Analysis of Immune System and Stress Parameters of *Oncorhynchus mykiss* in Response to Juvenile Thermal Preconditioning or Thermal-Based Parental Selection

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.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Chapter 2 – L. Whitehouse was involved in the design and validation of HSP70 and HSC70 qPCR primers. Other qPCR primers were previously designed and validated by S. Semple. L. Whitehouse assisted in performing the qPCR for various genes, as well as the statistical analysis.

Chapter 3 – qPCR primers were previously designed and validated by S. Semple. L.

Abstract

Higher water temperatures and pathogens are both significant factors that negatively affect the welfare of teleost fish. In aquaculture, compared to natural populations, these problems are especially exacerbated, as the animals have relatively limited mobility, and the higher density promotes faster spread of infectious diseases. Because of the potential harm these stressors can inflict, methods that can limit the damage of these stressors are particularly valuable. Two of the methods that have been explored as ways to improve the thermotolerance of aquacultured teleosts are juvenile-stage thermal preconditioning and thermotolerance-based parental selection. These methods have thus far demonstrated potentially effective improvements in the thermotolerance of animals, yet their potential effects on the immune system have not been explored, even though the heat-shock response and immune response are interconnected. In the first experiment, juvenile-stage thermal preconditioned rainbow trout (*Oncorhynchus mykiss)* were subject to a secondary thermal challenge in which animals were collected and sampled at the time of lost equilibrium. The effects of preconditioning were assessed on spleen and gill transcript profiles by quantifying IL-1β, IL-6, TNF-α, IFN-1, β2m, MH class I, HSP70, and HSC70 transcripts via qPCR, as well as assessment of the general stress response by measuring plasma cortisol levels. No changes in CTmax were observed between the preconditioned and control cohorts upon the second challenge. IL-1β and IL-6 transcripts were generally upregulated with increased temperature of the secondary thermal challenge, whereas IFN-1 transcripts were upregulated in the spleen, but downregulated in the gills, along with MH class I. The juvenile thermal preconditioning produced a series of changes in transcript levels for IL-1β, TNF-α, IFN-1, and HSP70, but the dynamics of these differences were inconsistent. Otherwise, plasma cortisol levels were lower in the pre-conditioned animals. In the second experiment, rainbow

trout offspring of crosses raised from thermally selected parents were co-stimulated with a thermal and pathogenic challenge. Animals held at different temperatures were injected with an inactivated *Vibrio anguillarum* vaccine, and spleen tissue was sampled at 6-, 24-, and 96-hours post-injection. Relative transcript analysis of splenic IL-1β, IFN-γ, tapasin, and IL-10 was conducted with qPCR. All four transcripts were upregulated in response to the vaccine at 6- and 24-hours post-injection, and IL-10 was upregulated at 96-hours post-injection. The vaccinated cohorts held at a higher temperature presented evidence of an accelerated regulation profile compared to the vaccinated cohort held at a lower control temperature. The thermal selection of parents had a few significant changes across the assessed transcripts, but no consistent trends between transcripts. In general, these two methods were able to induce significant changes in immune transcript expression at higher temperatures both with and without immune stimulation. However, the biological significance of these changes will require further analysis to understand. If these thermotolerance-based methods can confer additional protection to the immune response, the revelation will be relevant to a global aquaculture industry that is constantly struggling with environmental and pathogenic pressures.

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List of Abbreviations

AARS	alma aquaculture research station
ANOVA	analysis of variance
CTmax	critical thermal maximum
DAMP	danger associated molecular pattern
DNA	deoxyribonucleic acid
CT	Cycle threshold
EF1-α	elongation factor 1-alpha
ELISA	enzyme linked immune-sorbent assay
IFN-1	interferon-1
IFN-γ	interferon-gamma
IgM	immunoglobulin m
IL-8	interleukin-8
IL-10	interleukin-10
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IPNV	infectious pancreatic necrosis virus
IRF-1	interferon regulatory factor 1
HSP	heat shock protein
HSP40	heat shock protein 40
HSP60	heat shock protein 60
HSP70	heat shock protein 70
HSP90	heat shock protein 90
HSC70	heat shock cognate 70
LT	lower tolerance
MH	major histocompatibility
NF-κB	nuclear factor kappa-light-chain-enhancer of activated b cells
PAMP	pathogen associated molecular patterns
PBS	phosphate buffered saline
Poly(I:C)	polyinosinic:polycytidylic acid
Q_{10}	temperature coefficient
qPCR	quantitative polymerase chain reaction
RIG-I	retinoic acid-inducible gene i
RNA	ribonucleic acid
TAP	transporter associated with antigen processing
Th1	t helper type 1
Tm	melting temperature
TNF-α	tumor necrosis factor-alpha
TLR	toll-like receptor

UT	upper tolerance

Chapter 1 General Introductions

1.1 Climate Outlook and Effect of Climate Change on Global Water Temperatures

Globally, over 90% of the total energy produced by the greenhouse effect is absorbed by the oceans (Pachauri and Meyer, 2014). Consequently, the world's average ocean temperature has been increasing continuously since the 1920s (Laffoley and Baxter, 2016). The annual global sea surface temperatures of the last three decades were higher than the temperatures measured at any other period in recorded history (Laffoley and Baxter, 2016), and these temperatures are predicted to rise continually through 2100 with a predicted increase of 1.5^{o}C - 2^{o}C in global surface temperatures (IPCC, 2021). Furthermore, potential developments of extreme regional warming anomalies can be especially damaging, as the resulting fluctuation in temperatures is notably acute compared to routine seasonal changes (Amaya et al., 2016; Bond et al., 2015). These increases in water temperatures, sporadic or gradual, can have an extensive impact on the physiology and overall health of many aquatic species (Blaxter, 1991; Dallas, 2009; Pepin, 1991).

1.2 Aquaculture and the Threat of Pathogens on Aquatic Life

As the global demand for protein-based foods increases, so does the demand for aquaculture as a means of sustainable food productions in place of capture fisheries (Israngkura and Sae-Hae, 2002). For the aquaculture industry, infectious diseases are the most damaging sources of commercial loss for most species (Assefa and Abunna, 2018; Tavares-Dias and Martins, 2017). The tight quarters in which aquaculture products are raised means infections can spread with devastating speed compared to the dynamics found in a more natural setting. Moreover, the standards for the success of fish health and growth are defined with different stringencies when

analyzed from a conservation versus a commercial perspective. While a wild, unhealthy animal with active infections can still reach the mating age to reproduce and effectively participate as a functioning member of the population, the same animal in a commercial setting would not be deemed a success, as it would have limited profitability, if any at all. Contrasted to animals in nature, in aquaculture, diseases and pathogens can affect feed conversion, damage skin/muscle tissue, and cause mortalities outright (Tavares-Dias and Martins, 2017). Thus, even the non-fatal infections may cause the animals to be unviable as produce, due to the damage dealt by the pathogens in the infection process. Traditionally, vaccinations and anti-microbial treatments are effective methods to confer protection for an animal against pathogens. However, vaccination of animals against all possible pathogenic threats is costly and inefficient, and anti-biotic use can have damaging and irreversible impacts on the environment (Shinn et al., 2018). Therefore, other methods of raising the general immunity of animals to help them cope or respond to a pathogen are of particular interest.

1.3 Canadian Aquaculture and General Teleost Thermal Tolerance

Currently, Canada is one of the world's leading exporters of aquaculture products (Food and Agriculture Organization, 2018). The multibillion-dollar industry comprises a myriad of fish, mollusk, and crustacean products, but over 93% of the industry is based on production of just a few salmonid species such as trout and salmon (Fisheries and Oceans Canada, 2017). As ectothermic animals, a salmonid's surrounding water temperature has a direct and significant impact on the organism's physiology (Eliason et al., 2017; Steinhausen et al., 2008). The optimal temperature regarding the growth and feed conversion for rainbow trout is 13 - 18^{o}C (Aquaculture in North Carolina, 2001), and increases in water temperature beyond this optimal upper limit will result in decreased feeding activity (Breau et al., 2011). Accounts from Ontario

rainbow trout hatcheries have reported summer water temperatures as high as 21^{o}C. These temperature hikes have caused reductions in feeding behaviours for periods as long as 6 weeks in the summer months. This deficit has translated to reduced market fillet sizes and has contributed to commercial losses of up to 25%. Heat-induced thermal stress is an escalating challenge for salmonid growth and is a source of significant economic loss. Studying the physiological effects that heat stress has on salmonids and developing measures to mitigate the effects of high-water temperatures on fish are integral to continued growth in the industry.

1.4 The Concept and Significance of Thermal Tolerance

Intraspecific thermotolerance differences between individuals are interesting as they can be theoretically manipulated to confer protection for organisms. However, the mechanisms that cause these thermotolerant effects are complex and multi-faceted. For example, Heat Shock Protein (HSP) 70 is an evolutionarily conserved, integral chaperone protein in the response to heat stress. Its main purpose is to aid in the refolding of misfolded proteins. This protein is primarily expressed and regulated in two forms, a constitutive form (HSC70) that is constantly maintained, and an inducible form (HSP70) that can be dramatically upregulated in times of heat stress. However, while HSP70 is a good indicator for heat stress, it is not as good of an indicator for thermotolerance (Krebs and Bettencourt, 1999). Thermotolerant differences between individuals of the same species, often stemming from genetics-based differences, can be caused by a variety of difficult-to-trace factors. Examples such as the basal levels of HSC70, regulation of HSP70 translation, post-translational modifications, and the temperature at which HSP70 upregulation is induced in each individual animal, would all affect their thermotolerance status (Hassan et al., 2019). Additionally, researchers have suggested the idea that other signaling and regulatory molecules may be much more relevant to the thermotolerance of an individual than

HSP70. Ultimately, the concept of inferring the thermotolerance status of any individual or family from a specific designated molecular marker may be error-prone and misleading. Instead, the general idea of thermotolerant individuals would be better defined as an individual that can efficiently and robustly regulate gene expression and cellular mechanisms to minimize the effects of heat stress, while limiting the damage to self in the process (Hassan et al., 2019)

1.5 Heat Shock Response – Immune Interactions

Similar to mammals, the teleost immune and heat stress responses have been demonstrated to modulate each other (Chen et al., 2014; Forsyth et al., 1997; Han et al., 2017). Most of these currently known effects were identified by cause-and-effect studies where immune parameters were modulated by a thermal challenge, and vice versa. Thus, while the presence and general effects of these modulation patterns are understood, much of the mechanisms behind these regulatory changes have not yet been elucidated.

Most of the more specific studies on immune–heat stress co-modulation have approached the topic through the perspective of heat shock protein (HSPs) actions and regulations. As one of the most well studied HSPs, HSP70 has demonstrated patterns of upregulation in response to both bacterial (Ackerman and Iwama, 2001; Elibol-Flemming et al., 2009; Forsyth et al., 1997) and viral (Dang et al., 2010) pathogen types. The majority of these studied observed upregulations of HSP70 transcript in several immune relevant tissues in various species. Along with HSP70, other HSPs such as HSP40, HSP60, and HSP90 have also demonstrated upregulation in response to pathogenic stress in teleosts (Cha et al., 2013; Dong et al., 2005). However, a study of silver sea bream challenged with *Vibrio alginolyticus* observed a disease-progression-correlated decrease in HSP70 expression in the kidney at both the transcript and protein levels (Deane and Woo,

2005). This suggests an alternative narrative that HSP70 is not regulated positively with immune transcripts in certain study systems that researchers should be aware of.

1.6 Roles and Regulations of Cytokines and Immune Relevant Genes

The canonical mammalian proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) are versatile signaling molecules in fish. They play central roles in mediating the pro-inflammatory immune responses, and their vast physiological influence can often expand past the boundaries of immunology (Zou and Secombes, 2016). TNF-α is one of the earliest cytokines to be activated in the event of an infection. This fast-acting cytokine recruits leukocytes (Zou and Secombes, 2016), encourages phagocytosis (Garcia-Castillo et al., 2004), and induces apoptotic signals in damaged host cells (Xiao et al., 2007). Similarly, *IL-1β* can be upregulated in response to TNF-α activity (Zou et al.. 2003), as well as to cellular exposure to foreign antigens. Aside from sharing many of the same redundant pro-inflammatory effects with TNF-α, IL-1β has also been shown to dilates blood vessels (Costa et al., 2015), causes chronic inflammation (Bo et al., 2015), induces expression of *TNF-α* (Bo et al., 2015; Castro et al., 2011) and IL-6 (Castro et al., 2011), and upregulates genes relating the muscle growth (Pooley et al., 2013). Lastly, IL-6 is a potent proinflammatory cytokine and situational anti-inflammatory cytokine in mammals (Zou and Secombes, 2016). The proinflammatory properties of IL-6 include T helper cell differentiation (Chen et al., 2012), promotion of immunoglobulin M (IgM) response (Abos et al., 2016; Kaneda et al., 2012), and expression regulation of interferon regulatory factors and antimicrobial peptides (Costa et al., 2011). Conversely, IL-6's anti-inflammatory capabilities in fish are poorly understood and require further evaluation.

Type I interferons like interferon-1 (IFN-1) are cytokines that are produced in response to binding of retinoic acid-inducible gene I (RIG-I)-like receptors to viral pathogen associated molecular patterns (PAMP); membrane receptor interactions with IFN-1 will cause fish host cells to enter a protective anti-viral state (Chen et al., 2017). The type II interferon interferon-gamma (IFN-γ) is a potent macrophage activator (Zou and Secombes, 2016) in the event of an infection, and a promoter of the T helper type 1 (Th_1) response (Zou et al., 2005). Additionally, IFN-γ treatment demonstrated elevated expression in multiple genes related to the major histocompatibility (MH) class I antigen presentation pathway in rainbow trout (Martin et al., 2007). The MH class I antigen presentation pathway involves an array of different proteins that assemble and mediate MH class I functions (Peaper and Cresswell, 2008; Stet et al., 2003). One of these proteins of interest is tapasin (TAP binding protein), which is responsible for mediating the transformation of major histocompatibility complex to the cell surface (Jørgensen et al., 2007; Landis et al, 2006). The MH class I, consisting of alpha chain, and its associate molecule beta 2-microglobulin (β2m) are integral signaling molecules involved in self-modulating cellular mechanisms in association with the functions of cytotoxic T cells (Grimholt et al., 2015). Upregulations in MH class I related molecules can potentially increase the speed of clearance for pathogens and damaged self-cells (Raval et al., 1998).

After the activated immune response has sufficiently neutralized the pathogenic threat, interleukin-10 (IL-10) is one of the main anti-inflammatory signaling molecules responsible for the optimal resolution of the immune response. There are two paralogues of IL-10 in rainbow trout, both are regulated similarly in the presence of PAMPs and live bacterial challenges (Harun et al., 2011). IL-10 enacts its immune-suppression functions by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, this regulation is bi-directional as

NF-κB stimulates upregulation of IL-10 in a mode of negative feedback (Swain et al., 2012; Wei et al., 2015). The role of IL-10 is primarily anti-inflammatory, as demonstrated by its inversely proportional expression patterns compared to *IL-1β* during immune challenges (Seppola et al., 2008; Swain et al., 2012). However, IL-10 has also demonstrated some pro-inflammatory capabilities, in a display of its pleiotropic nature (Wu et al., 2021). Each cytokine/immune molecule is responsible for a myriad of effector functions, any significant modulation in the expression patterns of these cytokines/molecules can have highly relevant effects on other aspects of the immune response, not to mention the more indirect effects on other systems

1.7 Roles and Regulations of Heat Shock Relevant Genes

In addition to the immune markers, there are other biomarkers of interest that can demonstrate relevant information regarding the effects of temperature on an organism. Teleost HSP70 (heat shock proteins 70) are a class of ~70 kDa proteins that are upregulated significantly during stress (Basu et al., 2002; Roberts et al., 2010). The main responsibilities of HSP70 include protein folding, protein translocation, and refolding of misfolded proteins (Basu et al., 2002; Mayer and Bukau, 2005). While the prominent functions of HSP70 pertain to the maintenance of protein integrity, HSP70 can also be involved in a multitude of other roles (Roberts et al., 2010). Currently, the full scope of HSP70 functions in fish is not well established, but studies of HSP70 in mammals have demonstrated a plethora of immune related functions (Binder, 2014). For example, mammalian HSP70 has been shown to activate immature dendritic cells (Milani et al., 2002), and can trigger toll-like receptor (TLR) 2/4 as a danger associated molecular pattern (DAMP) to activate proinflammatory responses (Asea et al., 2000). Additionally, it is an important chaperone molecule in the endogenous antigen presentation pathway by transporting potential antigens to transporter associated with antigen processing (TAP) (Srivastava et al.,

1994). Considering the conservation of HSP70 across phyla (Iwama et al., 1999), it is not unlikely for HSP70 to perform some similar immune related functions in teleost.

1.8 Thesis Structure

Improving the thermotolerance of salmonid animals is one of the main focuses of aquaculture-related studies. Some of the developed methods have demonstrated the ability to confer protection against heat stress in various teleost models. However, how this improved thermotolerance potentially translates to changes in the immune response has not been explored. An applicable method for improving salmonid thermotolerance is the thermal preconditioning of animals in an early life stage to trigger or build up the heat-shock response mechanisms to prepare for later heat stress events. Another method for improving thermotolerance is the selection of parents based on parental thermotolerance, with the intent to selectively breed offspring with higher resistance to heat stress via selection over multiple generations. Both methods have demonstrated significant benefits to the animals under heat stress. However, their potential impacts on the adjacently modulated immune system remain unknown.

The primary goal of this thesis was to explore whether juvenile-stage thermal preconditioning and thermal-based parental selection methods affect the immune system. I hypothesized that these methods would have an enhanciing effect on the immune system on a transcript level via changes in the heat-shock response. Chapter 2 investigated the observable and molecular effects of juvenile-stage thermal preconditioning on the immune, heat-stress, and general stress responses of rainbow trout. Chapter 3 explored how thermal-based parental selection affects the transcriptional immune response at different temperatures while co-stimulated with a *Vibrio* vaccine. Finally, chapter 4 discusses the results of chapters 2 and 3 as a whole and provides

further insights into the impact of this research and how future studies can build on the current findings.

Chapter 2 Effects of Juvenile Thermal Preconditioning on the Heat-Shock, Immune, and Stress Responses of Rainbow Trout Upon a Secondary Thermal Challenge

2.1 Introduction

2.1.1 Literary Background of Preconditioning in Teleost

With the changes in climate trends causing fish mortality worldwide, early-life thermal preconditioning of organisms has been a method of interest in teleost fish. This method of preconditioning is a potential technique that can be applied by aquaculture producers to acclimatize animals to probable stressors and increase their overall resiliency. The concept of preconditioning involves the introduction of a controlled stimulus to a biological system, with the objective of triggering a long-lasting physiological response that will protect the organism from similar forms of stress in later life (Bolli, 2006). While a few recent publications have assessed the physiological impact and industrial applicability of thermal preconditioning in fish, the procedure has thus far eluded thorough analysis of the effects on immune responses that are often intertwined with and regulated by the heat-stress response. Any potential long-term effects that the thermal preconditioning methods can provide for the immune system of commercial species is both interesting and important for the viability of the preconditioning method.

