleen allowed for identification of the spleen as one of the likely tissues involved in their altered immune function just prior to infection-associated mortality. Chapter 4 validated this tissue-specificity since *miR-24a-5p* was impacted by ploidy in the cardiac ventricle, but in the cardiac atrium it was thermally responsive. Triploid fishes can have altered tissue architecture to maintain proper function (Small et al., 2024). Therefore, when investigating their responses to stressors, utilizing multiple tissues can provide some clarity on which tissue might be constraining these tolerances. Incidentally, in Chapter 3 this tissue appeared to be gill since its multifaceted function could result in worse immune performance after thermal stress, while in Chapter 4, the ventricle is more likely to be involved in their reduced cardiac performance.

The phenotypic impacts of these changes are largely predictive based on bioinformatic analysis and predictive pipelines. This is true across all three chapters, although the published data from Chapter 3 does include protein-level results (see Rogozynski et al., 2024). The nature of miRNA transcriptomic work makes it more difficult to go further than predictions due to the large number of potential gene targets that could be regulated. Therefore, the data from Chapter 2 provides the first step for further investigation and hypothesis generation to identify which pathways are dysregulated in triploid Chinook. For example, it has been identified that in Japanese flounder *miR-144-5p* directly regulates *hsp90α* abundance, which results in regulation of the Nlrp3 inflammasome (Li et al., 2023). In my thesis, splenic *miR-144-5p* (Chapter 2), ventricular *hsp90α* (Chapter 3 & 4), and gill and ventricular *nlrp3* (Chapter 3) were all impacted by ploidy. Following up this network could provide further clarity on epigenetic regulation in triploids. Similarly, the miR-24a data from Chapter 4 is predictive, but again the correlation between the *miR-24a-5p* and *myoglobin* provides some evidence for this miRNA's involvement in regulating cardiac oxygenation, and worthy of further exploration.

5.3 MicroRNA abundance changes were subtle

Interestingly, across all three Chapters the ploidy impacts on miRNA and mRNA abundances were fairly modest. The relatively low changes in mRNA could be gene dosage impacts (see Section 5.4), but low fold changes are common for miRNA due to redundancy in this epigenetic mechanism. Often, multiple miRNA binding sites are present within each mRNA 3' UTR, so changes in abundance across each of these miRNAs can exert stronger repression than if they worked independently (Bartel, 2009; Friedman et al., 2009). Maintaining miRNAs at consistent levels is likely adaptive since drastic changes in individual miRNA can have far reaching impacts on cellular function due to the diversity of binding sites they can regulate. The KEGG pathway data from Chapter 2 exemplifies this since many different pathways were overrepresented in the predicted gene list from the altered miRNAs. Although most have some immune connection, they also regulate other cell functions, and their widespread dysregulation would be detrimental. When stressors are induced, miRNA abundances change which can act to support cellular function.

Additionally, small changes in miRNAs could have greater phenotypic effects due to concentration-dependent changes in triploid cells. Cellular abundance of miRNAs correspond with changes in overall cellular RNA (Neilson et al., 2007), so total tissue miRNA concentration is likely

maintained in spite of ploidy-associated changes cellular volume. At the cellular level, this can become more complicated since these concentrations could alter the steady state relationships between miRNAs and their targets. Increases in the number of binding sites per cell can reduce miRNA-associated repression as miRNAs are sequestered to alternative targets (Arvey et al., 2010; Garcia et al., 2011). Therefore, investigation of the protein/cellular impacts of subtle changes in the identified miRNA would provide greater clarity on the phenotypic outcomes of these modest fluctuations

5.4 Gene dosage of microRNAs

Gene dosage effects, inverse dosage effects, and dosage compensation can all result in altered relative abundance of protein coding transcripts due to changes in abundance of regulatory complexes (reviewed by Birchler et al., 2001). Regulatory complexes include transcription factors that directly regulate genes of interest but could also include miRNA if they act upon transcripts that produce proteins involved in signal transduction pathways. Therefore, abundance changes identified in Chapter 2 & 4 can have far reaching impacts on gene expression. Across all three chapters I found that protein coding transcripts and miRNA responded to stress and, in specific cases, triploidy impacted this stressor-associated transcript abundance.