The viability of the preconditioning method has been explored in many fish species (Callaghan et al., 2018; Narum and Campbell, 2015; Pérez-Casanova et al., 2008; Scott and Johnson, 2012). In rainbow trout, thermal preconditioning has been shown to alter the rates of protein synthesis, and the regulation patterns of metabolic substrates (Callaghan et al., 2018). in comparison to thermal preconditioning, more studies have been conducted in respect to the related hypoxic preconditioning method. Rainbow trout studies on hypoxic preconditioning demonstrated

increased resiliency in cardiorespiratory function as a result of hypoxic and anoxic stimuli (Gamperl et al., 2001). However, the prevalence of the benefits of hypoxic-preconditioning mechanism on a species-wide level has been contended (Gamperl et al., 2004; Overgaard et al., 2004). Overall, while the current preliminary understanding has indicated that fish potentially have the plasticity and regenerative ability necessary to benefit from preconditioning (De Preux Charles et al., 2016; Gamperl and Farrell, 2004), the effects of thermal preconditioning on salmonids require further examination to determine the applicability of this method for the aquaculture industry.

2.1.2. Temperature-Related Regulation of Teleost Immune Transcripts and Goal of the Study

While recent publications have assessed the physiological impact and industrial applicability of preconditioning in fish, the procedure has thus not included thorough analyses of the effects on immune responses. Of course, any potential long-term effects that thermal preconditioning has on the immune system of the species of interest are crucially relevant to the viability of the thermal preconditioning method. For example, the regulation of the proinflammatory cytokine, *IL-1β*, has been assessed in various species with respect to thermal challenge. A study in Atlantic cod revealed significant upregulation of *IL-1β* at the mRNA level following a gradual thermal challenge of up to 19^{o}C (Pérez-Casanova et al., 2008). Furthermore, Costa et al. demonstrated the putative importance of IL-1β to blood vessel permeability and cardiovascular homeostasis when steelhead trout were exposed to supra-optimal water temperatures (Costa et al., 2015). Various other immune molecules such as β2m, MH class I, and IgM-light chain have also shown increased expression levels in response to higher water temperatures (Pérez-Casanova et al., 2008; Rodrigues et al., 1998). Ultimately, however, there remains a significant lack of

knowledge regarding the effect of thermal preconditioning on various physiological systems in teleost. This study attempts to explore the effects of juvenile thermal preconditioning on the pro-inflammatory immune system, anti-viral immune system, heat-shock response, and general stress response. I hypothesized that a juvenile thermal preconditioning regimen would enhance the regulation of immune-related transcripts upon a secondary acute thermal-stress.

2.2 Materials and Methods

2.2.1. Experimental Animals

Fish stocks used in this experiment were obtained from the alma aquaculture research station (AARS) in Guelph, ON. Juveniles were raised from hatch at 8°C for 4 months when they were separated into thermal preconditioning and non-preconditioning (control) cohorts. From 4 months post-hatch, experimental animals of the preconditioning cohort were subjected to thermal preconditioning treatment.

2.2.2 Juvenile Thermal Preconditioning

The thermal preconditioning cohort was subjected to designed temperature cycles twice a week over 6 weeks, for 12 cycles total. The first 2 of 12 cycles entailed; i) moving the fish to holding tanks previously set at 11°C, and held for 10 minutes, ii) raising the temperature of holding tanks from 11°C to 19°C, and held for 30 minutes, iii) from 19°C back to 11°C, held for 10 minutes, iv) return to original housing tanks. The remaining 10 of 12 cycles were adjusted to increase the 19°C stages from 30 to 60 minutes (**Fig. 2.1.A.**).

2.2.3 Thermal Challenge and Sample Collection

A subsequent thermal challenge trial was performed 16 weeks after the end of the last round of preconditioning at the Hagen Aqualab of the University of Guelph. Both preconditioned and non-preconditioned control cohorts were held at and acclimatized to 12°C before the trial. Throughout the trial, individuals were visually monitored for the loss of equilibrium, or critical thermal maximum (CTmax), as described with the evaluation methods described by Morgan et al., (2018). They were then collected and euthanized with MS-222 (400 mg/L) before they were sampled for tissues of interest. At the start of the trial, temperatures were increased at a rate of ~2°C/hour until temperatures reached 24°C. The temperature was then maintained at 24°C for 4 hours before increased again at the previous rate until all subjects were processed and collected (**Fig. 2.1.B.)**.

Animals were collected and processed at 3 points during the trial for subsequent mRNA and protein analysis. Prior to the thermal challenge, 10 preconditioned and 10 non-preconditioned individuals were sampled at 12°C to establish a non-stressed baseline. 12 preconditioned and 12 non-preconditioned individuals were collected before the 24°C hold and were designated as the "lower tolerance" (LT) group. A further 12 preconditioned and 12 non-preconditioned individuals were collected after the 24°C hold and were designated as the "upper tolerance" (UT) group. The purpose of the 4-hour temperature hold at 24°C was to ensure the animals collected in the LT and UT groups were accurately representative. For each sampled animal, gill and spleen tissues were aseptically collected and stored in 1.5mL tubes containing RNAlater. Blood was collected from caudal vein and stored tubes with 100 μL of heparin, completed to a final concentration of 20 units of heparin/1mL of blood where stock heparin was prepared at 200 units/mL. Heparinized blood was processed, plasma was extracted and stored at -80°C. Tissues were stored at -80°C after RNAlater was removed.

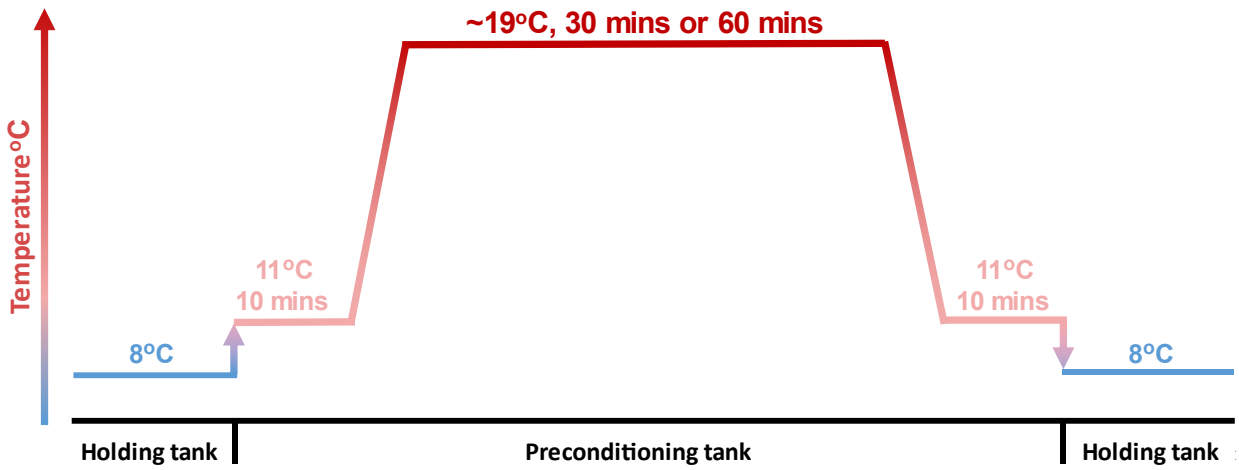

Fig. 2.1.A. Juvenile thermal preconditioning protocol For each individual thermal preconditioning cycle, animals were transferred to a holding tank set to 11°C for 10 minutes, then the temperature was increased to 19°C and held for either 30 or 60 minutes, depending on the week number in the protocol. The temperature was then brought down to 11°C for 10 minutes, before being transferred back to their original tanks.

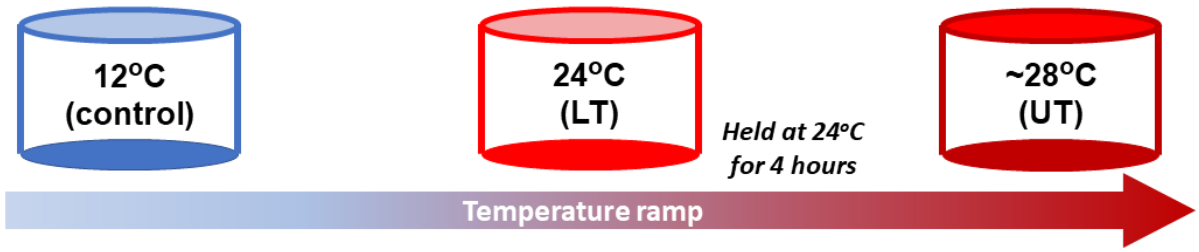

Fig. 2.1.B. Acute secondary thermal challenge protocol Designated thermal trial cohorts were subjected to a gradual increase in water temperature at ~2°C/hour. Once the temperature reached 24°C, the temperature increase was suspended for 4 hours, at the end of which the increase continued at the original pace. Animals were observed for their loss of swimming equilibrium (CTmax) and collected at that time. The animals collected prior to the 4-hour temperature hold was grouped as the lower tolerance (LT) cohort, whereas animals that reached CTmax after the 4-hour temperature hold was grouped as the upper tolerance (UT).

2.2.4 reverse transcription quantitative real-time PCR analysis (RT-qPCR) of mRNA levels

RNA and proteins were extracted from the tissue samples with the RNA/Protein purification plus kit (Norgen Biotek) as per manufacturer's instructions and stored at -80°C. qPCR primers were

designed or inherited for the transcript quantification of *IL-1β, IL-6, TNF-α, IFN-1, β2m, MH class I, HSP70, HSC70,* and *elongation factor 1-alpha (EF1-α)* (Table 2.1). Primers were optimized with Primer3 and Net Primer, and were selected for amplicon production of between 150bp and 200 bp. Standard curves were performed to optimize for cDNA template, primer melting temperature (Tm), and primer concentration.

Table 2.1 qPCR primer information for primers used in relative gene expression analysis. Forward (F) and reverse (R) primer sequence information for each transcript of interest, as well as the respective accession number for each sequence.

Primer ID	Dir.	Sequence (5'- 3')	Accession No.
EF1-α	F	CGCACAGTAACACCGAAACTAATTAAGC	XM_024396038.1
	R	GCCTCCGCACTTGTAGATCAGATG	
IL-1β	F	CCACAAAGTGCATTTGAAC	AJ298294.1
	R	GCAACCTCCTCTAGGTGC	
TNF-α	F	GTGCAAAAGATACCCACC	AJ278085.1
	R	CACTGCACGGTGTCAG	
IL-6	F	CTTCTACACGCTATCTCTCACTC	NM_001124657.1
	R	CGTCTGTCCCGAGCT	
IFN-1	F	AAAACTGTTTGATGGGAATATGAAA	CAM28541.1
	R	CGTTTCAGTCTCCTCTCAGGTT	
MH class I	F	AAAGTCCGTCCCTCAGTGTCTCT	FR688119.1
	R	ATGCTGTTCTTGTCCGTCTTTCT	
B2m	F	CAGACAGACCTGGCCTTCGA	AY217450.1
	R	GGCGGACTCTGCAGGTGTAC	
HSP70	F	CTCCTGGGGAAGTTTGAGC	AB062281.1
	R	TGGCTCTCTCTCCCTCGTAG	
HSC70	F	CGGTGCTGAAAGGAATGTC	NM_001124232.1
	R	CTGCGATGAAGTGGTTGA	

2.2.5 cDNA Synthesis and qPCR Protocol

Extracted total RNA from gill, muscle, head kidney, and spleen tissues were quantified with the Take3 plate quantification kit (Biotek® Synergy H1 Hybrid Multi-Mode Reader). RNA was stored at -80°C until further use. cDNA was synthesized with the qScript cDNA SuperMix (QuantaBio) according to the manufacturer's instructions.

qPCR was performed for all biomarkers transcripts of interest listed in section Table 2.1. mRNA levels for each gene were determined using a LightCycler® 480 instrument II (Roche Molecular Systems). 20uL qPCR reactions were run under the following conditions: 1 initial denaturing cycle of 95°C for 10 mins, followed by 40 cycles at of denaturation at 95°C for 10 seconds, annealing at the optimal temperature determined in section 2.2.1 for 5 seconds, and 72°C for 8 seconds for extension. *EF1-α* was used as the referenced housekeeping gene to demonstrate normalized expression. Every sample was performed in triplicate. Controls without template were included in all runs and melt curve analysis was performed to confirm the presence of a single amplicon. In addition, plate to plate variance was accounted for by running an inter-run calibrator sample on each plate.

2.2.6 Quantification of Plasma Cortisol Levels

Plasma cortisol concentrations were quantified using the Neogen® Cortisol ELISA Kit. The assay was performed as per the manufacturer's instructions with two exceptions. The samples were diluted at 1:100 in the designated buffer without performing the cortisol extraction step, and each sample was assayed in triplicate instead of duplicate.

2.2.7 Statistical Analysis

qPCR data from Section 2.2.5 was analyzed statistically with a Poisson-lognormal generalized mixed model and a Markov Chain Monte Carlo procedure through the MCMC.qPCR package (Matz et al., 2013) implemented with RStudio, which infers changes in transcript levels of all genes from the joint posterior distributions of parameters. The reference gene (*EF1-α*) added as priors to the model function to account for variations in cDNA and were allowed 1.2-fold changes (Matz et al., 2013). Data is presented as mean $\log_2$ fold change compared to a global

control cohort where fish were subjected to neither thermal preconditioning nor a subsequent thermal trial. Data represent the posterior means ± 95% credible intervals. Differences between treatment groups and the global control were accepted as significant if the credible intervals did not cross zero. Differences between treatment groups were accepted as significant if the 95% credible intervals did not overlap. Significant differences between cohorts are denoted by alphabetical letters.

Statistical analysis of the time of lost equilibrium was determined by an independent T-test at $P<0.05$ using Graphpad Prism 9.0. Assumptions of the independent T-test were met without transformations. Statistical analysis of plasma cortisol levels was performed by a two-way ANOVA with Tukey's post hoc test at $P<0.05$ using Graphpad Prism 9.0. Measured values were transformed to meet the assumptions of two-way ANOVA.

2.3 Results

2.3.1 Pro-inflammatory cytokine transcript profiles of *IL-1β, TNF-α,* and *IL-6*

The mRNA transcripts of *IL-1β* and *IL-6* showed similar trends, with upregulation in response to temperature in most of the thermally challenged groups of both control and preconditioned cohorts (**Fig. 2.2.a**, **Fig. 2.2.b**, **Fig. 2.2.e**). The most significant of these upregulations reached ~60 fold from the respective global control expression level. More specifically, splenic *IL-1β* of the preconditioned LT cohort did not demonstrate any upregulation compared to the global controls, whereas the control LT cohort was significantly upregulated, while both UT groups showed upregulated *IL-1β* (**Fig. 2.2.a**). *IL-1β* transcripts in the gills showed significant differences between the LT and UT cohorts of the control groups, but no differences between preconditioned and control animals (**Fig. 2.2.b**). *TNF-α* transcripts were largely unaffected by

exposure to thermal stress. The transcript displayed slight upregulation in the control LT cohort in gill tissue compared to the global control, and no changes at all were observed in the spleen. (**Fig. 2.2.c, Fig. 2.2.d**). *IL-6* transcripts were only consistently detectable in the spleen (**Fig. 2.2.e**). Splenic IL-6 displayed upregulation in all thermally challenged cohorts. Between these cohorts, the control LT cohort was significantly less upregulated than the UT cohort, but no differences were demonstrated between preconditioned and control fish (**Fig. 2.2.e**).

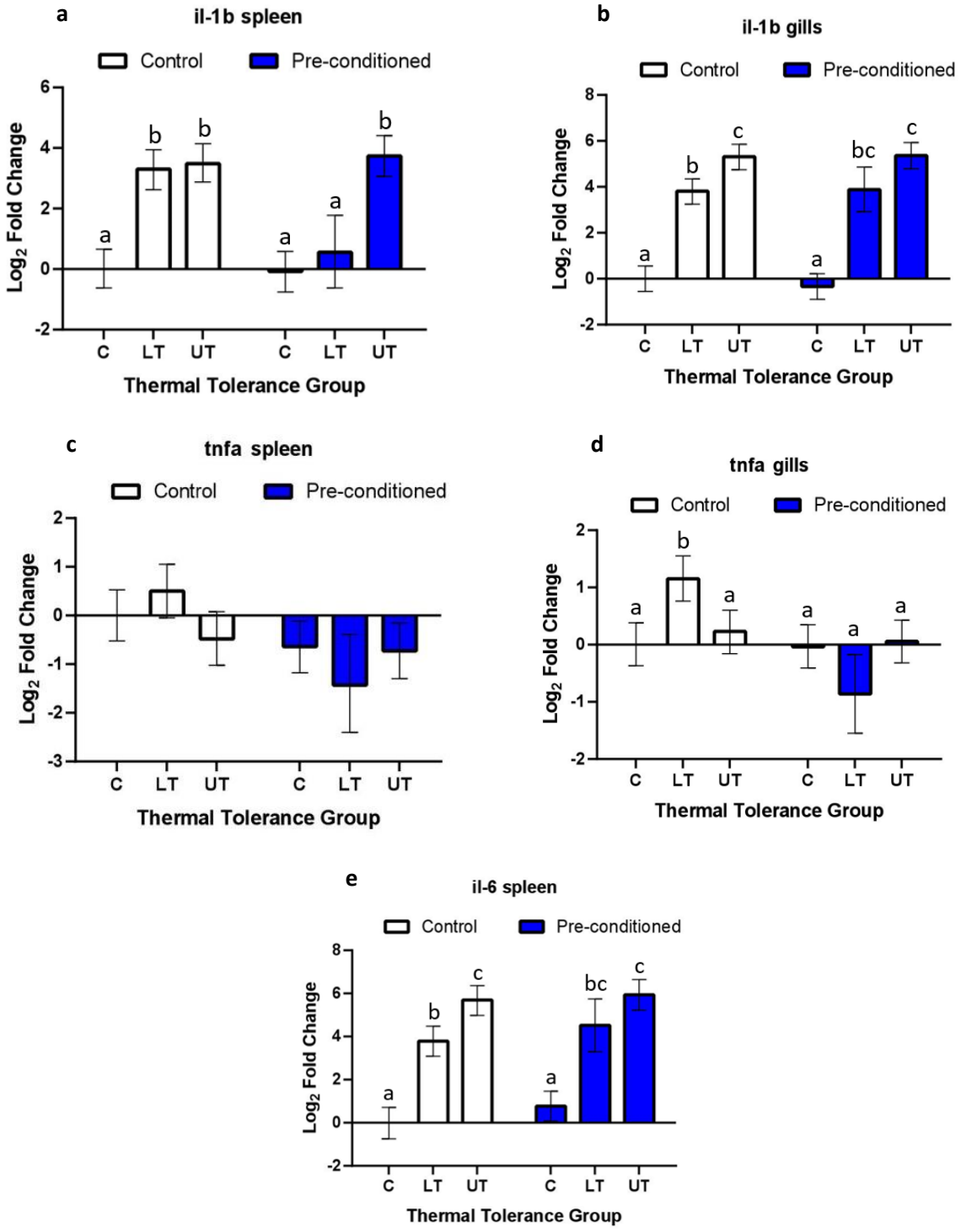
a
il-1b spleen
Control
Pre-conditioned
Log2 Fold Change
Thermal Tolerance Group
C
LT
UT
b
il-1b gills
c
tnfa spleen
d
tnfa gills
e
il-6 spleen

Fig. 2.2 Expression of *IL-1β, TNF-α,* and *IL-6* transcripts of both control and thermally preconditioned cohorts after a lethal heat stress challenge. Splenic and gill *IL-1β* (a, b), *TNF-α* (c, d), *and IL-6* (e) (spleen only) mRNA levels (Log_2 Fold Change) were assessed for control (non-preconditioned) and preconditioned juvenile rainbow trout following a thermal tolerance trial (n=7). Thermal tolerance groups were as follows: C (Control, not thermally challenged), LT (Lower Tolerance), and UT (Upper tolerance). Data were modelled with a Poisson-lognormal generalized mixed model fitted with a Bayesian Markov Chain Monte Carlo model, as part of the 'MCMC.qpcr' package. Data is presented as a log fold change compared to a global control cohort where fish were subjected to neither thermal preconditioning nor a subsequent thermal trial. Data represent the posterior means ± 95% credible intervals. Differences between treatment groups and the global control were accepted as significant if the credible intervals did not cross zero. Differences between treatment groups were accepted as significant if the 95% credible intervals did not overlap. Significant differences between cohorts are denoted by alphabetical letters.