Additionally, these dosage impacts are likely to have greater impacts due to changes in cytosol volume identified in triploids. In particular, the role of miRNA as a translational threshold to protein coding transcripts could be impacted when cytosolic concentrations are altered (Mukherji et al., 2011). Further exploration of how increased ploidy impacts miRNA abundance utilizing methods that allow for specific cell expression (e.g. single-cell sequencing) could elucidate how changes in cell size and cell number impact regulation. Although I focused on miRNA, other regulatory RNAs (e.g. long non-coding RNAs) are important for the immune and thermal stress responses and are likely impacted by changes in gene dosage (Zhou et al., 2023). Utilizing other -omics techniques (such as for mRNAs, other non-coding RNAs, and proteins) to map the molecular landscape of triploids would provide a greater understanding of how changes in gene dosage and cell size impact expression of genes and therefore, cellular function.

5.5 Further Confounding Factors

Across all three chapters, limitations were reported for individual studies. However, there are additional factors that could have resulted in the differences observed between ploidies and therefore need to be commented on. Firstly, the rearing temperatures that fish were maintained at were within or below the ideal range for Chinook salmon. In the wild, Chinook salmon can experience temperatures up to 22°C during salmon runs but prefer water temperatures between 14.4°C and 18.0°C (Baird et al., 2018; Keefer et al., 2018). However, these reported temperatures are for diploid Chinook salmon, but triploid salmonids tend to have lower thermal optima when compared to diploids (Altimiras et al., 2002; Atkins and Benfey, 2008). Although the acclimation temperatures reported (Chapter 2 10°C, Chapter 3 & 4 12°C) are likely within the thermal optima for triploids, differences in acclimation could have impacted how the fish responded to the stressors. Secondly, these studies did not replicate rearing units prior to stressor exposures. It has been well established that utilizing replicate tanks should be used to account for differences in exposures due to “tank effects” (Speare et al., 1995). The infection in Chapter 2 utilized two tanks, one for diploids and one for triploids since space was limited at the time of the trial. In Chapters 3 & 4, fish were similarly cultured in two separate tanks, but replicating rearing tanks was not possible due to the size of the fish and trough availability. Therefore, differences in ploidies could have been confounded by the tanks themselves and future work should validate whether these differences are replicated in a more robust experimental setup. Thirdly, the diploids used in Chapters 3 & 4 were over two times the mass of triploids, despite being the same age. This increased growth rate would likely impact nutrient availability, metabolism, and the response to stressors. It is unfortunate that the different rearing conditions in these trials accounted for extensive confounding factors between ploidies, but these fish were the only available stocks of the size sufficient for the logger size. Ideally, triploids would have also been reared in net pens and returned to freshwater at the same time as diploids but the reduced infrastructure due to government mandate made this impossible. Therefore, these results should be validated in similarly reared ploidies.

5.6 Implications for aquaculture

Climate change will increase pressure on all aspects of our society, but aquaculture will have to adapt. Finding ways to reduce pressure on wild fish populations while also maintaining the health of their

own stocks is vital to maintain economic viability of fish farming. Utilizing triploids will help aquaculture overcome the first issue, but their poor stress tolerance could make triploids less profitable due to losses over production cycles. Production of strains that are more tolerant of common stressors (pathogens and thermal stress) can support implementing these fish in aquaculture. The data presented here identifies molecular impacts of increased ploidy. Specifically, I have identified genes that could be screened in parents to generate strains that have molecular responses more similar to diploid fishes. Alternatively, companies could utilize this data to create transgenic triploid strains that have more ideal responses to stress, similar to the generation of AquAdvantage Salmon that have a shorter production cycle (Cook et al., 2000).

Additionally, this data can be used to develop treatments that can benefit farming of diploid fishes as well. MicroRNA-based therapy has been utilized in rats and grass carp to improve pulmonary hypertension and lipid metabolic disorders (Chen et al., 2022; Lu et al., 2021). Although these techniques are currently expensive, identification of dysregulated genes is the first step in developing therapeutics that could become more economical in the future. Treatment with specific drugs at specific times after pathogen exposure or prior to expected heat waves could protect aquaculture stocks from mass mortality events. If the lower oxygen availability is in fact what results in reduced survival of triploids, increasing the oxygen availability during heat waves would reduce mortality and protect stocks.