2.3.2 Anti-Viral Response Transcript Profiles of *IFN-1, MH class I,* and *β2m*

Anti-viral and antigen-presentation related transcripts *IFN-1, MH class I,* and *β2m* were measured at the mRNA level in the spleen and gill tissues of all cohorts. *IFN-1* transcript levels in the spleen were upregulated in all heat stressed cohorts, with the exception of the control LT cohort (**Fig. 2.3.a**). This control LT cohort expressed significantly less *IFN*-1 transcripts compared to its respective UT cohort, as well as the preconditioned LT cohort (**Fig. 2.3.a**). Conversely, the regulation of *IFN-1* in the gills of all heated cohorts was significantly downregulated with increased water temperatures, except the control, LT cohort (**Fig. 2.3.b**). The patterns of these *IFN-1* downregulations in the gills mirrored the regulation of *IFN-1* upregulation in the spleen, with the control LT cohort being the group differently regulated than the three other thermally stressed cohorts (**Fig. 2.3.b**). Like *IFN-1, MH class I* transcript levels in gills were also downregulated in all heat-stress cohorts except for the preconditioned LT cohort, which was not significantly different from the global control (**Fig. 2.3.d**). *MH class I* transcripts were not differentially regulated in the spleen (**Fig. 2.3.c**). Changes for expression of *MH class I* were relatively minor (~3-5 fold) compared to the differences seen for *IFN-1*(~16 fold). Lastly,

transcripts of *β2m* in both spleen and gill tissues did not demonstrate any differential regulation in response to the thermal challenge (**Fig. 2.3.e, Fig. 2.3.f**).

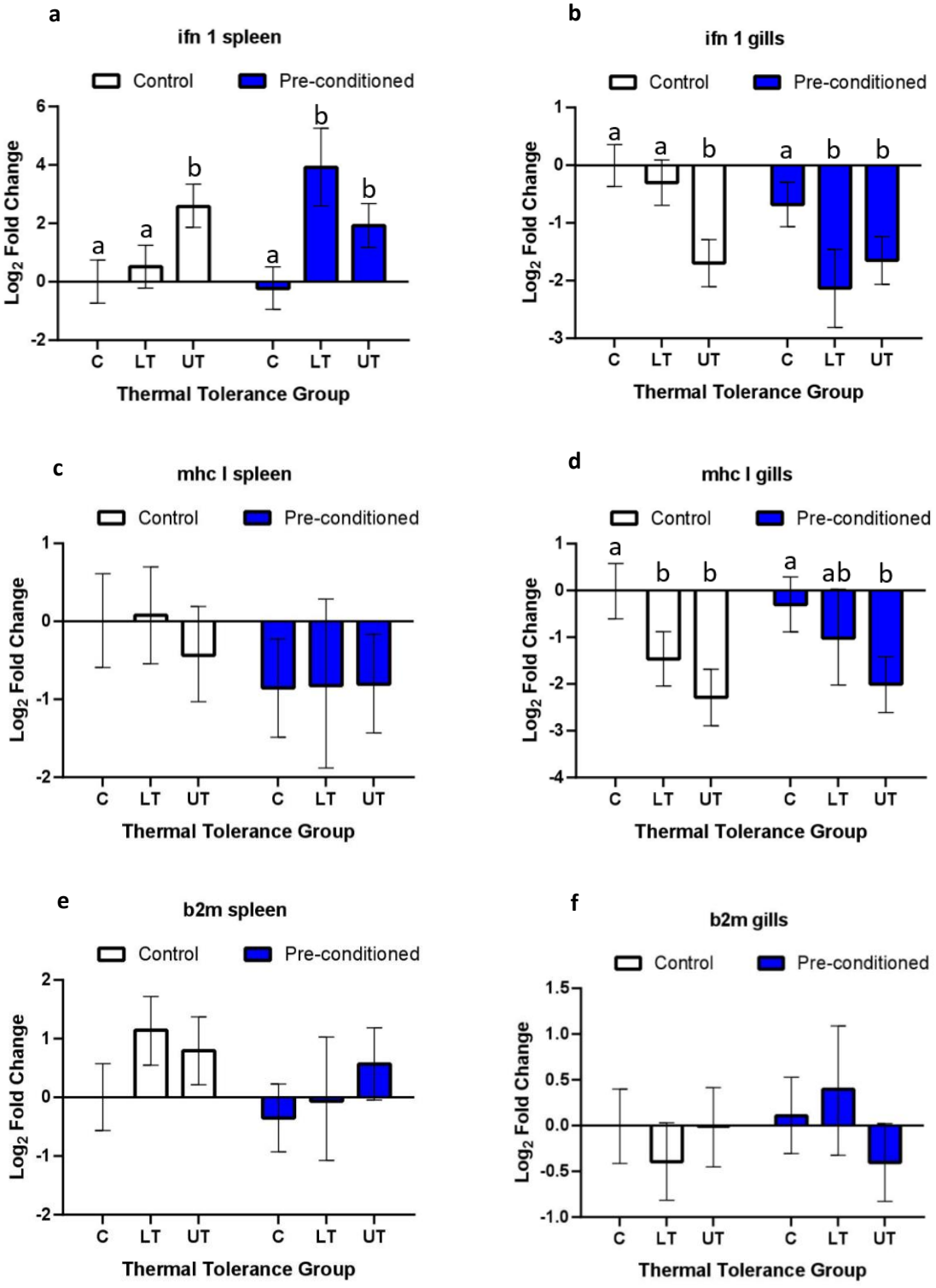

Fig 2.3 Expression of *IFN-1, MH class I,* and *β2m* transcripts of both control and thermally preconditioned cohorts after a lethal heat stress challenge. Splenic and gill *IFN-1*(a, b), *MH class I* (c, d) and *β2m* (e, f) mRNA levels (Log_2 Fold Change) were assessed for control (non-preconditioned) and preconditioned juvenile rainbow trout following a thermal tolerance trial (n=7). Thermal tolerance groups were as follows: C (Control, not thermally challenged), LT (Lower Tolerance), and UT (Upper tolerance). Data were modelled with a Poisson-lognormal generalized mixed model fitted with a Bayesian Markov Chain Monte Carlo model, as part of the 'MCMC.qpcr' package. Data is presented as a log fold change compared to a global control cohort where fish were subjected to neither thermal preconditioning nor a subsequent thermal trial. Data represent the posterior means ± 95% credible intervals. Differences between treatment groups and the global control were accepted as significant if the credible intervals did not cross zero. Differences between treatment groups were accepted as significant if the 95% credible intervals did not overlap. Significant differences between cohorts are denoted by alphabetical letters.

2.3.3 Heat Stress Response Transcript Profiles of *HSP70* and *HSC70,* and Time of Lost Equilibrium Analysis

HSP70 and *HSC70* transcript levels were measured in spleen and gill tissue to assess the heat stress response in the differently treated cohorts. Splenic *HSP70* transcripts were very strongly upregulated in all heat stressed cohorts to levels of $2^{10} – 2^{15}$ compared to the global control cohort (**Fig. 2.4.a**). More specifically, the LT preconditioned cohort *HSP70* transcripts were less upregulated than the other three heat-stressed cohorts which were similarly upregulated (**Fig. 2.4.a**). In gills, HSP70 transcripts in the LT control cohort were expressed at a lower level than the other three heated cohorts, which were all similarly upregulated (**Fig. 2.4.b**). The cognate *HSC70* transcript levels were not significantly different in any experimental heat-stressed cohort when compared to the global control cohort, for both spleen and gill tissues (**Fig. 2.4.c, Fig. 2.4.d**).

The time of lost equilibrium/CTmax of the animals during the thermal incline challenge was analyzed to investigate whether the thermal preconditioning protocol influenced the functional thermal tolerance of the subjected animals. Between the control (non-preconditioned) and

preconditioned LT cohorts, the CTmax recorded were not significantly different from each other (**Fig. 2.5**). Similarly, no significant differences were observed when comparing the CTmax in the respective UT cohorts (**Fig. 2.6**).

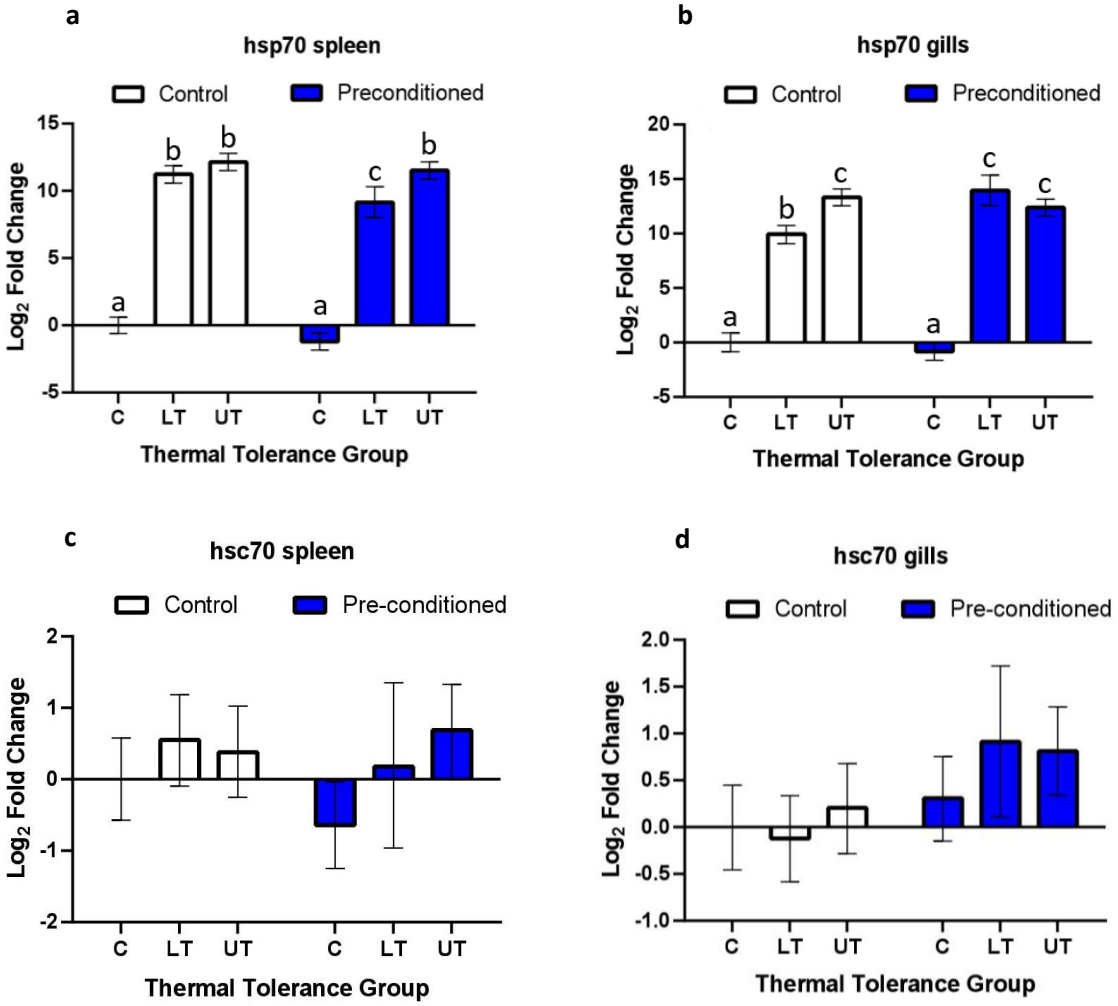

Fig 2.4 Expression of *HSP70* and *HSC70* transcripts of both control and thermally preconditioned cohorts after a lethal heat stress challenge. Splenic and gill *HSP70* (a, b) and *HSC70* (c, d) mRNA levels (Log_2 Fold Change) were assessed for control (non-preconditioned) and preconditioned juvenile rainbow trout following a thermal tolerance trial (n=7). Thermal tolerance groups were as follows: C (Control, not thermally challenged), LT (Lower Tolerance), and UT (Upper tolerance). Data were modelled with a Poisson-lognormal generalized mixed model fitted with a Bayesian Markov Chain Monte Carlo model, as part of the 'MCMC.qpcr' package. Data is presented as a log fold change compared to a global control cohort where fish were subjected to neither thermal preconditioning nor a subsequent thermal trial. Data represent the posterior means ± 95% credible intervals. Differences between treatment groups and the global control were accepted as significant if the credible intervals did not cross zero.

Differences between treatment groups were accepted as significant if the 95% credible intervals did not overlap. Significant differences between cohorts are denoted by alphabetical letters.

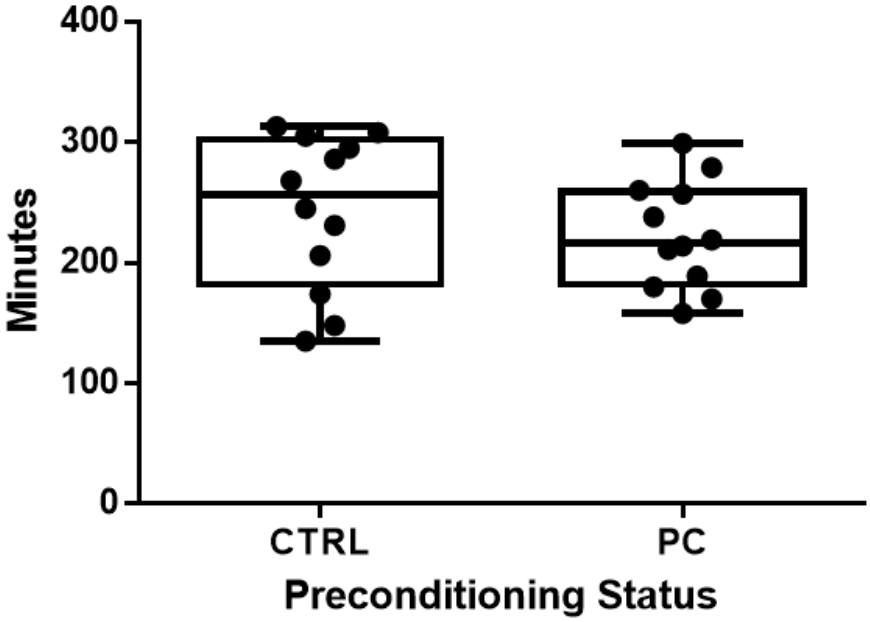

Fig 2.5 Time of lost equilibrium of lower tolerance (LT) rainbow trout. Observed time of lost equilibrium (minutes) for sampled rainbow trout in the designated lower tolerance (LT) cohort for both control (CTRL) and preconditioned (PC) group. (n=12)

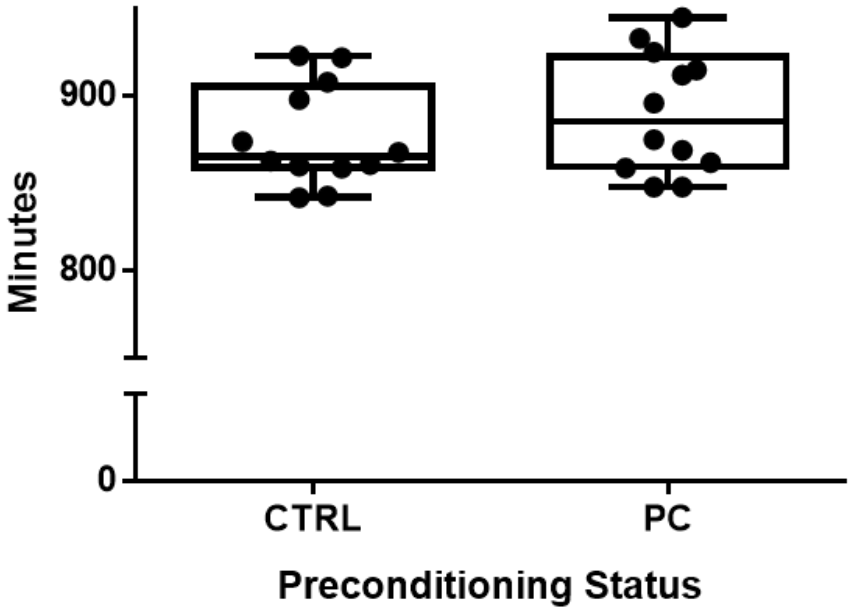

Fig 2.6 Time of lost equilibrium of upper tolerance (UT) rainbow trout. Observed time of lost equilibrium (minutes) for sampled rainbow trout in the designated upper tolerance (UT) cohort for both control (CTRL) and preconditioned (PC) groups. (n=12)

2.3.4 Plasma Cortisol Analysis

Plasma cortisol levels were measured and analyzed with a two-way ANOVA, with the level of heat stress (LT, UT), and preconditioning (C, PC) as the two factors, both factors were found to be significant influencers of plasma cortisol concentration (**Fig. 2.7**). The two control temperature cohorts showed no basal differences in cortisol levels. The preconditioned UT cohort had significantly lower levels of plasma cortisol than the respective non-preconditioned individuals, this trend is not significant in the lower tolerance cohorts. Additionally, the preconditioned UT cohort also had significantly lower levels of cortisol than the preconditioned LT cohort. Finally, the control temperature cohorts showed no basal differences in cortisol level (**Fig. 2.7**).