5.7 Future directions

This thesis provides preliminary evidence on the molecular background that drives reduced stress performance of triploid salmon. I have investigated the miRNA dynamics in three immune tissues, but further work could expand this investigation through understanding cellular-level differences. T cell maturation occurs in the thymus and involves selection of cells that recognize novel peptides, while inducing apoptosis in cells that recognize self-antigens (Nossal, 1994; Takaba and Takayanagi, 2017). Triploids have a reduced cellular repertoire relative to diploids (Benfey, 1999); therefore, T cell negative selection could result in triploids having significantly reduced T cell counts. Measuring T cell counts before and during an immune response could provide further insight into the immune incompetence of triploids. Additionally, the predicted miRNA-mRNA relationships should be investigated to provide further clarity on phenotypic effects of increased ploidy. Investigation of more

timepoints (and alternative infections such as parasites or viruses) could expand on the miRNA identified as dysregulated. For Chapter 2, an intraperitoneal injection was utilized to confirm that each fish was receiving the same bacterial load. However, utilizing natural infection routes might provide a more realistic molecular response, such as an oral gavage or bath infection since invading mucosal surfaces is the first barrier infections must overcome. The data in Chapter 3 provides some insight into the immunological molecular changes occurring after thermal stress; however, without any pathogen exposure it is unclear if these responses are adaptive or maladaptive. Combining thermal and pathogenic stressors would also provide a more aquaculturally-relevant study to identify whether triploid fishes will be more at risk as climate change progresses. Finally, investigating the cardiac impacts of increased ploidy would provide further evidence for this tissue governing their reduced thermal performance. I chose to investigate genes involved in cardiac oxygenation because in salmonids, oxygen capacity governs their thermal performance (Eliason et al., 2011; Eliason et al., 2013; Farrell et al., 2008). Recent work has shown that cardiac oxygenation might not be the sole determinate of cardiac upper limits (Schwieterman et al., 2023). Cardiac excitability also fails at higher temperatures and is associated with cardiac arrhythmias (Haverinen and Vornanen, 2020). Therefore, investigating how altered triploid neural excitability or plasma membrane parameters impact their cardiac function at high temperatures could provide explanation for why they have earlier onsets of cardiac arrhythmia.

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Appendix A

Supplemental Data for Chapter 2

Supplemental data for this chapter can be found online at <https://doi.org/10.1016/j.cbd.2023.101121> and <https://doi.org/10.6084/m9.figshare.22802816>

Table A.1: Two-way ANOVA table for Ploidy impacts on sequencing metadata

Initial testing whether ploidy had a significant impact on the total reads generated, reads after trimming, proportion of reads aligned, proportion of reads associated with miRNA, or total miRNA loci identified. Significant factors ($p \leq 0.05$) are represented in bold.

	DF	F	P value
Total number of reads			
Interaction	2, 50	0.6653	0.5186
Ploidy	1, 50	0.001769	0.9666
Tissue	2, 50	0.4278	0.6543
Reads after trimming			
Interaction	2, 50	1.759	0.1827
Ploidy	1, 50	1.134	0.292
Tissue	2, 50	119.8	<0.0001
Proportion of reads aligned			
Interaction	2, 50	1.326	0.2747
Ploidy	1, 50	0.3253	0.571
Tissue	2, 50	5.584	0.0065
Proportion of reads associated with miRNA			
Interaction	2, 50	0.2251	0.7992
Ploidy	1, 50	0.6455	0.4255
Tissue	2, 50	31.37	<0.0001
Total miRNA loci identified			
Interaction	2, 50	0.6699	0.5163
Ploidy	1, 50	0.3935	0.5333
Tissue	2, 50	41.83	<0.0001

Table A.2: One-way ANOVA table for tissue impacts on sequencing metadata

Testing whether tissue had a significant impact on the total reads generated, reads after trimming, proportion of reads aligned, proportion of reads associated with miRNA, or total miRNA loci identified. Significant factors ($p \leq 0.05$) are represented in bold.

	DF	F	P value
Total number of reads			
Tissue	2, 53	0.4417	0.6453
Reads after trimming			
Tissue	2, 53	116.7	<0.0001
Proportion of reads aligned			
Tissue	2, 53	6.04	0.0043
Proportion of reads associated with miRNA			
Tissue	2, 53	33.56	<0.0001
Total miRNA loci identified			
Tissue	2, 53	42.96	<0.0001

Table A.3: Two-way ANOVA table for qPCR validation of differentially expressed miRNA
Testing whether ploidy or sampling timepoint had a significant impact on specific miRNA abundance. Significant factors ($p \leq 0.05$) are represented in bold.