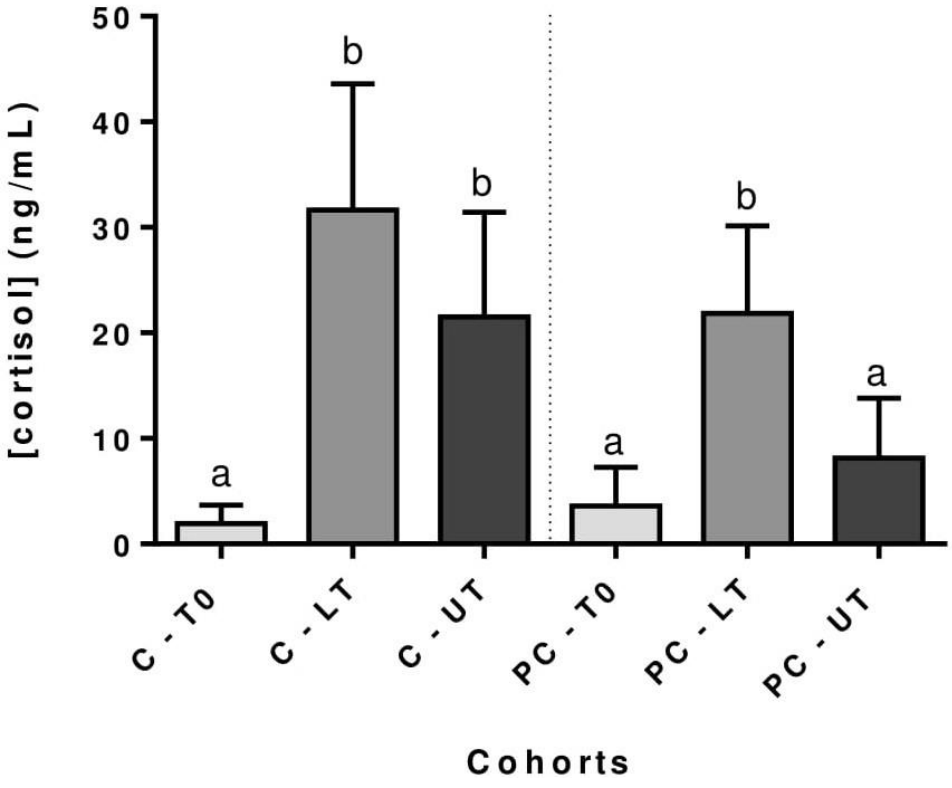

Fig 2.7 Plasma cortisol concentrations of all experimental cohorts. Plasma cortisol concentrations of control (C) and thermally preconditioned (PC) cohorts for baseline (T0), lower tolerance (LT), and upper tolerance (UT) treatments (n = 7). Two-way ANOVA with Tukey's post hoc test ($p < 0.05$).

2.4 Discussion

2.4.1. Proinflammatory Transcript Expression Profiles in Response to Acute Heat Stress

IL-1β has been demonstrated to be one of the most pleiotropic immune-signaling molecules and has long been understood to be co-regulated with the heat-shock response, as also confirmed in the current study (Chen et al., 2014; Khosravi-Katuli et al., 2021). Adjacently, however, *TNF-α*, in theory, is also upregulated by the heat-shock response and the upregulation of *IL-1β*, but this *TNF-α* upregulation was not observed in the current study in either of the assessed tissues (Oladiran and Belosevic, 2009; Zou and Secombes, 2016). A possible cause for this discrepancy could be that the acute nature of sampling in this protocol, and consequently the lack of time for observable *TNF-α* upregulations to occur. Additionally, this idea is supported by previous observations that heat-shock-induced immune signaling often presents a muted/delayed upregulation of *TNF-α* compared to *IL-1β* (Shahi et al., 2018; Zhang et al., 2015).

Detectable differences of *IL-6* transcripts between experimental cohorts were observable in the spleen, but not gill tissue. This absence of *IL-6* transcript detection in gill tissues could be attributed to tissue-based differences of constitutive expression. Since the current study system did not use a pathogenic stressor, the *IL-6* transcripts in gills may not have been as significantly induced as they would have been in a pathogenic study system (Iliev et al., 2007). Consequently, demonstrated differences in constitutive expression levels of *IL-6* between tissues could explain why the transcript was not consistently detectable in gill tissue compared to spleen tissue (Iliev et al., 2007). Additionally, while both spleen and gill tissues are sites of immune activity, the

purpose of their immune response is notably different. The immune activity taking place in the gills are mostly for the purpose of barricading and early recognition of potential pathogens at a mucosal site. Whereas the spleen is a central lymphoid organ that oversees a broader response. This distinction between the two immune functions in the tissues could explain the discrepancy in *IL-6* expression. Another notable observation in the proinflammatory transcripts profiles is the diminished upregulation of *IL-1β* and *IL-6* in the LT cohorts compared to their respective UT cohorts. These differences may have been regulated by the more intensified heat-shock response in the UT cohorts compared to the LT cohorts. However, *TNF-α* transcripts once again did not respond similarly to *IL-1β* and IL-6. This contrasts with most related studies where increases of *IL-1β* and *TNF-α* occur concurrently in response to immune stimulation (Bo et al., 2015; Raida et al., 2011; Taechavasonyoo et al., 2013), although scenarios do exist where the protocol induces one but not the other proinflammatory transcript (McBeath et al., 2007).

2.4.2 Heat Shock Response Transcript Analysis

HSP70 expression differences were observed in both splenic and gill tissues, where the LT cohorts were situationally less upregulated than the respective UT cohorts. However, these changes alone did not present a clear idea of the effect of thermal preconditioning on HSP70 regulation, as the patterns observed were not consistent between the two tissues. Physiologically, different tissues have been shown to regulate HSP70 differently under varying severities of heat-stress conditions (Wang et al., 2007). Additionally, there have been reported study systems where higher HSP70 expression both did and did not correlate with observable increases in heat-resilience of the animal. (Ahmad et al., 2020; Fangue et al., 2006; Healy et al., 2010; Kelly et al., 2018; Liu et al., 2017). In many of these studies, more “tolerant” individuals displayed a delayed and/or depressed HSP70 upregulation compared to more “susceptible” individuals at higher

temperatures. Moreover, measuring HSP70 at a protein level may also help with a better understanding of the induced heat shock response, as temperatures of transcription and translation of HSPs have been shown to be significantly different from each other (Lewis et al., 2016; Mottola et al., 2020). Lastly, HSP70 analysis alone may be insufficient to represent the heat-shock response, regulation of other HSPs such as HSP90 could supplement the understanding of the heat-induced regulatory activities (Fangue et al., 2006; Healy et al., 2010; Kelly et al., 2018; Quinn et al., 2011). Taken together, while the heat-shock analysis in this study provided a sufficient baseline of reference for the immune-focused analysis, more heat-shock-specific analysis would have to be performed to make conclusion about the heat-shock response to this specific pre-conditioning experiment regimen.

2.4.3 Anti-Viral Response Transcript Expression Profiles

The general regulation trends of anti-viral transcripts were temperature and tissue specific. The observed down-regulation of *IFN-1* and *MH class I*, and the lack of changes observed in *β2m* transcript levels in gill tissues were not congruent with previous leukocyte studies (Pérez-Casanova et al., 2008; Rodrigues et al., 1998). Specifically, cell culture studies have generally demonstrated attenuation of *IFN-1* transcripts at lower temperatures, and the opposite at higher temperatures (Abram et al., 2019a; Arguedas Cortés et al., 2015; Sever et al., 2014). However, more specific temperature-related studies of *IFN-1* expression have not yet been conducted with gill tissue in *in vivo* studies. The difference in regulatory direction of *IFN-1* between splenic and gill tissue suggests the transcript may be regulated in a tissue-specific manner in response to acute heat stress.

Additionally, this downregulation of *IFN-1* seen in gill tissue may be related to the similar regulation patterns seen in *MH class I* transcripts in gill tissue, as type I interferons have been

shown to regulate members of the endogenous antigen presentation pathway (Hobart et al., 1997; Sever et al., 2014). The downregulation of constitutive *MH class I* and *β2m* transcript levels at higher temperatures have also been shown in one previous instance, where a walleye skin fibroblast cell line downregulated the two respective transcripts at higher temperatures (Abram et al., 2019b). Taken together, previous studies of *MH class I* and *β2m* regulation at supra-optimal temperatures have at times come to contradictory conclusions, and more detailed analyses would be needed to find the reasons behind these discrepancies.

2.4.4 Thermal Preconditioning-Based Differences in Transcript Expression and Plasma Cortisol

The identification of any potential differences in transcripts expression caused by the preconditioning method was the main objective of this study. In the splenic transcript analyses, *HSP70* expression levels in the control LT cohorts were significantly higher than the preconditioned LT cohorts. This differential regulation could be related to the similar differences seen in splenic *IL-1β* expression, as HSP70 induction is commonly associated with an immune-stimulated profile, as the chaperone protein has been linked to increases in both the expression and synthesis of IL-1β and TNF-α (Chen et al., 2014; Zhang et al., 2015). However, similar differences were not observed in *TNF-α* and *IL-6* expression, possibly due to the different regulatory pathways in which HSP70 regulates these different transcripts (Zhang et al., 2015). The assessed anti-viral profile did not demonstrate clear effects of thermal preconditioning across the tissues, or each transcript. Although some differences were observed as discussed in section 2.4.3. between the pre-conditioned and control animals, specifically for *IFN-1*, it is hard to interpret the biological significance of these changes with the lack of consistent observations between these anti-viral transcripts. Ultimately, this study is the first attempt at elucidating

potential immunological benefits of juvenile thermal-preconditioning, and more detailed studies are necessary to further explore and elaborate on the presently observed differences.

2.4.5 Plasma Cortisol Changes in Response to Thermal Preconditioning and Acute Secondary Thermal Challenge

The preconditioned UT cohort demonstrated lower cortisol levels than the respective LT cohort. Previous studies involving increasing temperature stressors have observed similar patterns with a plateau or decrease of plasma cortisol levels as temperatures approach the upper limits of the animals of interest (Panase et al., 2019; Pérez-Casanova et al., 2008; Strange, 1980). Additionally, peak levels of cortisol often occur quickly after the initial onset of the stressor and start to decrease hours after the start of stress (Redding and Schreck, 1983, Strange, 1980). When comparing the cortisol levels across the preconditioning factor, the preconditioned UT cohort had lower levels of cortisol than the control UT cohort, but the same trend was not significant among the LT cohorts. When comparing the cortisol levels between the LT and UT cohorts of the respective control and preconditioned groups, the UT cohorts overall presented lower levels of cortisol than the LT cohorts. As cortisol have well known immunosuppressive effects on cytokine regulation, many of the instances observed in the immune transcript analysis where the UT cohorts expressed higher levels of the transcript could potentially be related to stress-induced immunosuppression. Overall, interpreting plasma cortisol as a general stress marker, the preconditioned cohort in the current preconditioning regimen demonstrated significant changes in the general stress response to a second stressor 16 weeks after the conclusion of juvenile preconditioning. This reduction in cortisol could potentially have enhanced expression of some of the assessed immune transcripts.

2.4.6. Analysis of the Preconditioning Method

In the current study, juvenile-stage thermal-preconditioning demonstrated some transcript level differences for transcripts relevant to both the heat-shock response and the immune response, as well as the general stress response via plasma cortisol analysis. However, in this study, these changes did not translate to differences in terms of mortality data and the critical thermal temperature. This study serves as one of the first studies to explore the chronic effects of early-life thermal preconditioning in fish on the heat-shock response, and the first of such studies for the immune response.

Teleost studies related to hypoxic pre-conditioning have demonstrated significant protective effects to subsequent exposures to hypoxia (Gillis and Johnson, 2017; Rees et al., 2001). However, thermal preconditioning has been relatively less explored, and the conclusions made thus far have also been less clear. Even rarer, are the thermal-preconditioning studies that aim to identify long-term effects of juvenile pre-conditioning, as most thermal-preconditioning studies in teleost have designs involving short periods (minutes – days) between the initial preconditioning and the subsequent second challenge. Of the available thermal-preconditioning teleost studies, some did not observe a clear improvement of thermal tolerance from the performed preconditioning regime (Gallant et al., 2017; Tunnah et al., 2017), while other studies did demonstrate beneficial effects of preconditioning (Callaghan et al., 2016; Feminella and Matthews, 1984).

This lack of understanding in the field is accompanied by the unclear definitions between the ideas of preconditioning and acclimation. Some of these related studies have been labeled as preconditioning but could also be categorized as temperature acclimation. Noticeably, many of these related studies observed clearer and stronger effects of thermal preconditioning when the secondary challenge followed shortly after, or at an unspecified time (Ahmad et al., 2020;

Callaghan et al., 2016; Gallant et al., 2017; Kelly et al., 2018). To understand these results, it is possible that the protection provided by non-lethal thermal exposure seen in these studies are only temporary, and these animals will return to pre-preconditioned physiological status over time (Ahmad et al., 2020; Buckley and Hofmann, 2002; Kelly et al., 2018). Understandably, it is unreasonable/too optimistic to assume that the potential resistance to adult stage heat-stress developed via juvenile stage thermal preconditioning will maintain the same level of protective efficacy during the entire lifetime of the animal. However, a dedicated study to explore both the short- and long-term effects of thermal-preconditioning will be needed to confirm this hypothesis.

2.5 Conclusions

The thermal preconditioning of juvenile rainbow trout in this experiment induced various measured changes upon a secondary thermal challenge. Various transcriptional regulatory changes were demonstrated for both pro-inflammatory and anti-viral immunity, as well as heat-shock response. Taken together, these results were not consistent enough between each other to suggest whether the thermal preconditioning was beneficial to the animals, as suggested by the similar mortality data between the two treatments. However, these transcript level data can serve as a baseline point of comparison for future studies. Additionally, the *IFN-1* downregulation in gills at higher temperatures, and the lower level of plasma cortisol observed as a result of preconditioning are novel discoveries presented here for the first time.

Chapter 3: Effect of Thermal-Based Parental Selection on Splenic Immune and Heat-Shock Regulation

3.1 Introduction

The effects of thermal selection on offspring thermal performance and heat-stress-related regulation have long been studied in both natural (Stitt et al., 2014; Yu et al., 2018) and experimental selection systems (Ineno et al., 2005). The majority of available data demonstrates a profile of decreased sensitivity/increased tolerance to thermal challenges in cohorts from populations that have undergone thermal-based selection (Fangue et al., 2006; Liu et al., 2017). However, the effects of thermal-based selection on the subsequent immune response are not well elucidated in the literature.

From the immunological perspective, the effects of pathogen-resistance-based selection on subsequent pathogenic challenges are comparatively better understood (Marancik et al., 2015). Selective breeding methods against both viral and bacterial pathogens have yielded significant changes in both differential immune transcript regulation (Cofre et al., 2014; Jørgensen et al., 2011; Zhang et al., 2011), and reduced disease susceptibility (Purcell et al., 2010). However, there have been conflicting reports about the immune response profiles between selected "resistant" and "susceptible" families, and how they are differentially regulated to combat pathogens. For example, two similar studies exploring the effect of disease-based selective breeding on infectious pancreatic necrosis virus (IPNV) infection in head kidney transcript expression came to different conclusions on how the immune response is regulated for better protection (Cofre et al., 2014; Reyes-López et al., 2015). Reyes-López et al. (2015) demonstrated an intense and short immune reaction in the susceptible animals, while the resistant animals presented a more tapered, longer-lasting resolution. Contrastingly, Cofre et al. (2014) found a

correlation between higher upregulation of immune genes with the resistant family relative to the susceptible family. These confounding results demonstrate the potential complexity in the molecular interactions behind family-based pathogen resistance and the methods of action behind it.

Additionally, the concept of resistance itself is also hard to define and should not be reductively interpreted by up- or down-regulation of immune-relevant genes and other measurable humoral factors. A study that explored the differential expression between high and low susceptible Atlantic salmon families to salmon louse found that the families most resistant to the immunosuppressive effect caused by the parasite were more successful (Holm et al., 2015). Ultimately, an immune response profile that infers resistance to a specific pathogen or subtype of pathogens may not contribute to a reality of a holistically more productive immune system against all pathogen types.

As described in Chapter 1.4, the heat-stress and immune responses have been demonstrated to be connected via several signaling molecules in teleost fishes. This study is the first attempt to explore whether temperature-based selection has any cross-response effects on immunity upon a secondary pathogenic stimulus. This experiment hypothesizes that both temperature- and family-based effects will differentially regulate the immune response presented through the splenic transcript profiles of *IL-1β*, *IFN-γ*, *tapasin,* and *IL-10*. *IL-1β* and *IFN-γ* are key mediators of the pro-inflammatory response to bacterial PAMPs. Although *Tapasin* is mainly involved in the anti-viral response, the temperature-dependent regulation of *tapasin* is also a point of interest. Lastly, *IL-10* transcript levels were analyzed to represent the course of the anti-inflammatory response. Altogether, analysis of these four genes should give a meaningful representation of how the immune system responds to the factors of interest on a transcript level.

3.2 Materials and Methods

3.2.1 Experimental Animals and Animal Selection

Rainbow trout used in this trial were the offspring of four parental crosses. Two high thermal tolerance females, previously determined from the selection of CTmax measurements, were crossed with a low thermal tolerance male, and a male of unknown origin. The offspring of the four different crosses were each assigned a letter for simplicity of reference (Table 3.1). Offspring crosses Q and R were fertilized on October 10th, 2018, while, crosses S and T were fertilized on September 26th, 2018 at Alma Aquaculture Research Station (AARS) Guelph, Ontario, Canada. Post-fertilization, juveniles were held at AARS and fed with a maintenance level diet.

Table 3.1. Parental crossed of experimental animals and assigned symbols. The table displays the parental cross designs performed to rear offspring for the experiment between two high thermal tolerance females with a low thermal tolerance male and a male of unknown thermal tolerance. Crosses were designated as Q, R, S, and T for simplicity of representation and discussion.

Crosses	Representative Symbol
High tolerance female 1 x Unknown male	Q
High tolerance female 1 x Low tolerance male	R
High tolerance female 2 x Unknown male	S
High tolerance female 2 x Low tolerance male	T

3.2.2 Animal Tagging, Transportation, and Thermal Incline Regiment

On October 1st, 2019, all animals were injected with visible implant elastomer tags (Northwest Marine Technology, Inc) in the clear tissue under the jaw or above the eye for identification purposes. On November 21st, 2019, all animals were transported to the Hagen Aqualab (University of Guelph, Guelph, ON, Canada). Upon arrival, the four different crosses were evenly mixed and distributed into eight 750-liter tanks, with 77-78 individuals in each tank,

recirculated with 12°C water. On January 6th, 2020, four of the eight holding tanks were started on a temperature increase regimen; over a period of two weeks, the water temperatures in these tanks were raised to 19°C, at a rate of 0.5°C/day. For the duration of the period when the animals were housed at the Hagen Aqualab, the animals were subjected to a light/dark cycle system designed to mirror the natural light/dark cycle at that time of year.

3.2.3 Vaccination Trial

On Jan 21st, 2020, 48 animals were initially collected as part of the pre-injection baseline cohorts. The remaining animals (621) were injected with 0.1mL of either phosphate-buffered saline (PBS) as a sham control or VIBROGEN 2 vaccine (Elanco Canada Limited, Charlottetown, PE, Canada), which contained formalin-killed *Vibrio anguillarum* serotypes I & II and *Vibrio ordalii*. The injection was made intraperitoneally on the ventral side of the animals with pre-loaded 0.5mL syringes, between the pelvic and anal fins. 100mg/L of MS-222 was used as an anesthetic dose to immobilize the animals.

3.2.4 Tissue Sampling and Processing

Feeding of the animals ceased 48 hours prior to the start of the trial, as instructed by the vaccine manufacturers. A time-0 control cohort was sampled prior to the vaccine trials to establish a baseline group. Following the vaccination process, animals were sampled at 6, 24, and 96 hours post-injection. At each sampling timepoint, 3 individuals from each cross were collected from each tank (12 fish/tank) and euthanized with 400 mg/L of MS-222. The spleen of each animal was extracted and stored in RNAlater and stored long-term at -20°C. Sampling was performed evenly amongst the tanks at each timepoint, and in the same order, to eliminate tank effects and maintain equal stocking density among the tanks throughout the trial process.

3.2.5 RNA Extraction and cDNA Synthesis

25mg of splenic tissue from sampled animals was homogenized per the manufacturer's instructions (Omni International). Total RNA was extracted with Trizol (Ambion, Inc) according to the manufacturer's instructions. Total RNA was quantified with Take3 micro-volume plate (Biotek Instruments, Inc) after the RNA pellet was redissolved in RNase-free water, then genomic DNA in the extracts was eliminated with DNase (Invitrogen) according to manufacturer's instructions. Samples were then loaded onto an RNA spin column and washed (3x, 400uL, 3 500g – 1 minute) with wash buffer A (Norgen Biotek, Cat#: 48200), then eluted with RNase-free water. Total RNA was quantified with Take3 micro-volume plate (Biotek Instruments, Inc), and reverse transcribed with qScript cDNA supermix (Quantabio) according to the manufacturer's instructions.

3.2.6 qPCR Primer Validation

Primers were designed with consideration for primer dimerization and positioning regarding intron-exon borders to eliminate amplification of gDNA contamination (Table 3.2). Standard curves were performed with each set of qPCR primers to verify primer efficiencies. Primer analysis was performed with pooled samples to determine the optimal annealing temperature for each set of primers.

Table 3.2. qPCR primer information for primers used in relative gene expression analysis. Forward (F) and reverse (R) primer sequence information for each transcript of interest, as well as the respective accession number for each sequence.

Primer ID	Dir.	Sequence (5'- 3')	Accession No.
EF1-α	F	CGCACAGTAACACCGAAACTAATTAAGC	XM_024396038.1
	R	GCCTCCGCACTTGTAGATCAGATG	
IL-1β	F	CCACAAAGTGCATTTGAAC	XM_024418276.1
	R	GCAACCTCCTCTAGGTGC	
IFN-γ	F	GAAGGCTCTGTCCGAGTTCA	XM_036945593.1
	R	TGTGTGATTTGAGCCTCTGG	
tapasin	F	ACTATTACGCGTGCTCCCAC	DQ092322.1
	R	AACCCAGAAGCTGCAATCCA	
IL-10	F	GCCTTCTCCACCATCAGAGAC	XM_024405117.1
	R	GATGCTGTCCATAGCGTGAC	

3.2.7 qPCR

qPCR was performed with a LightCycler® 480 Instrument II (Roche Diagnostics). Each individual qPCR reaction totaled 10 μL. Each reaction consisted of 1.0 μL of cDNA template diluted 1:10 in molecular grade water (Corning), 5 μL of WISENT ADVANCED^{tm} qPCR mastermix with SUPERGREEN^{tm} dye (Wisent Bioproducts), 0.25 μL of both forward and reverse primers at a concentration of 10 μM (Sigma-Aldrich), and 3.5 μL of molecular grade water (Corning). The qPCR protocol consisted of a pre-incubation step of 95°C for 2 min, 40 cycles of 95°C for 5 secs, 60°C for 30 secs, and 72°C for 8 secs with the acquisition of signal. A melt curve step was performed after thermocycling, and involved ramping to 97°C at 0.11°C/sec. The plate was cooled to 60°C before the conclusion of the protocol. cDNA samples were run in triplicate wells, each plate included an inter-plate control performed with pooled samples to control for inter-plate variation, and a negative control with no cDNA to control for contamination. Melting curve data was analyzed to confirm the uniform length of generated amplicons.

3.2.8 Statistics and Data Analysis

3.2.8.1 *IL-1β*, *IFN-γ*, and *tapasin* Transcript Analysis

Triplicate CT values were averaged and converted to relative fold change data via the $2^{-\Delta\Delta CT}$ method using *ef1-α* as the housekeeping gene (Livak and Schmittgen, 2001). Statistical significances between factors of interest were determined using a general linear univariate model, relative fold change data was transformed to fit the assumptions of the model. Bonferroni corrections were applied for the multiple comparisons in the analysis of the effect of selective crosses to appropriately reduce type I error rates. Figures were generated and statistical analysis was performed with SPSS (IBM). When applied, statistical significance was denoted with symbols (*, #) where * or # uniformly represented a P-value < 0.05, and ** or ## uniformly represented a P-value <0.001. Otherwise, statistical significance was denoted by lettering when suitable.

3.2.8.2 *IL-10* Transcript Analysis

The qPCR output CT values for *IL-10* splenic gene expression were below the reliable limit of detection for all non-VIBROGEN II injected cohorts (including all PBS injected control cohorts and all 0-hour pre-injected cohorts). As a result, the relative fold change for the detectable cohorts was calculated with the aforementioned $2^{-\Delta\Delta CT}$ method using an assigned CT value of 35 to represent all non-detected samples, and *ef1- α* as the housekeeping gene. A Kruskal–Wallis test was performed by combining the water temperature and timepoint factors into a single factor to compare the relative gene expression between the VIBROGEN II injected cohorts of different temperature and timepoints. Bonferroni corrections were applied to adjust for potential type I errors caused by multiple comparisons (**Fig. 3.10**). A non-parametric test was used as the data

was not transformable to pass the assumptions of a parametric, One-way ANOVA. A univariate general linear model was used to determine the difference in *IL-10* expression between crosses (**Fig. 3.11**, **Fig. 3.12**), Bonferroni corrections were applied where applicable, and letters were used to denote statistically significant differences.

3.3 Results

3.3.1 Analysis of *IL-1β* Transcript Expression on the Factors of Vaccination, Temperature, and Selection

3.3.1.1 Effects of Vaccine Challenge and Injection Procedure on Splenic *IL-1β* Expression

For the splenic *IL-1β* transcript (**Fig. 3.1**), the VIBROGEN II vaccine caused a significant upregulation in both the 6- and 24-hour post-injection cohorts (**Fig. 3.1.A**). By the 96 hours post-injection timepoint, transcript levels returned to close to basal level, although expression in some cohorts was still significantly higher in the vaccinated cohorts compared to the PBS-injected controls. Regarding the PBS-injected control cohorts, many cohorts at 6-hours post-injection were notably upregulated compared to the pre-injected, time-0 controls, but this upregulation transitioned into a down-regulation by 96-hours post-injection.

3.3.1.2 Effects of Water Temperature on Splenic *IL-1β* Expression

When the data is reanalyzed to assess the effects of water temperature, *IL-1β* transcript levels in the pre-injected cohorts did not differ between the temperatures (**Fig. 3.1.B**). In the PBS-injected animals, all three post-injection timepoints demonstrated higher *IL-1β* expression in the 19^{o}C cohorts relative to their respective 12^{o}C control cohorts. This pattern could be indicative of temperature-related regulation of basal levels of *IL-1β,* but the lack of differences in the pre-

injected cohorts would be contradictory to this idea. In the vaccinated animals, however, the 12^{o}C cohorts expressed higher levels of *IL-1β* than the 19^{o}C cohort at all three post-injection timepoints. While this could be interpreted as a simple suppression of *IL-1β* expression at higher temperatures, it is also plausible that *IL-1β* expression peaked before 6-hours post-injection at this temperature, and the relationships seen in figure 3.1.B is evidence of a faster resolution of the immune response at 19^{o}C.

3.3.1.3 Effects of Thermally-Selected Crosses on Splenic *IL-1β* Expression

The potential effects of selective parental crosses on *IL-1β* expression were assessed in **Fig. 3.1.C**, where differences in *IL-1β* expression between crosses were observed in the 6-hour, PBS-injected, 12^{o}C cohort, the 24-hour, PBS-injected, 19^{o}C cohort, and amongst both temperatures in the 96-hour vaccine-injected cohorts. In general, the determined differences, although statistically significant, were inconsistent across different factors. Additionally, the specific crosses that were different from each other in each case were also inconsistent between different combinations of factors. Overall, it is difficult to state a clear effect that selective parental crosses had on *IL-1β* expression.

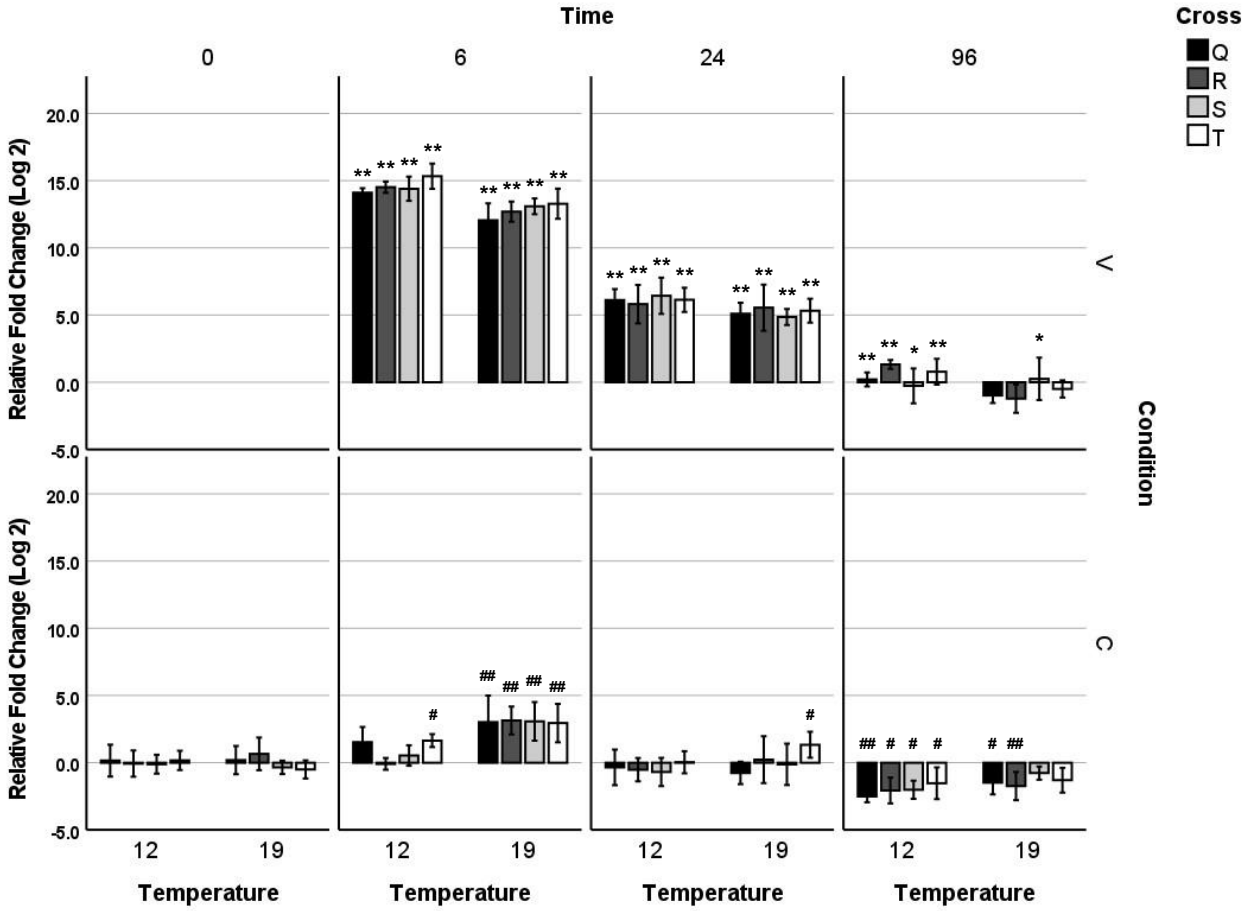

Figure 3.1.A Relative transcript levels of splenic *IL-1β* from four crosses of rainbow trout treated with different conditions and temperatures, collected at 4 different timepoints. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Each panel presents relative transcript fold changes for all four crosses at both experimental temperatures. The upper left panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Asterisks (*) denote statistically significant differences between a VIBROGEN II injected cohort (V) compared directly to its respective PBS injected control (C) where * denotes a P-value < 0.05, and ** denotes a P-value < 0.001. Number signs (#) denote statistically significant differences between a PBS injected control cohort and its respective 0-hour (pre-injected) control cohort, where # denotes a P-value < 0.05, and ## denotes a P-value < 0.001. (n=6)

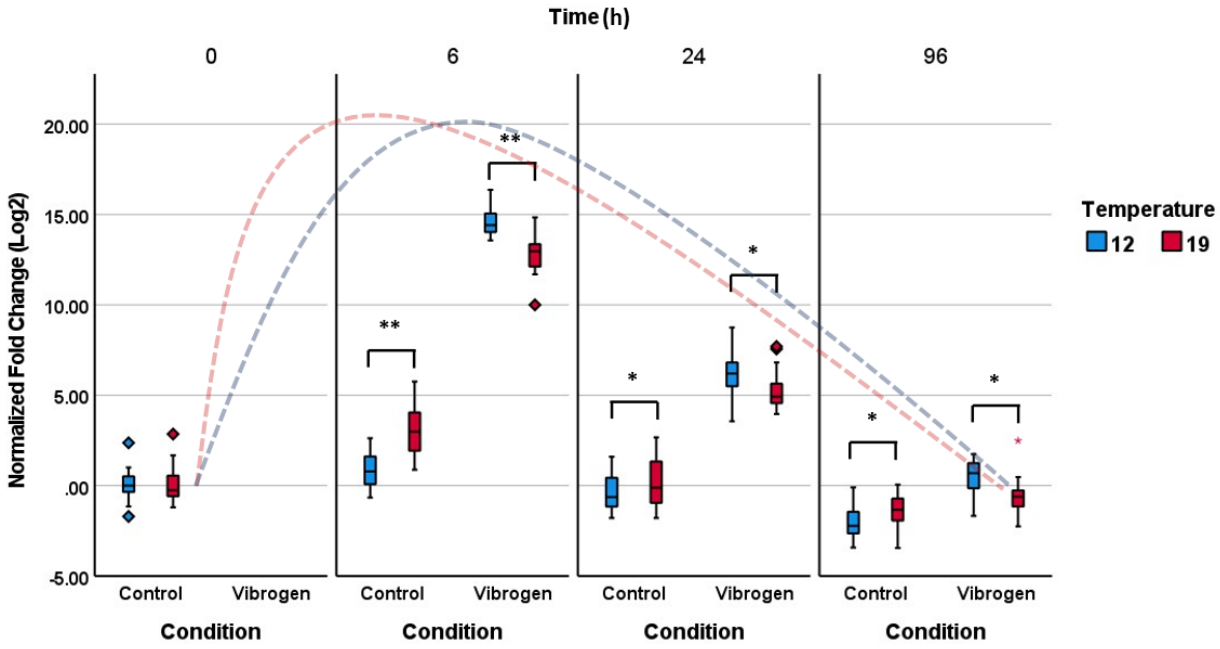

Figure 3.1.B Relative transcript levels of splenic *IL-1β* of rainbow trout between experimental temperatures, treated to different water temperatures and injection conditions, collected at 4 different timepoints. Animals were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Splenic *IL-1β* transcripts are presented regardless of crosses to elucidate the effects of water temperature. The "Vibrogen" cluster in the 0-hour panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Asterisks (*) denote statistically significant differences between the two experimental temperatures in otherwise identical treatment conditions where * denotes a P-value < 0.05, and ** denotes a P-value < 0.001. A diamond indicates a sample value greater than 1.5 IQRs outside the IQR, an asterisk value indicates a sample value greater than 3 IQRs outside the IQR. The dotted lines represent the possible regulation kinetics of splenic *IL-1β* at 12ºC (blue) and 19ºC (red) respectively, these visual aids are estimated projections and not calculated. (n=24)

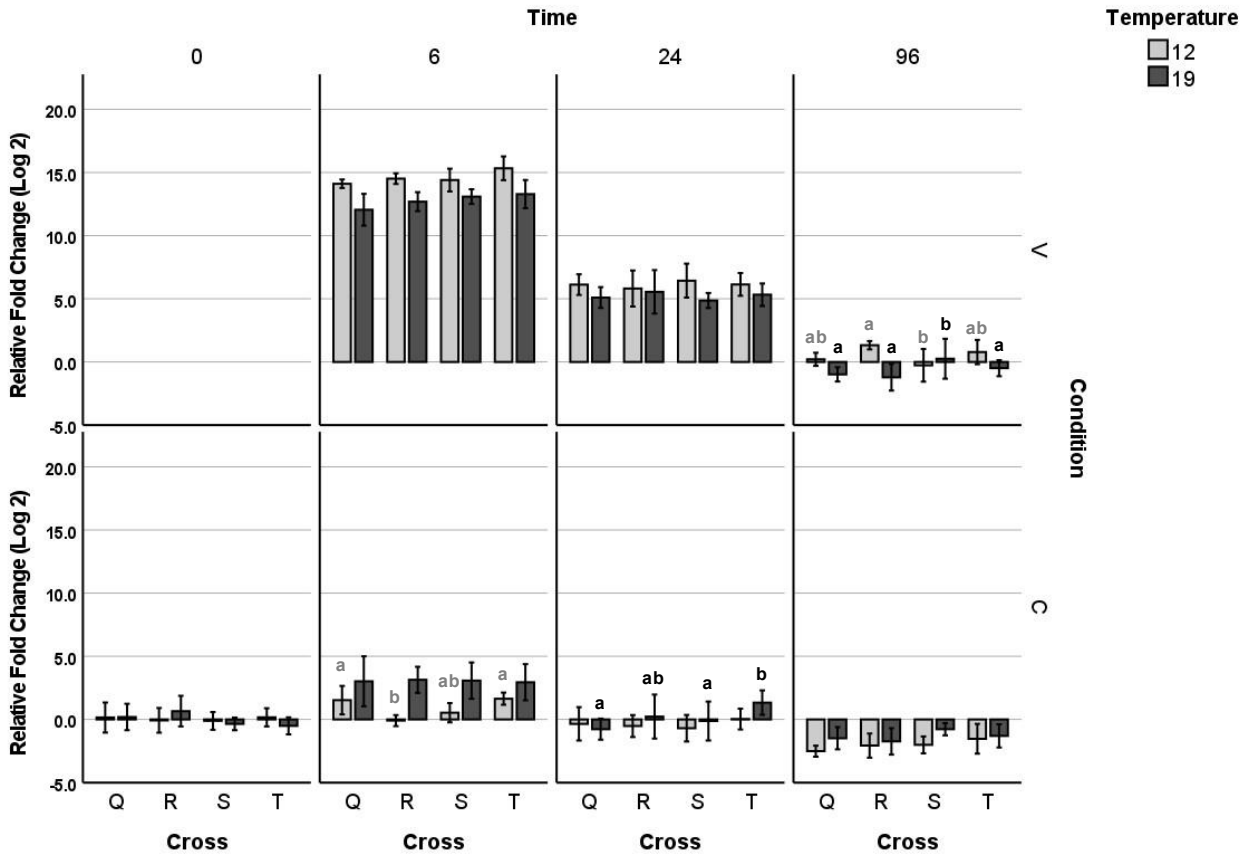

Figure 3.1.C Relative transcript levels of splenic *IL-1β* comparing four crosses of rainbow trout treated to different conditions and temperatures, collected at 4 different timepoints. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Each panel presents relative transcript fold changes for all four crosses, clustered by both experimental temperatures. The upper left panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Cross-based differences were only contrasted with other cross cohorts with the same value in all other factors (within each panel). Significant differences are denoted by letters where applicable, grey lettering represents differences amongst the crosses at 12ºC, black lettering represents differences amongst the crosses at 19ºC. (n=6)

3.3.2 Analysis of *IFN-γ* Transcript Expression on the Factors of Vaccination, Temperature, and Selection

3.3.2.1 Effects of Vaccine Challenge and Injection Procedure on Splenic *IFN-γ* Expression

When analyzing *IFN-γ* expression levels relative to vaccination status, significant upregulations of *IFN-γ* were observed in all vaccine-injected cohorts at both 6- and 24-hours compared to their respective PBS-injected controls (**Fig. 3.2.A**). Moreover, the vaccine-injected 96-hour cohort demonstrated a decreased state of transcript upregulation, where a few specific cohorts had returned to pre-injection basal levels. Additionally, a few differences were observed when comparing the PBS-injected controls to the pre-injected controls, the cross S cohort 19^{o}C cohort upregulated *IFN-γ* at 6-hour, and the cross Q cohort in 12^{o}C water at 24-hour downregulated *IFN-γ* when compared to their respective pre-injected controls (**Fig. 3.2.A**).