	DF	F	P value
miR-1-3p hindgut			
Interaction	1, 20	0.1071	0.7469
Day	1, 20	0.6708	0.4224
Ploidy	1, 20	0.4369	0.5162
miR-1-3p head kidney			
Interaction	1, 20	0.9507	0.3412
Day	1, 20	7.364	0.0134
Ploidy	1, 20	2.966	0.1004
miR-1-3p spleen			
Interaction	1, 20	2.18	0.1554
Day	1, 20	0.01937	0.8907
Ploidy	1, 20	0.001529	0.9692
let-7e-5p spleen			
Interaction	1, 20	0.09357	0.7629
Day	1, 20	1.998	0.1728
Ploidy	1, 20	0.01251	0.9121
miR-146d-5p spleen			
Interaction	1, 20	0.1495	0.7031
Day	1, 20	39.84	<0.0001
Ploidy	1, 20	1.129	0.3006
miR-2188-3p spleen			
Interaction	1, 20	12.74	0.0019
Day	1, 20	0.01197	0.914
Ploidy	1, 20	4.562	0.0452

Appendix B

Supplemental Data for Chapter 3

Table B.1: Two-way ANOVA table for heat shock response genes in gill and ventricle

Testing whether ploidy or sampling treatment (control or thermal stress recovery) had a significant impact abundance on heat shock response genes. FDR corrections were completed using the two-stage step-up method, and significant factors (p adjusted ≤ 0.05) are represented in bold.

	Gill				Ventricle			
	DF	F	P value	P adj	DF	F	P value	P adj
<i>hsp70</i>								
Interaction	1, 28	0.7261	0.4014	0.28098	1, 26	0.8864	0.3551	0.18643
Ploidy	1, 28	0.8393	0.3674	0.28098	1, 26	1.68	0.2063	0.12997
Treatment	1, 28	587.8	<0.0001	<0.0001	1, 26	1289	<0.0001	<0.0001
<i>hsp90aa1</i>								
Interaction	1, 27	4.91	0.0353	0.04942	1, 26	2.823	0.1049	0.08261
Ploidy	1, 27	1.787	0.1925	0.20213	1, 26	6.972	0.0138	0.01449
Treatment	1, 27	167.1	<0.0001	<0.0001	1, 26	238.1	<0.0001	<0.0001

Table B.2: Two-way ANOVA table for pattern recognition receptor genes in gill and ventricle
Testing whether ploidy or sampling treatment (control or thermal stress recovery) had a significant impact abundance on pattern recognition receptor genes. FDR corrections were completed using the two-stage step-up method, and significant factors (p adjusted ≤ 0.05) are represented in bold.

	Gill				Ventricle			
	DF	F	P value	P adj	DF	F	P value	P adj
<i>tlr2</i>								
Interaction	1, 28	0.819	0.3733	0.2039	1, 26	0.3587	0.5544	0.4657
Ploidy	1, 28	16.71	0.0003	0.0009	1, 26	2.439	0.1304	0.13692
Treatment	1, 28	6.285	0.0183	0.0192	1, 26	0.1361	0.7152	0.50064
<i>nlrp3</i>								
Interaction	1, 28	1.195	0.2837	0.2039	1, 26	3.47	0.0738	0.10332
Ploidy	1, 28	11.12	0.0024	0.0038	1, 26	50.39	<0.0001	<0.0001
Treatment	1, 28	0.768	0.3884	0.2039	1, 26	9.281	0.0053	0.01113

Table B.3: Two-way ANOVA table for MHC class I genes in gill and ventricle

Testing whether ploidy or sampling treatment (control or thermal stress recovery) had a significant impact abundance on MHC class I genes. FDR corrections were completed using the two-stage step-up method, and significant factors (p adjusted ≤ 0.05) are represented in bold.

	Gill				Ventricle			
	DF	F	P value	P adj	DF	F	P value	P adj
<i>tapasin</i>								
Interaction	1, 28	0.089	0.7676	0.7164	1, 26	0.002	0.9627	0.89852
Ploidy	1, 28	6.508	0.0165	0.0693	1, 26	19.43	0.0002	0.00168
Treatment	1, 28	0.615	0.4395	0.5274	1, 26	5.864	0.0227	0.09534
<i>mhc1a</i>								
Interaction	1, 28	0.192	0.6643	0.6975	1, 26	0.491	0.4896	0.58752
Ploidy	1, 28	2.289	0.1415	0.2972	1, 26	1.163	0.2908	0.40712
Treatment	1, 28	0.803	0.378	0.5274	1, 26	1.888	0.1812	0.40712
<i>b2m</i>								
Interaction	1, 28	1.620	0.2135	0.35868	1, 26	1.483	0.2342	0.40712
Ploidy	1, 28	38.29	<0.0001	<0.0001	1, 26	0.049	0.8268	0.86814
Treatment	1, 28	5.013	0.0333	0.09324	1, 26	1.242	0.2752	0.40712

Table B.4: Two-way ANOVA table for MHC class II genes in gill and ventricle

Testing whether ploidy or sampling treatment (control or thermal stress recovery) had a significant impact abundance on MHC class II genes. FDR corrections were completed using the two-stage step-up method, and significant factors (p adjusted ≤ 0.05) are represented in bold.

	Gill				Ventricle			
	DF	F	P value	P adj	DF	F	P value	P adj
<i>mhc2a</i>								
Interaction	1, 28	2.787	0.1061	0.1892	1, 26	0.4778	0.4955	0.7151
Ploidy	1, 28	12.47	0.0015	0.0079	1, 26	0.1728	0.681	0.7151
Treatment	1, 28	2.755	0.1081	0.1892	1, 26	6.932	0.0141	0.0888
<i>mhc2b</i>								
Interaction	1, 28	0.1262	0.7251	0.6345	1, 26	0.3076	0.5839	0.7151
Ploidy	1, 28	1.647	0.2099	0.2755	1, 26	0.3769	0.5446	0.7151
Treatment	1, 28	0.5686	0.4571	0.48	1, 26	0.4411	0.5124	0.7151

Table B.5: Two-way ANOVA table for cytokine genes in gill and ventricle

Testing whether ploidy or sampling treatment (control or thermal stress recovery) had a significant impact abundance on cytokine genes. FDR corrections were completed using the two-stage step-up method, and significant factors (p adjusted ≤ 0.05) are represented in bold.

	Gill				Ventricle			
	DF	F	P value	P adj	DF	F	P value	P adj
<i>il1b</i>								
Interaction	1, 28	0.9385	0.341	0.15345	1, 26	0.028	0.8684	0.91182
Ploidy	1, 28	19.84	0.0001	<0.0001	1, 26	6.345	0.0183	0.07686
Treatment	1, 28	140.9	<0.0001	<0.0001	1, 26	156.2	<0.0001	<0.0001
<i>il8</i>								
Interaction	1, 28	7.765	0.0095	0.0050	1, 26	0.360	0.554	0.6648
Ploidy	1, 28	26.61	<0.0001	<0.0001	1, 26	2.342	0.138	0.2898
Treatment	1, 28	36.1	<0.0001	<0.0001	1, 26	3.403	0.0765	0.2142
<i>tgfb</i>								
Interaction	1, 28	0.0186	0.8925	0.3124	1, 26	4.08E-05	0.995	0.92867
Ploidy	1, 28	9.072	0.0055	0.0035	1, 26	0.5866	0.4506	0.63084
Treatment	1, 28	0.7195	0.4035	0.1589	1, 26	0.7885	0.3827	0.63084

Appendix C

Supplemental Data for Chapter 4

Table C.1: Permutation two-way ANOVA for $CT_{\max}$

A Permutation two-way ANOVA was used (50 000 iterations) to test whether ploidy or treatment had a significant impact on $CT_{\max}$. Significant factors ($p \leq 0.05$) are represented in bold.

	DF	Iter	Pr (Prob)
Interaction	1, 37	50000	0.807
Treatment	1, 37	50000	0.933
Ploidy	1, 37	50000	0.057

Table C.2: Two-way ANOVA table for plasma metabolites

Testing whether ploidy or treatment had a significant impact on circulating plasma metabolites. Significant factors ($p \leq 0.05$) are represented in bold.