3.3.2.2 Effect of Water Temperature on Splenic *IFN-γ* Expression

When the data is reorganized for the effects of water temperature on *IFN-γ* expression, the only groups that showed a significant difference in *IFN-γ* expression were the vaccine-injected cohorts of the 6- and 24-hour post-injection groups (**Fig. 3.2.B**). Interestingly, the patterns of differential regulation are opposite between the two timepoints. In the 6-hour, vaccine-injected cohorts, the 19^{o}C group was upregulated to a significantly higher level than the 12^{o}C group, but the opposite was true in the 24-hour, vaccine-injected cohorts where the 12^{o}C group expressed much higher levels of *IFN-γ* than the 19^{o}C group (**Fig. 3.2.B**)..

3.3.2.3 Effect of Thermally-Selected Crosses on Splenic *IFN-γ* Expression

Finally, the parental cross factor demonstrated significant differences in the PBS-injected, 19^{o}C temperature groups at all four timepoints (**Fig. 3.2.C**). Among these observations, cross T is the

parental cross that consistently expressed a lower level of *IFN-γ* than other crosses, especially cross Q, and, interestingly, crosses Q and T do not share a common parent (**Fig. 3.2.C**). When the crosses are analyzed regardless of timepoint, crosses S and T are significantly downregulated across the non-vaccinated cohorts compared to cross Q (**Fig. S1**). Additionally, the crosses were different between each other in the 6-hour vaccine-injected cohort for both temperatures, and in both cases, cross T was interestingly the highest-expressed parental cross for *IFN-γ* (**Fig. 3.2.C**).

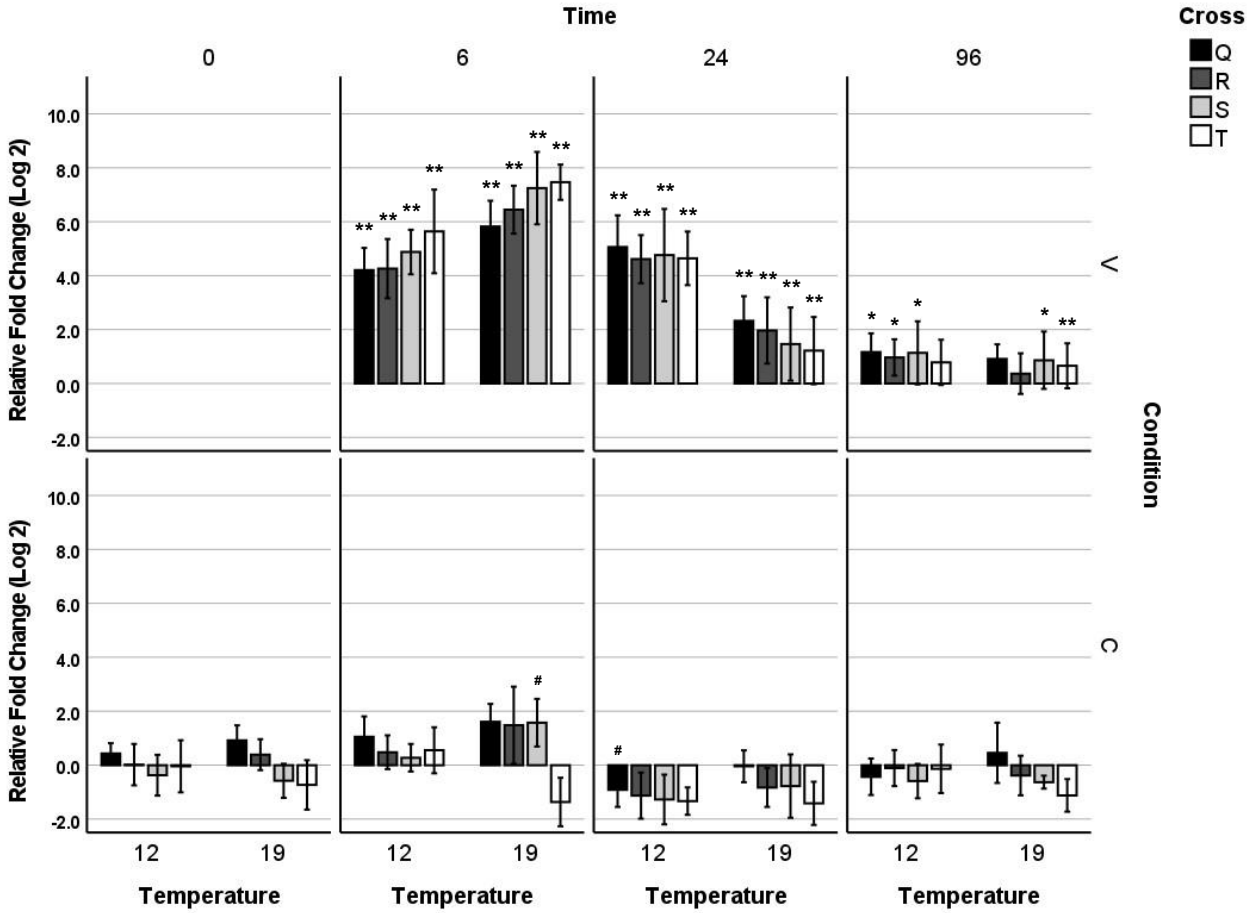

Figure 3.2.A Relative transcript levels of splenic *IFN-γ* from four crosses of rainbow trout treated to different conditions and temperatures, collected at 4 different timepoints. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Each panel presents relative transcript fold changes for all four crosses at both experimental temperatures. The upper left panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Asterisks (*) denote statistically significant differences between a VIBROGEN II injected cohort (V) compared directly to its respective PBS injected control (C) where * denotes a P-value < 0.05, and ** denotes a P-value < 0.001. Number signs (#) denote statistically significant differences between a PBS injected control cohort and its respective 0-hour (pre-injected) control cohort, where # denotes a P-value < 0.05, and ## denotes a P-value < 0.001. (n=6)

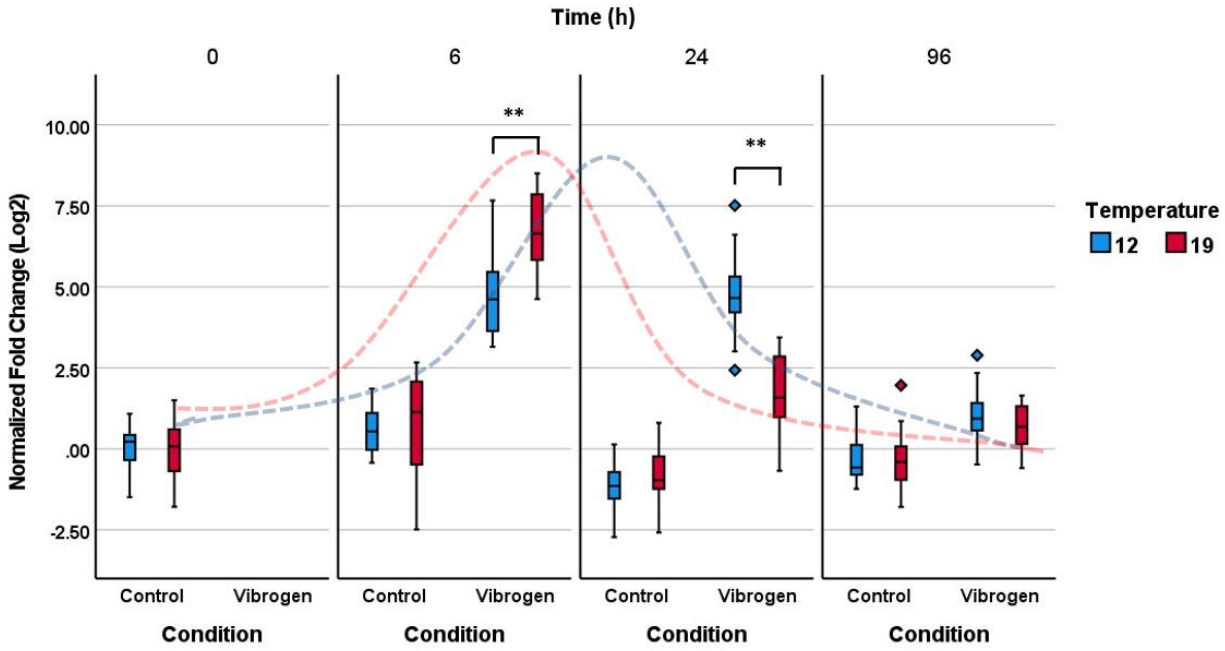

Figure 3.2.B Relative transcript levels of splenic *IFN-γ* of rainbow trout between experimental temperatures, treated to different water temperatures and injection conditions, collected at 4 different timepoints. Animals were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Splenic *IFN-γ* transcripts are presented regardless of crosses to elucidate the effects of water temperature. The "Vibrogen" cluster in the 0-hour panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Asterisks (*) denote statistically significant differences between the two experimental temperatures in otherwise identical treatment conditions where * denotes a P-value < 0.05, and ** denotes a P-value < 0.001. A diamond indicates a sample value greater than 1.5 IQRs outside the IQR. The dotted lines represent the possible regulation kinetics of splenic *IL-1β* at 12ºC (blue) and 19ºC (red) respectively, these visual aids are estimated projections and not calculated. (n=24)

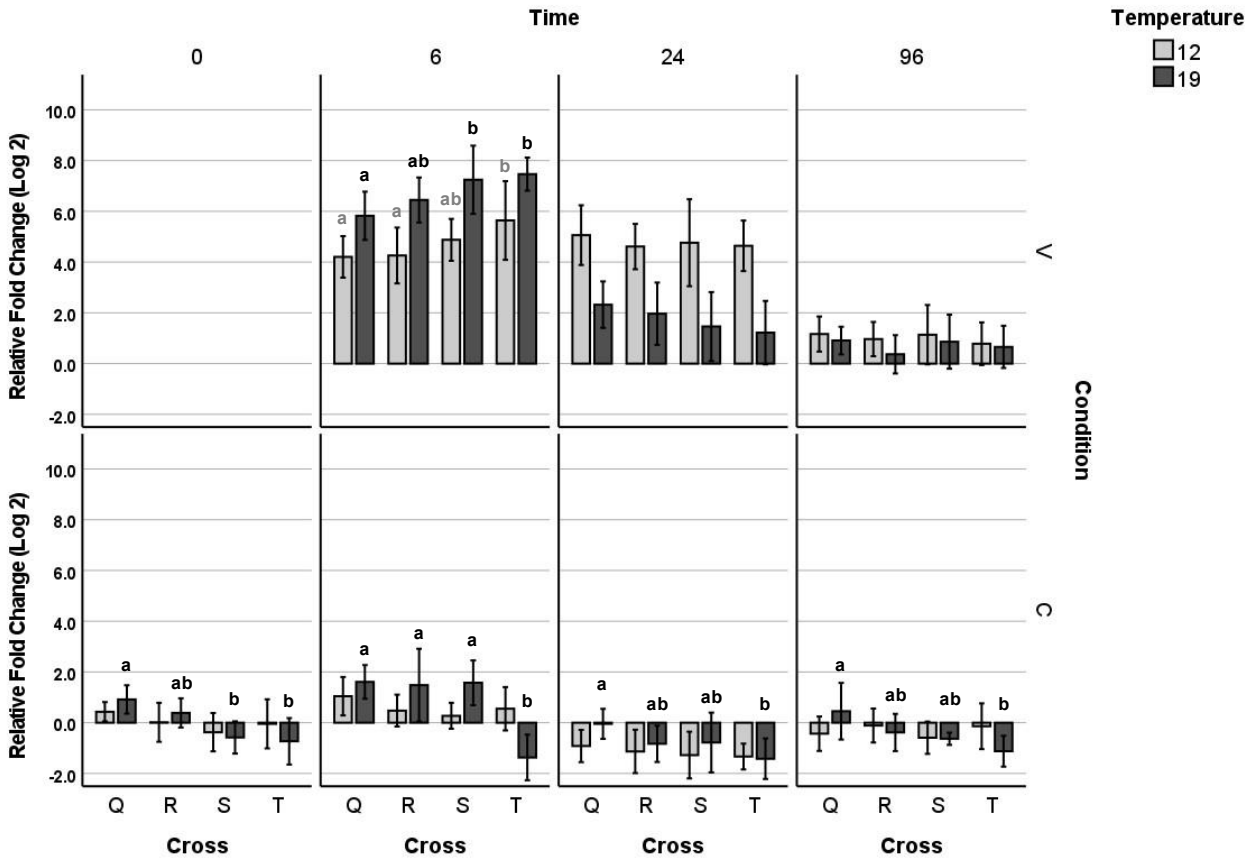

Figure 3.2.C Relative transcript levels of splenic *IFN-γ* comparing four crosses of rainbow trout treated to different conditions and temperatures, collected at 4 different timepoints. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Each panel presents relative transcript fold changes for all four crosses, clustered by both experimental temperatures. The upper left panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Cross-based differences were only contrasted with other cross cohorts with the same value in all other factors (within each panel). Significant differences are denoted by letters where applicable, grey lettering represents differences amongst the crosses at 12ºC, black lettering represents differences amongst the crosses at 19ºC. (n=6)

3.3.3 Analysis of *tapasin* Transcript Expression on the Factors of Vaccination, Temperature, and Selection

3.3.3.1 Effects of Vaccine Challenge and Injection Procedure on Splenic *tapasin* Expression

For the *tapasin* transcript, expression levels in individually analyzed vaccine-injected-cohorts at 6- and 24-hour were significantly upregulated compared to their respective PBS-injected control cohorts (**Fig. 3.3.A**). However, the clear peak expression level was not observed at the 6-hour timepoint, but at 24 hours. By the 96-hours post-injection timepoint, *tapasin* expression in vaccinated cohorts had subsided, and a few cohorts were no longer significantly upregulated compared to the respective controls. For the PBS-injected control groups, a few cohorts were significantly upregulated compared to their pre-injected controls, specifically the 12^{o}C, cross T cohorts at the 6-, 24- and 96-hour timepoints, as well as the 12^{o}C, cross S cohort at the 24-hour timepoint. No significant *tapasin* downregulations were observed (**Fig. 3.3.A**).

3.3.3.2 Effects of Water Temperature and Thermally-Selected Crosses on Splenic *tapasin* Expression

Water temperature had a significant effect on *tapasin* expression (**Fig. 3.3.B**). Of the seven comparisons made between cohorts of different temperatures at each set of combinations of factors, five pairs observed a significant difference between the temperatures. In each of the significantly different pairs, the 12^{o}C cohorts were upregulated compared to the 19^{o}C cohorts. Of the five cohort pairs, the vaccine-injected cohorts at 24-hour post-injection were the most different. However, the basal pre-injected controls did not express *tapasin* differently between the two temperatures, the same was true for the vaccine-injected 6-hour cohort. Regarding the effects of parental crosses on *tapasin* expression, relatively few differences were observed, only

in the 6-hour post-injection cohorts for the 12ºC vaccinated crosses, and the 19ºC PBS-injected crosses (**Fig. 3.3.C**).