	DF	F	P value
Cortisol			
Interaction	3, 67	1.201	0.3162
Treatment	3, 67	30.49	<0.0001
Ploidy	1, 67	10.39	0.002
Lactate			
Interaction	3, 67	0.5457	0.6528
Treatment	3, 67	139.9	<0.0001
Ploidy	1, 67	0.6155	0.4355
Glucose			
Interaction	3, 63	1.821	0.1525
Treatment	3, 63	30.25	<0.0001
Ploidy	1, 63	0.7996	0.3746

Table C.3: Two-way ANOVA table for relative abundance of genes in atrium

Testing whether ploidy or treatment (control or CT_{max} recovery) had a significant impact on relative abundance of heat shock and cardiorespiratory genes. Significant factors ($p \leq 0.05$) are represented in bold.

	DF	F	P value
<i>hsp90aa1</i>			
Interaction	1, 27	0.6494	0.4274
Ploidy	1, 27	7.397	0.0113
Treatment	1, 27	162.7	<0.0001
<i>hsp70</i>			
Interaction	1, 27	0.07884	0.781
Ploidy	1, 27	1.099	0.3039
Treatment	1, 27	640.2	<0.0001
<i>miR-24a-5p</i>			
Interaction	1, 27	0.804	0.3778
Ploidy	1, 27	0.004762	0.9455
Treatment	1, 27	31.18	<0.0001
<i>ca4</i>			
Interaction	1, 27	2.602	0.1184
Ploidy	1, 27	3.887	0.059
Treatment	1, 27	0.2987	0.5892
<i>mb</i>			
Interaction	1, 27	2.275	0.1431
Ploidy	1, 27	10.89	0.0027
Treatment	1, 27	4.941	0.0348

Table C.4: Two-way ANOVA table for relative abundance of genes in ventricle

Testing whether ploidy or treatment (control or CT_{max} recovery) had a significant impact on relative abundance of heat shock and cardiorespiratory genes. Significant factors ($p \leq 0.05$) are represented in bold.

	DF	F	P value
<i>hsp90aa1</i>			
Interaction	1, 26	3.237	0.0836
Ploidy	1, 26	5.944	0.0219
Treatment	1, 26	385.7	<0.0001
<i>hsp70</i>			
Interaction	1, 26	1.121	0.2994
Ploidy	1, 26	0.2246	0.6395
Treatment	1, 26	1216	<0.0001
<i>miR-24a-5p</i>			
Interaction	1, 26	0.4677	0.5001
Ploidy	1, 26	8.576	0.007
Treatment	1, 26	0.7409	0.3972
<i>ca4</i>			
Interaction	1, 26	1.992	0.17
Ploidy	1, 26	22.03	<0.0001
Treatment	1, 26	2.71	0.1117
<i>mb</i>			
Interaction	1, 26	0.6715	0.42
Ploidy	1, 26	39.34	<0.0001
Treatment	1, 26	2.013	0.1678

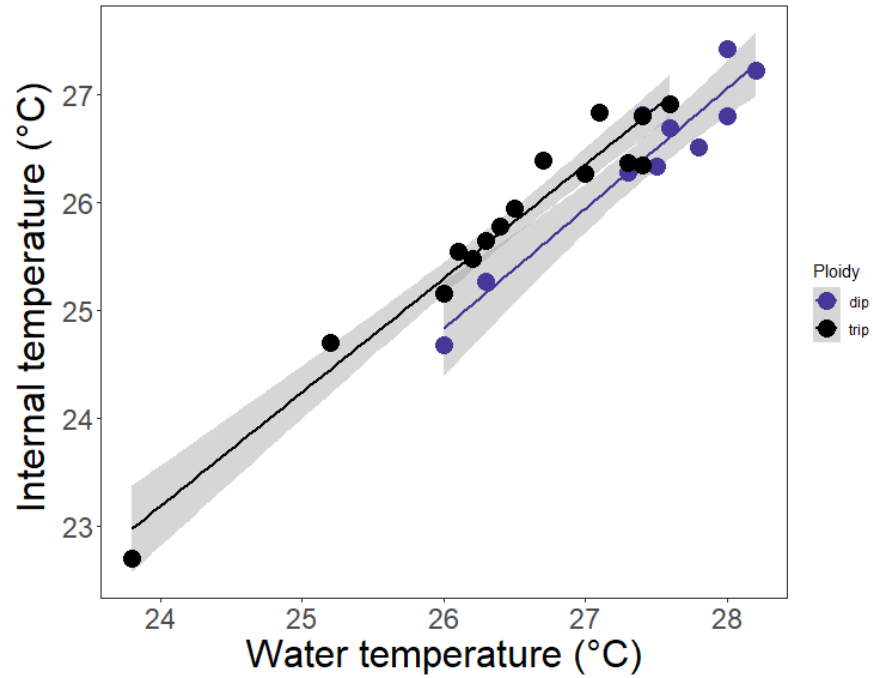


Figure C.1: Water temperature (°C) at CT_{max} compared to the logger-recorded temperature (°C) at CT_{max}

Each point represents an individual fish's CT_{max} measurement, with lines representing the linear relationship (95% confidence interval in grey) within each ploidy.

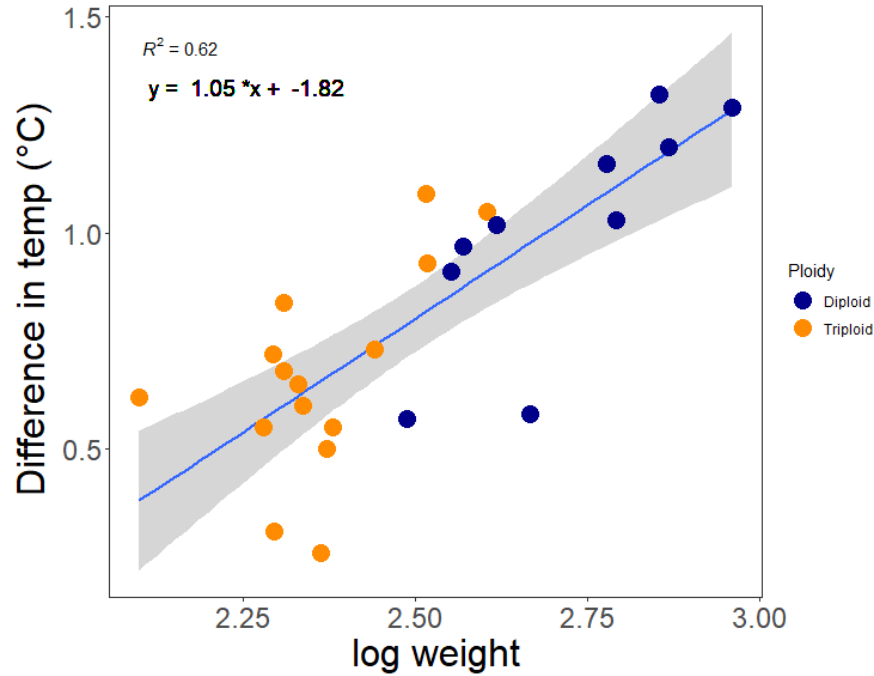


Figure C.2: Model representing the mass (g) compared to the difference in temperature between water and internal logger-recorded temperature (°C)

Difference in temperature was calculated for each logger-implanted fish by subtracting the CT_{max} water temperature from the internal recorded CT_{max} temperature. Each point represents an individual fish (coloured based on ploidy), and the resulting linear model (95% confidence interval in grey) was used to calculate the predicted internal thermal tolerance measurement for all CT_{max} -exposed individuals.