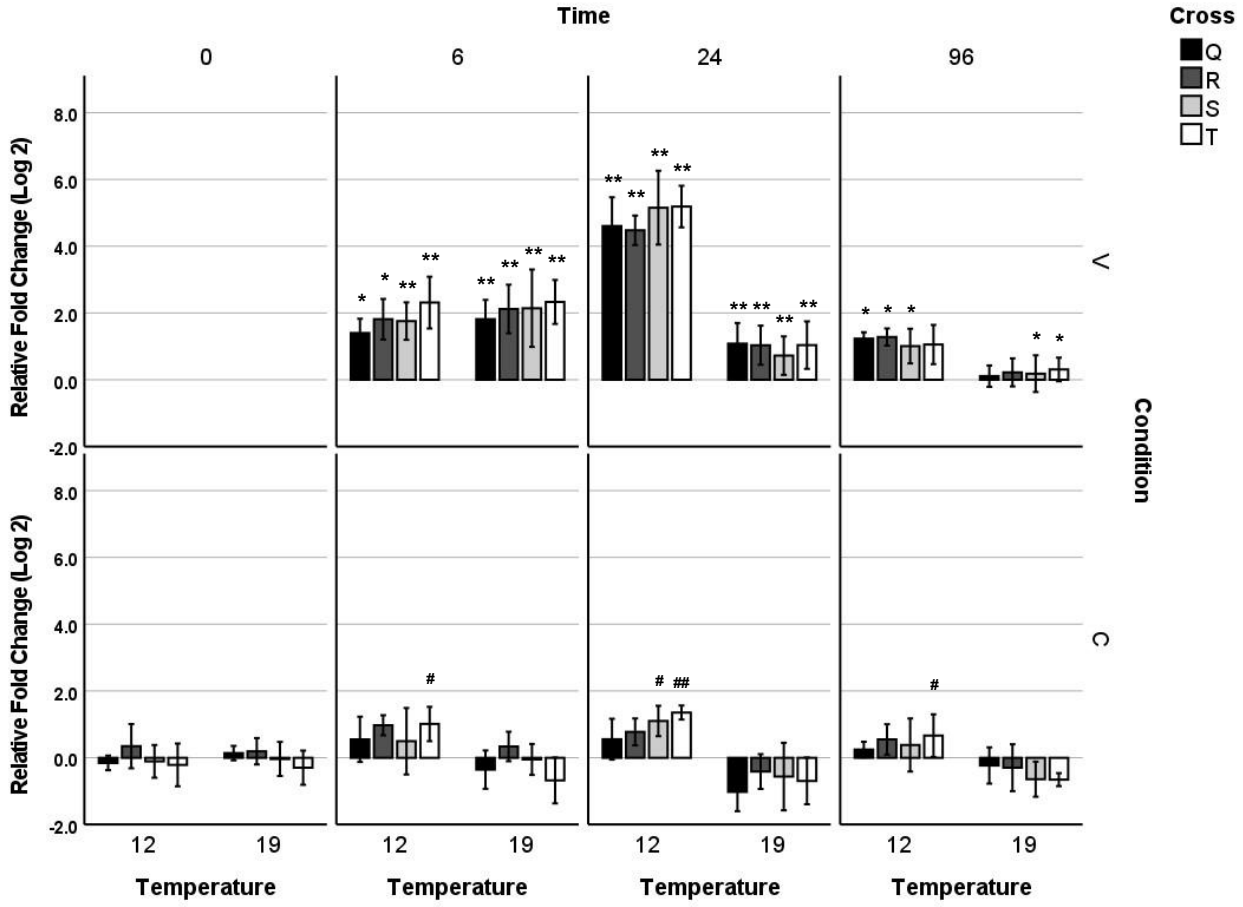

Figure 3.3.A Relative transcript levels of splenic *tapasin* from four crosses of rainbow trout treated to different conditions and temperatures, collected at 4 different timepoints. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Each panel presents relative transcript fold changes for all four crosses at both experimental temperatures. The upper left panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Asterisks (*) denote statistically significant differences between a VIBROGEN II injected cohort (V) compared directly to its respective PBS injected control (C) where * denotes a P-value < 0.05, and ** denotes a P-value < 0.001. Number signs (#) denote statistically significant differences between a PBS injected control cohort and its respective 0-hour (pre-injected) control cohort, where # denotes a P-value < 0.05, and ## denotes a P-value < 0.001. (n=6)

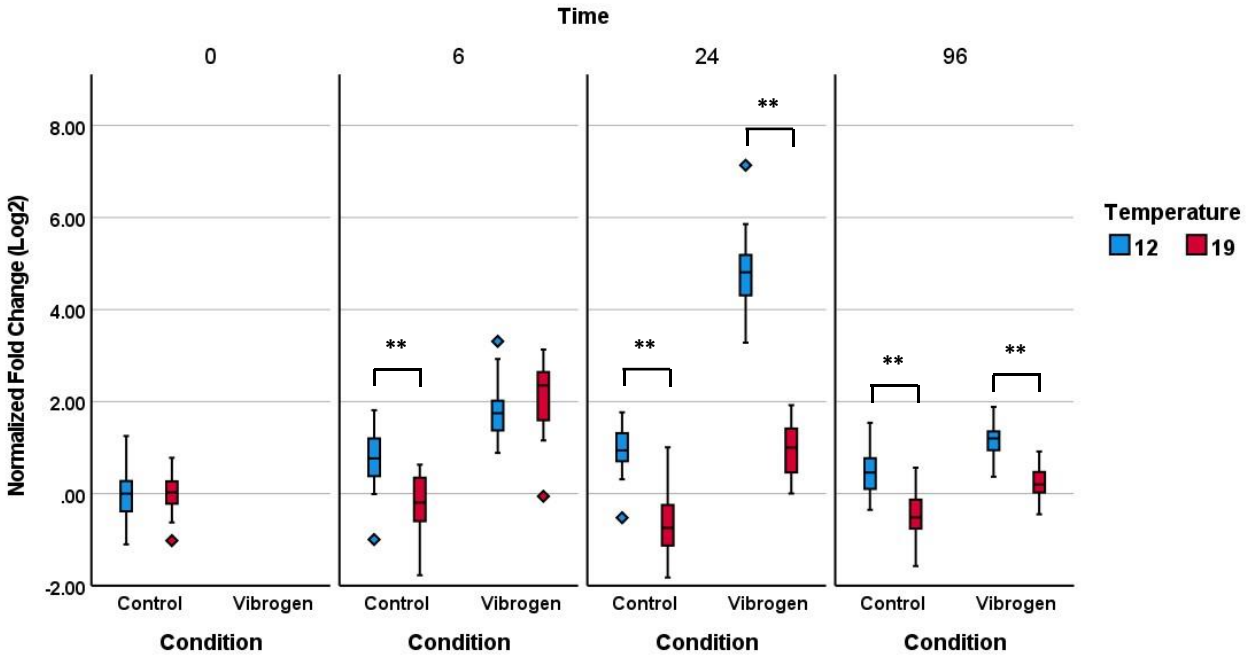

Figure 3.3.B. Relative transcript levels of splenic *tapasin* of rainbow trout between experimental temperatures, treated to different water temperatures and injection conditions, collected at 4 different timepoints. Animals were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Splenic *tapasin* transcripts are presented regardless of crosses to elucidate the effects of water temperature. The "Vibrogen" cluster in the 0-hour panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Asterisks (*) denote statistically significant differences between the two experimental temperatures in otherwise identical treatment conditions where * denotes a P-value < 0.05, and ** denotes a P-value < 0.001. A diamond indicates a sample value greater than 1.5 IQRs outside the IQR. (n=24)

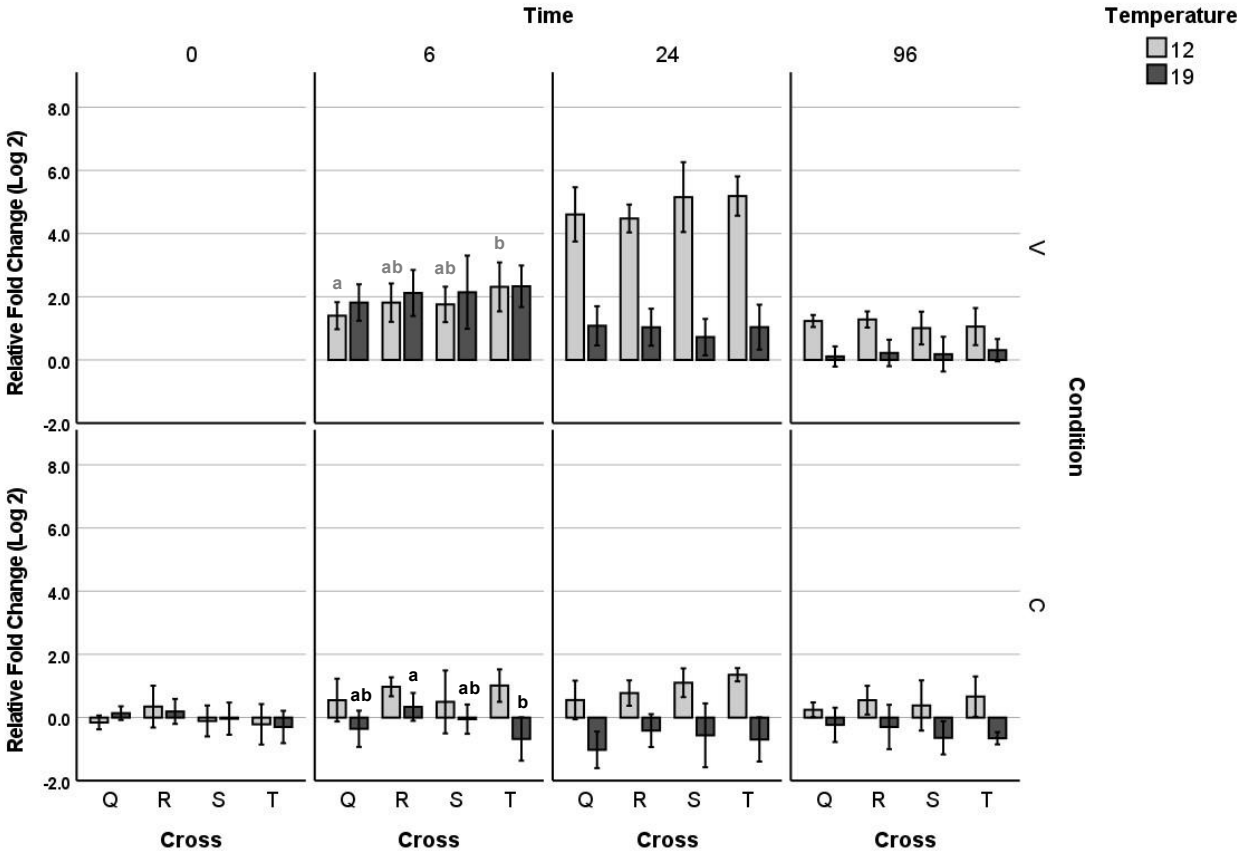

Figure 3.3.C Relative transcript levels of splenic *tapasin* comparing four crosses of rainbow trout treated to different conditions and temperatures, collected at 4 different timepoints. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C) or VIBROGEN II vaccine (V). Spleen samples were collected at 0, 6, 24, and 96 hours post-injection. Each panel presents relative transcript fold changes for all four crosses, clustered by both experimental temperatures. The upper left panel is empty as the 0-hour timepoint was sampled prior to the injection protocol. Cross-based differences were only contrasted with other cross cohorts with the same value in all other factors (within each panel). Significant differences are denoted by letters where applicable, grey lettering represents differences amongst the crosses at 12ºC, black lettering represents differences amongst the crosses at 19ºC. (n=6)

3.3.4 Analysis of *IL-10* Transcript Expression on the Factors of Vaccination, Temperature, and Selection

3.3.4.1 Effects of Vaccine Challenge and Water Temperature on Splenic *IL-10* Expression

Finally, the analysis of splenic *IL-10* expression was performed differently than the other transcripts assessed, as the non-vaccinated cohorts did not express *IL-10* at a detectable level (as detailed in section 3.2.8.2), thus the data presented in these sections only reflects comparisons between the vaccinated cohorts. When focused on the effects of water temperature and time, *IL-10* was highly upregulated to a similar level at 6-hour in both the 12^{o}C and 19^{o}C cohorts (**Fig. 3.4.A**). The 12^{o}C cohort at 24-hour was upregulated similar to the 6-hour cohorts, but the 19^{o}C, 24-hour cohort was significantly less upregulated than the three aforementioned groups. The 96-hour cohorts at different temperatures were similarly upregulated compared to each other, but the 19^{o}C, 96-hour cohorts were downregulated compared to the 19^{o}C cohort at 24-hour, whereas the 12^{o}C 96-hour cohort was not. Additionally, it is interesting to note that the *IL-10* expression at 96-hour was still notably expressed at a higher level than the limit of detection (where the control cohorts are presumed to be expressed).

3.3.4.2 Effects of Thermally-Selected Crosses on Splenic *IL-10* Expression

When investigating the differences in IL-10 expression based on crosses, discriminated by time and temperature, the only notable difference between the crosses was the 24-hour cohort in 19^{o}C water, where cross S was significantly less upregulated than crosses Q and R (**Fig. 3.4.B**). Observing the other groups in the same figure, there is a trend where cross S appears to be less upregulated for *IL-10* expression in each respective group of comparison, although any suggested difference was not statistically significant. However, when the fold change data was

analyzed based on the effects of crosses regardless of time and water temperature, cross S was found to be significantly less upregulated than the other three crosses across all vaccinated cohorts (**Fig. 3.4.C**).

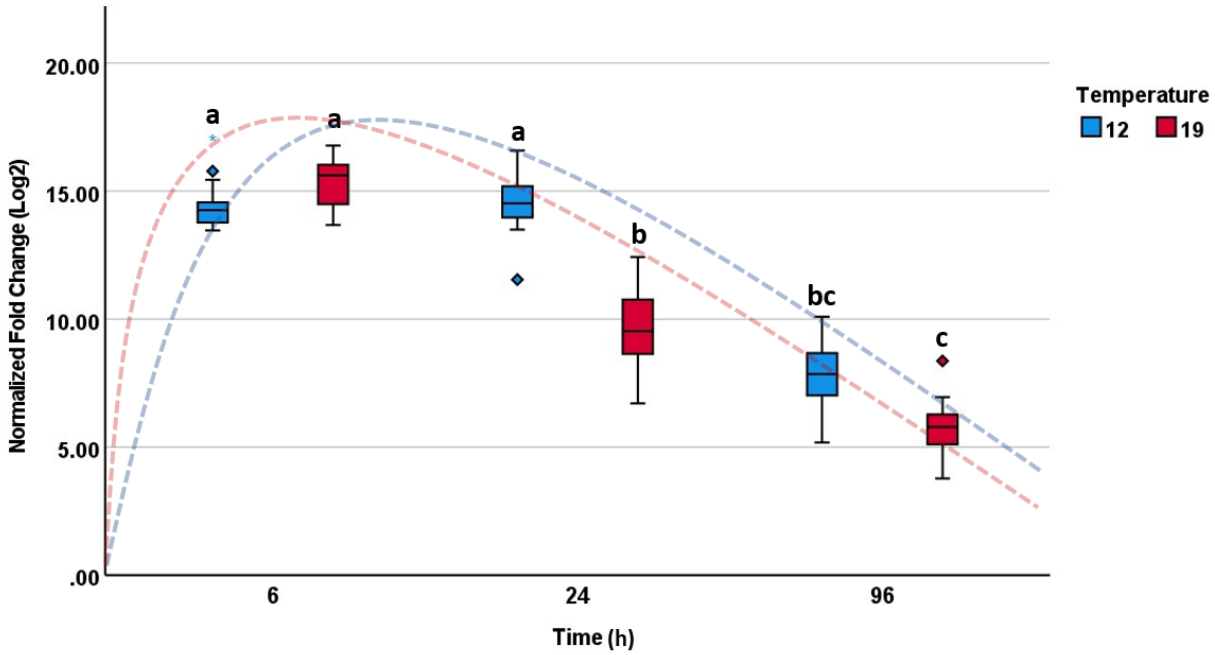

Figure 3.4.A. Relative transcript levels of splenic *IL-10* of VIBROGEN II injected rainbow trout between different experimental temperatures, collected at 3 different timepoints. Animals were subjected to either 12ºC or 19ºC water temperatures and injected with the VIBROGEN II vaccine. Spleen samples were collected at 6, 24, and 96 hours post-injection. Splenic *IL-10* transcripts are averaged regardless of crosses to elucidate the effects of water temperature. Significant differences were denoted with letters. A diamond indicates a sample value greater than 1.5 IQRs outside the IQR, an asterisk value indicates a sample value greater than 3 IQRs outside the IQR. The dotted lines represent the possible regulation kinetics of splenic *IL-1β* at 12ºC (blue) and 19ºC (red) respectively, these visual aids are estimated projections and not calculated. (n=24)

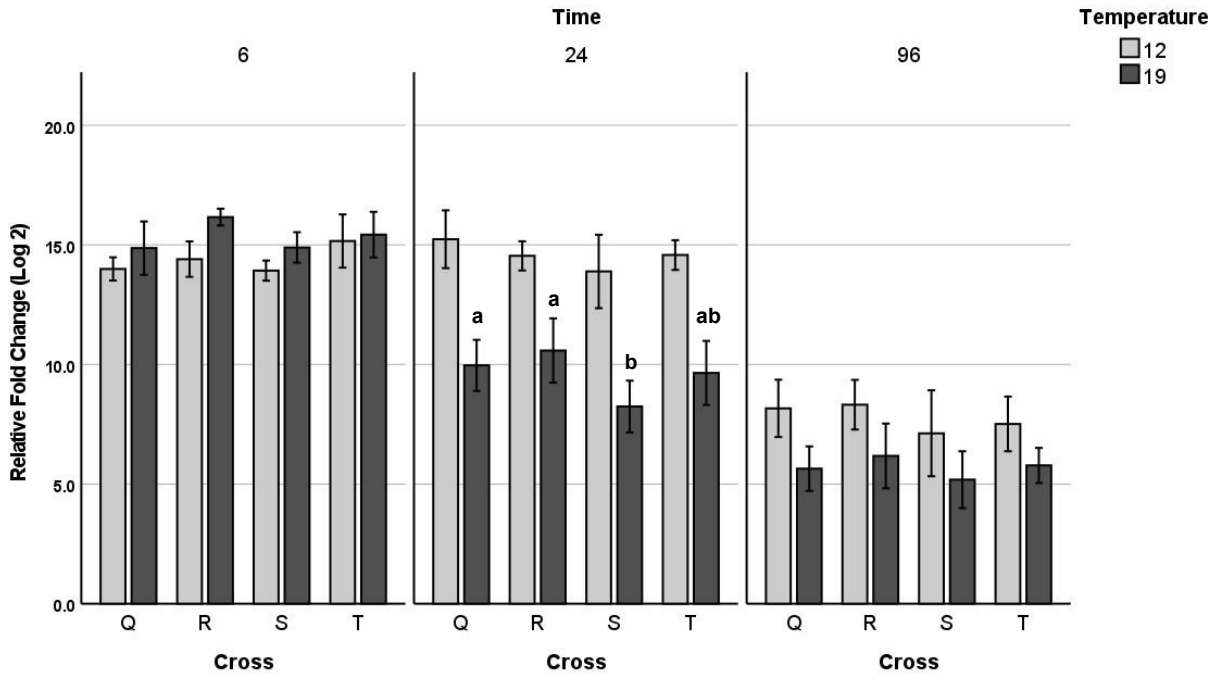

Figure 3.4.B. Relative transcript levels of splenic *IL-10* comparing four crosses of VIBROGEN II injected rainbow trout treated to different temperatures, collected at 3 different timepoints.
Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with the VIBROGEN II vaccine (V). Spleen samples were collected at 6, 24, and 96 hours post-injection. Each panel presents relative transcript fold changes for all four crosses, clustered by both experimental temperatures. Cross-based differences were only contrasted with other cross cohorts with the same value in all other factors (within each panel). Significant differences are denoted by letters where applicable, grey lettering represents differences amongst the crosses at 12ºC, black lettering represents differences amongst the crosses at 19ºC. (n=6)