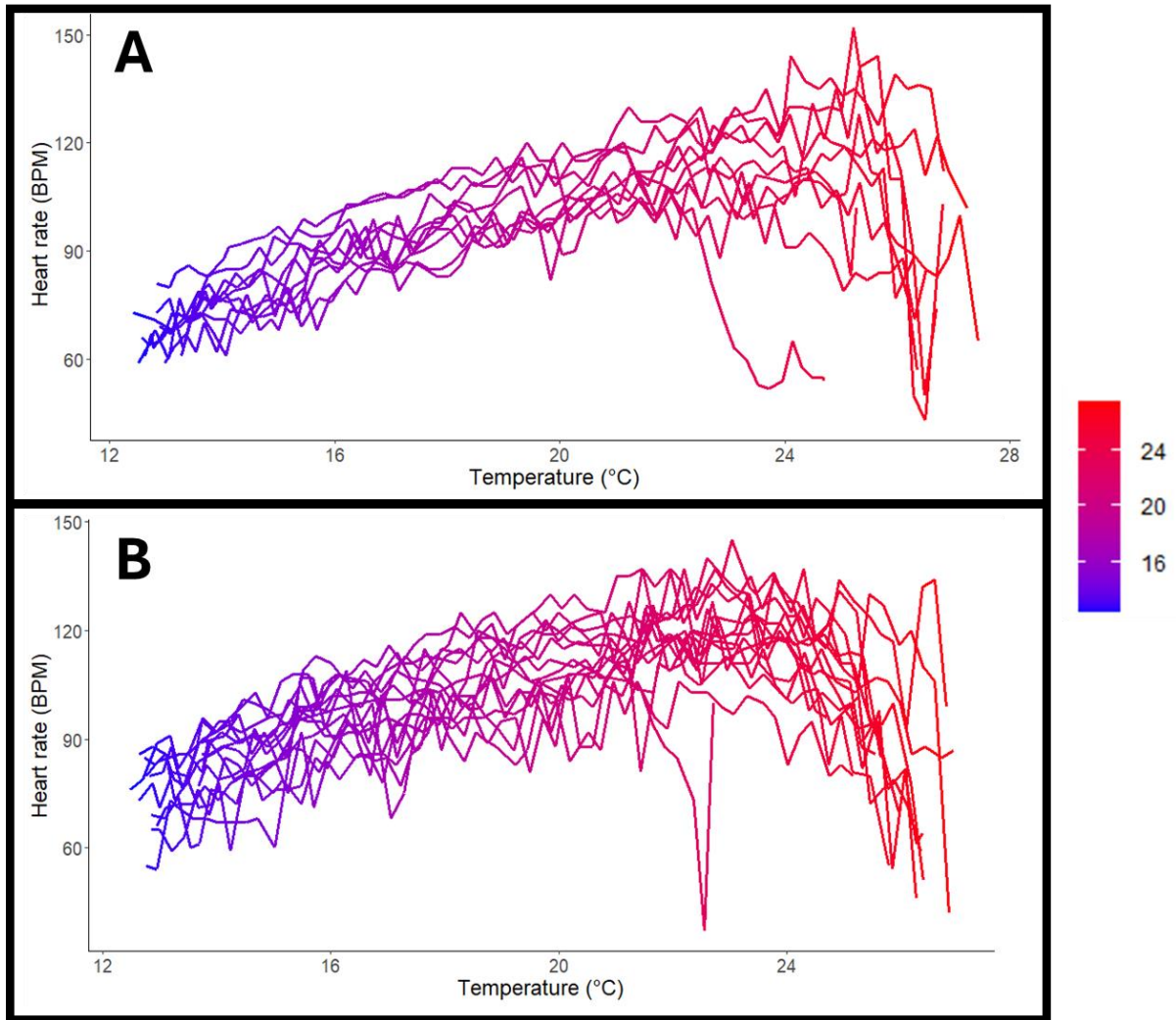


Figure C.3: Individual fish heart rates during CT_{max} trial for diploid and triploid Chinook salmon

Heart rate of diploid (A, $n = 10$) and triploid (B, $n = 15$) over the CT_{max} trial. Heart rates were determined using electrocardiogram data generated by Star-Oddi heart rate and temperature loggers. Each line represents an individual fish, and only fish that were upright one hour after recovery from CT_{max} and had no signs of disease are represented. Colour represents the temperature (°C) for that associated heart rate measurement.

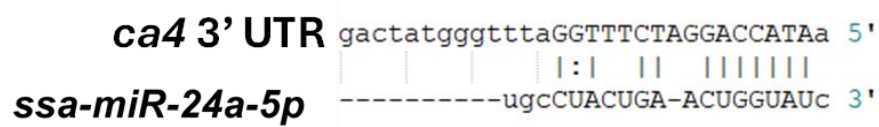


Figure C.4: Carbonic anhydrase 4 (*ca4*; XM_024442862.2) 3' UTR binding site for miR-24a-5p
 Binding site relationship was predicted using miRanda (v3.3a) and has an alignment score of 143 and a minimum free energy of -258.33 kcal/mol.