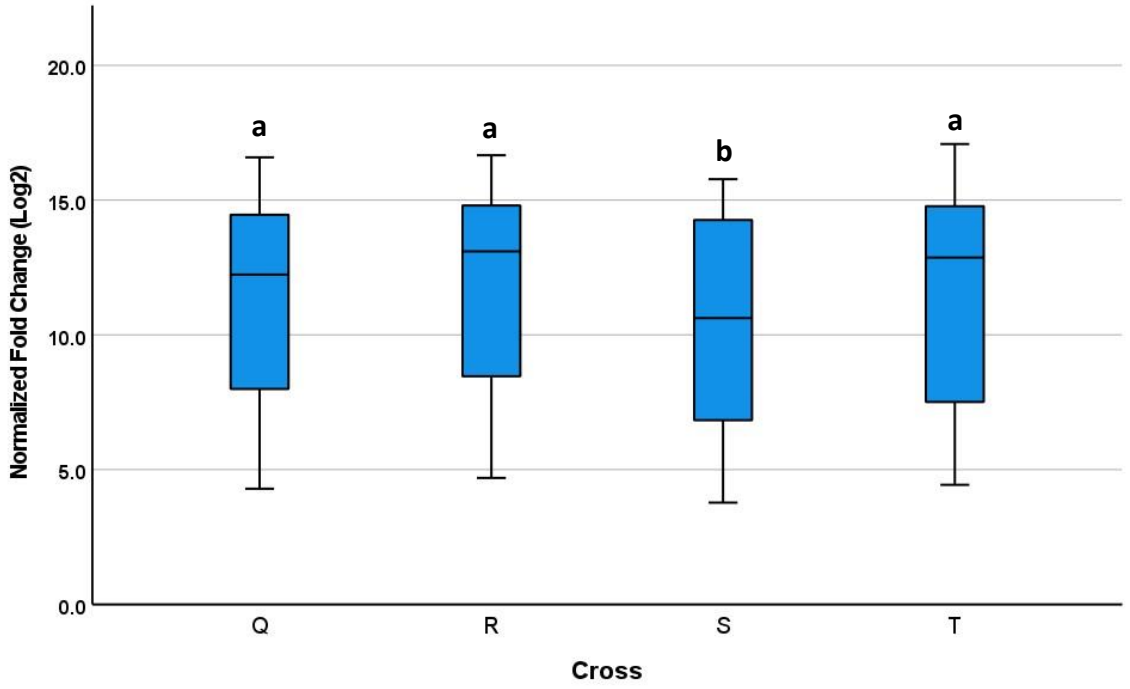

Figure 3.4.C Relative transcript levels of splenic *IL-10* comparing four crosses of VIBROGEN II injected rainbow trout. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12°C or 19°C water temperatures and injected with the VIBROGEN II vaccine. Spleen samples were collected at 6, 24, and 96 hours post-injection. This figure visualized these assessed cohorts based on crosses, and regardless of the temperature and timepoint factors. Significant differences are denoted by letters where applicable. (n=36)

3.4 Discussion

3.4.1 General Induction of Expression Changes

IL-1β and *IFN-γ* transcript levels were upregulated in response to the vaccine, with peak upregulated levels at 6-hours post-injection, decreasing towards the resolution of the response at 96 hours post-injection (**Fig. 3.1.A**, **Fig. 3.2.A**). These are expected patterns of regulation for these transcripts, similar to previous whole organism studies involving stimulation with killed bacterial pathogens (Furnes et al., 2009; Zanuzzo et al., 2015). Although the peak upregulation time of *IFN-γ* seems to be delayed compared to the peak upregulation time of *IL-1β* (Zhang et al., 2017). This difference is understandable if contextualized through the idea that IL-1β is crucially involved in the initial inflammation and recruitment, whereas *IFN-γ* is more related to cellular activation and the subsequent antigen presentation process. Additional to the vaccinated cohorts, the PBS injected control cohorts demonstrated clear upregulation of *IL-1β* at 6-hour post-injection that transitioned to a clear downregulation by 96-hour post-injection. These differences could be attributed to acute-stress-based regulation of *IL-1β*, which has been previously reported in several species in response to acute stressors (Hoseini et al., 2019; Magouz et al., 2021). It is notable that while these differences were biologically significant, they did not affect the statistical analysis of the vaccinated cohorts as each vaccinated cohort was compared directly to its respective PBS-injected control cohort.

Teleost *tapasin* gene expression in response to bacterial pathogens has not been exhaustively assessed, as it is more often studied in conjunction with viral challenges. Despite this lack of literature, an LPS-exposed rainbow trout study observed upregulation of splenic *tapasin* at 48 hours post-exposure, which is consistent with the finding of the current study (Djordjevic et al., 2009). The *tapasin* expression data is also consistent with the current *IFN-γ* expression data, as

IFN-γ has been shown to directly upregulate *interferon regulatory factor 1* (*IRF-1*) and *IFN-1* expression in rainbow trout spleen, both of which are primary regulators of the endogenous antigen presentation pathway (Abram et al., 2019a; Cao et al., 2016a; Cao et al., 2016b; Hansen and La Patra, 2002). Comparatively, while there was significant upregulation of *tapasin* at 6- and 24-hour timepoints of the vaccinated cohorts, the overall regulation time-course of *tapasin* was not as distinctly interpretable as the patterns seen in *IL-1β* and *IFN-γ*. It is possible the designed timepoints in this experiment did not optimally capture the expression kinetic of *tapasin* in response to the vaccine. Combined with the limited amount of literature on the subject, it is difficult to infer the biological significance of these regulatory changes for *tapasin*.

Lastly, the anti-inflammatory signaling cytokine IL-10 was significantly upregulated throughout the time-course of the vaccine response (Fig 3.4.A). The regulation kinetics of IL-10 across the assessed timepoints were relatively similar to what was observed in the aforementioned *IL-1β* and *IFN-γ*, with peak upregulated levels at 6-hours post-injection. However, the *IL-10* response was still very significantly upregulated at 96-hours post-injection. This was not a trend seen in the other genes analyzed, as the differential regulation caused by the vaccine had mostly subsided by the 96-hour timepoint in *IL-1β, IFN-γ, and tapasin* expression. This finding of delayed attenuation of *IL-10* upregulation is not unprecedented and is typical of the dynamic between an anti-inflammatory response and the pro-inflammatory respond preceding it. Live animal studies involving a bacterial challenge across various teleost species have also reported a delayed reversion to constitutive *IL-10* transcript levels compared specifically to *IL-1β* and *IFN-γ* (Ellingsen et al., 2011; Seppola et al., 2008; Tu et al., 2019). Mechanistically, *IL-10* regulation and the anti-inflammatory response in general trails the active pro-inflammatory response to

ensure optimal resolution of a successful immune response, which reduces unnecessary damage to self (Raida and Buchmann, 2008).

3.4.2 Effect of Temperature on the Regulation Kinetics of Assessed Genes

One of the main purposes of this study was to explore how water temperature co-stimulated with a pathogenic challenge can affect the teleost immune response. This is a relevant topic of study, as genes can be modulated in response to heat stress during an immune challenge (Han et al., 2017). As fish are ectothermic animals, the effects of temperature on fish immunity have been a topic of interest. Despite these efforts, however, much of the more nuanced relationships remain unknown. These gaps in knowledge may be caused by the specificity of interactions observed between water temperature and its physiological effects. For example, a non-natural water temperature can have a seemingly enhancing effect on one area of the immune response but suppress a different area of an immune response (Köllner and Kotterba, 2002; Wentworth et al., 2018). Likewise, while a certain temperature can stimulate a respective immune expression profile, the patterns of differential regulations can alter significantly when co-stimulated with a pathogen challenge (Abram et al., 2019a; Kales et al., 2006).

Supra-optimal, yet non-lethal water temperatures have been shown to enhance teleost immunity (Inkpen et al., 2015; Raida and Buchmann, 2007; Thanasaksiri et al., 2015). In contrast, sub-optimal water temperatures have been observed to attenuate immune responses (Abram et al., 2019b; Nath et al., 2006; Raida and Buchmann, 2007). These relationships between temperature and immune responses are congruent with fundamental chemical kinetics concepts such as collision theory, and measurements like the temperature coefficient (Q_{10}). These relationships are also more specifically in conjunction with evolved behaviours such as behavioural fever in teleosts (Bennoit and Craig, 2020; Boltaña et al., 2013; Gräns et al., 2002). Behavioural fever is

an observable behavioural change in the temperature preference of fish when they recognize that they are under pathogenic threat. When fighting infections, fish prefer higher water temperatures than their non-infected optimal temperature, and this change in preference translates to higher survival rates compared to fish subjected to lower temperatures (Boltaña et al., 2013).

In the present study, all four assessed transcripts were regulated in a temperature-dependent manner. When interpreted with the available context in literature, *IFN-γ*, *IL-1β*, and *IL-10* all exhibited indications of a more robust response in the higher temperature cohorts. A more robust immune expression profile can be understood as both i) an earlier, faster activation and initial up- or down-regulation of the transcript, and ii) an increase in the peak magnitude of the up- or down-regulation. In these results, the acceleration of initial upregulation was more clearly demonstrated. Whereas the lack of more timepoints made it difficult to compare peak upregulation intensity and magnitude. These accelerations were also closely matched with patterns seen in previous efforts to identify temperature-related kinetic patterns of immune transcripts. An *in vitro* experiment on southern bluefin tuna observed accelerated upregulation of *IL-1β*, *TNF-α*, and *IL-8* transcripts at higher temperature when induced with LPS, these effects were observed in both head kidney and splenic cultures (Polinski et al., 2013). To a similar effect, a variety of immune-related transcripts in Atlantic cod were upregulated more rapidly in higher-temperature cohorts in response to the viral mimic poly(I:C) (Hori et al., 2012). Interestingly, Raida and Buchmann, (2007) observed a faster and stronger regulation pattern for *IL-10* and *IFN-γ* in response to a *Yersinia ruckeri* vaccination at higher temperatures, but not for *IL-1β* (Raida and Buchmann, 2007). Similarly, a study designed to evaluate the effect of chronic heat stress on Atlantic salmon innate immunity also did not observe a different *IL-1β* expression profile that was observed in other transcripts (Zanuzzo et al., 2020). In summary, while there is a

relationship between higher-than-normal water temperatures and a more robust immune response. These effects could be transcript specific, as well as specific to other aspects of the study system, as seen in the inconsistent results of *IL-1β* expression patterns in these experiments. Further investigations should be conducted to elucidate the cause behind these inconsistencies in *IL-1β* expression patterns in response to high water temperatures. Overall, in a live infection scenario, these regulatory changes in higher temperatures can feasibly increase the speed of resolution, reduce damage to self, and produce an overall more effective immune response.

As a transcript encoding a protein involved in the MH class I response, *tapasin* has previously demonstrated the capability to be regulated by bacterial challenges, in addition to the traditionally related viral challenges (Abarca-Heidemann et al., 2002; Djordjevic et al., 2009). A few previous cell-culture studies have examined the effects of temperature on *tapasin* expression, with and without pathogenic exposure. Viral treatments of rainbow trout and walleye fibroblast cells demonstrated significant upregulation of *tapasin* at supra-optimal temperatures compared to cells kept at sub-optimal temperatures (Abram et al., 2019a; Abram et al., 2019b). These patterns were also displayed at the protein level (Sever et al., 2014). However, constitutive levels of *tapasin* transcripts in response to different temperatures, and not pathogens, observed signs of downregulation at higher temperatures compared to *tapasin* expression at lower temperatures (Abram et al., 2019a; Abram et al., 2019b). These previous results demonstrate that low-temperature mediated attenuation of immunity does not affect *tapasin* expression in a non-infected state. This is congruent with findings in the current study where the non-vaccinated cohorts displayed lower *tapasin* expression levels at higher temperatures at all timepoints except the pre-injected controls. Contrastingly, the vaccinated cohorts in this study did not demonstrate

a clear pattern of up- and down-regulation, especially in the 24-Hr cohorts where the 12^{o}C cohort was significantly upregulated compared to the 19^{o}C cohort. Possibly related, *IFN*-γ expression was also diminished in the 19^{o}C cohort at 24-Hr. Given the interferon and MH class I pathways are often similarly regulated, these results may be linked through common regulation factors. This suppression of *tapasin* also seems to contrast with the previous cell culture study that demonstrated higher *tapasin* levels at a supra-optimal temperature (26^{o}C) than a sub-optimal temperature (4^{o}C) in an immune-stimulated state (Abram et al., 2019a). However, it is also possible that both supra-optimal and sub-optimal temperatures suppress *tapasin* expression compared to expression at optimal temperatures, but a study investigating all three temperature-optimums has not yet been conducted (Abram et al., 2019a; Sever et al., 2014). Ultimately, this study explored, for the first time, the effects of temperature on teleost *tapasin* expression in a whole-animal study system, as well as the use of bacterial treatments instead of viral stimulants.

3.4.3 Effect of Parental Crosses and Family Effects on Immune Gene Expression

Studies of selection for higher tolerance and performance to thermal and pathogenic stress have demonstrated significant and biologically relevant protective effects for each respective stressor. However, the effects of thermal selection on the immune response has not been an area of focus, even though the heat shock and immune responses are potentially interconnected. The solely identified publication that investigated this specific subject challenged regionally distinct rainbow trout lines with a thermal trial and analyzed the transcriptome of gill tissue (Rebl et al., 2013). The generalized observations concluded that whereas the local cohort activated the innate response, the imported cohort presented a more adaptive response. Rebl et al., (2013) demonstrated differential regulation of the immune system during heat stress between selected lines of rainbow trout in constitutive conditions. Comparatively, the current study is the first to

explore the effects of thermal-based selection on the immune response in conjunction with a pathogen challenge. Selection-based statistical differences were identified in all four assessed transcripts, especially in *IFN-γ* and *IL-10*, which demonstrated differences in transcript expression between crosses from different parents. However, the biological significance of these changes are unclear at this stage. Perhaps a more extensive selection process over more generations will present a clearer pattern of transcriptional changes.

3.5 Conclusions

Ultimately, the obtained transcript data presented expected regulation patterns of the assessed transcripts in the context of previously available literature. The results also presented significant evidence that rainbow trout immune transcripts were regulated in a temperature-dependent manner, tentatively suggesting that higher water temperatures accelerated the kinetics of transcript regulation, and perhaps inhibit expression of the *tapasin* gene. More detailed analysis and more frequent sampling timepoints are needed to clearly demonstrate the change in regulatory magnitude in response to different temperatures. Lastly, parental thermal-selection resulted in some splenic transcriptional differences, although the biological relevance of these differences is difficult to interpret from these results. Taken together, supra-optimal water temperatures induced a more robust transcriptional immune response, and parental thermal-selection had a significant impact on transcriptional immune regulation.

Chapter 4: General Discussion and Future Directions

Both water temperature and pathogens are significant sources of stress that can affect the welfare of teleost to varying degrees of severity. From both commercial and conservation perspectives, there is considerable interest in the idea of preparing animals for these stressors to hopefully improve how effectively they can respond to them throughout their lifetimes, and limit the harm these stressors can cause. The main motivations behind the experiments and research of this thesis were to take previously established methods for improved thermal tolerance in fish and assess the effects of these methods through the perspective of immunology. Both thermal-preconditioning, and heat-tolerance-based parental selection have demonstrated varying protective effects in fish against heat-stress throughout the animal's lifetime (Callaghan et al., 2016; Fangue et al., 2006; Feminella and Matthews, 1984; Liu et al., 2017). Since the regulation of the heat-shock response and the immune response are interconnected, and as pathogens are a large source of loss for the aquaculture industry, it was determined that investigating the impact of these methods on not only the heat-shock response, but also the immune system, would also be relevant to the overall viability of these methods. This chapter will discuss some of the common transcription level effects that were observed across the two studies, how thermal tolerance translates to immune system changes, the efficacy of the applied methods, and future directions.

4.1 Analysis of Spleen and Gill Anti-Viral Immune Transcripts in Response to Temperature

Transcripts involved in the endogenous antigen presentation pathway and anti-viral defense (*MH class I, β2m, tapasin, IFN-1*, and *IFN-γ*) were assessed in both studies in response to both optimal and supra-optimal water temperatures. One of the more notable connections between

these transcripts across the two experiments is perhaps their seemingly downregulated nature at higher temperatures compared to more optimal temperatures. *IFN-1* and *MH class I* transcripts in chapter 2, and *tapasin* transcripts in chapter 3 all exhibited relative downregulation at higher temperatures. Though these differences were not consistently observed across all related transcripts, as *IFN-1* was shown to increase with temperature in the spleen, and *β2m* and *IFN-γ* were largely unchanged in response to temperature. Non-pathogenic stimulated analyses of these transcripts at different temperatures are rare, and especially so at the higher rather than the lower temperature extremes notably due to how poorly the anti-viral response functions at lower temperatures (Abram et al., 2017; Kim and Leong, 1999; Sever et al., 2014). It is possible that the lower constitutive expressions of these transcripts were evolutionarily developed to direct resources to other more affected physiological systems during heat-stress. After all, higher temperatures generally produce a more robust and stronger anti-viral immune reaction, and viral activities themselves also tend to be attenuated with increasing water temperature (Hawley and Garver, 2008; Hershberger et al., 2013; Oidtmann et al., 2018).

4.2 Assessment of Applied Methodology and General Conclusions

This thesis employed two animal rearing strategies with the aim of developing tolerance in the heat-shock response, and subsequently modulating the immune response. The juvenile thermal-preconditioning experiment (chapter 2) demonstrated notable changes between the expression levels of the immune transcript, mostly between the respective LT cohorts, as well as differences in the general stress response indicated by plasma cortisol levels. The thermal-based parental selection experiment (chapter 3) demonstrated some local-level differences at specific combinations of factors for *IL-1β, IFN-γ*, and *tapasin*, but more distinct differences across entire parental crosses were only observed for *IL-10*.

When holistically comparing the outcome of the two experiments, the degree of observed differences caused by the employed method was similar. Though both studies presented differences in immune transcripts by the effect of their respective methods, these differences were often not consistent enough to draw conclusions about any produced alterations to the immune response as a whole. These differences, while novel and significant, could possibly be clearer or more well defined with some adjustments to the protocols. For the preconditioning method, animals in the experiment were exposed to the thermal cycling at a juvenile life-stage, other studies have presented results to suggest that initial exposure to stress closer to the embryonic stage could induce more significant changes (Farago et al., 2017; Scott and Johnson, 2012; Schnurr et al., 2014). For the thermal-based parental selection experiment, the selection process was performed over three generations. More generations of thermal selection could induce larger changes in both thermotolerance and the immune response (Jesus et al., 2013; Nakano and Iwama, 2002). It is also important to consider that the genes of interest assessed in the two present experiments were assessed solely at the transcript level. The reliability and accuracy of predicting protein levels from transcript expression data are prone to inconsistencies (Bauernfeind and Babbitt, 2017; Fortelny et al., 2017). In the future, similar studies should try to incorporate more protein level analysis for higher confidence of biological relevance of potential changes caused by designed experimental factors.

4.3 Analysis of Rearing Method for Increases of Both Thermal and Pathogenic Tolerance for Aquaculture

The efficacy of thermal preconditioning is still relatively uncertain. The effectiveness of the technique in regard to the conferred protection seems to heavily depend on the specific protocol

of the pre-conditioning cycles and the life-stage of the animals. With the large number of animals that are usually associated with aquaculture production, scaling up the apparatus for cycles of thermal preconditioning may not be as feasible as when attempted at a smaller scale. In comparison, thermotolerance-based parental selection has fewer logistical issues, the protective strength of this method can increase over generations of selection, but genetic diversity issues could be of concern. In addition to thermal preconditioning and thermal-based parental selection, dietary supplementation and nutrition have also shown significant effectiveness as a method for improving both thermal and pathogenic tolerance (Dawood and Koshio, 2016; Gupta et al., 2010; Ragaza et al., 2015; Rodrigues et al., 2020). A well-developed feed system could be a worthwhile way to improve both responses simultaneously. Additionally, of course, significant protection against pathogens can be achieved through antibacterial drug use and vaccinations (Aly and Albutti, 2014; Sommerset et al., 2005). However, improving non-specific immune mechanisms through methods used in the current study can still help animals ward off pathogens for which there are no vaccines and improve their overall welfare.

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Appendix

Supplemental Figures

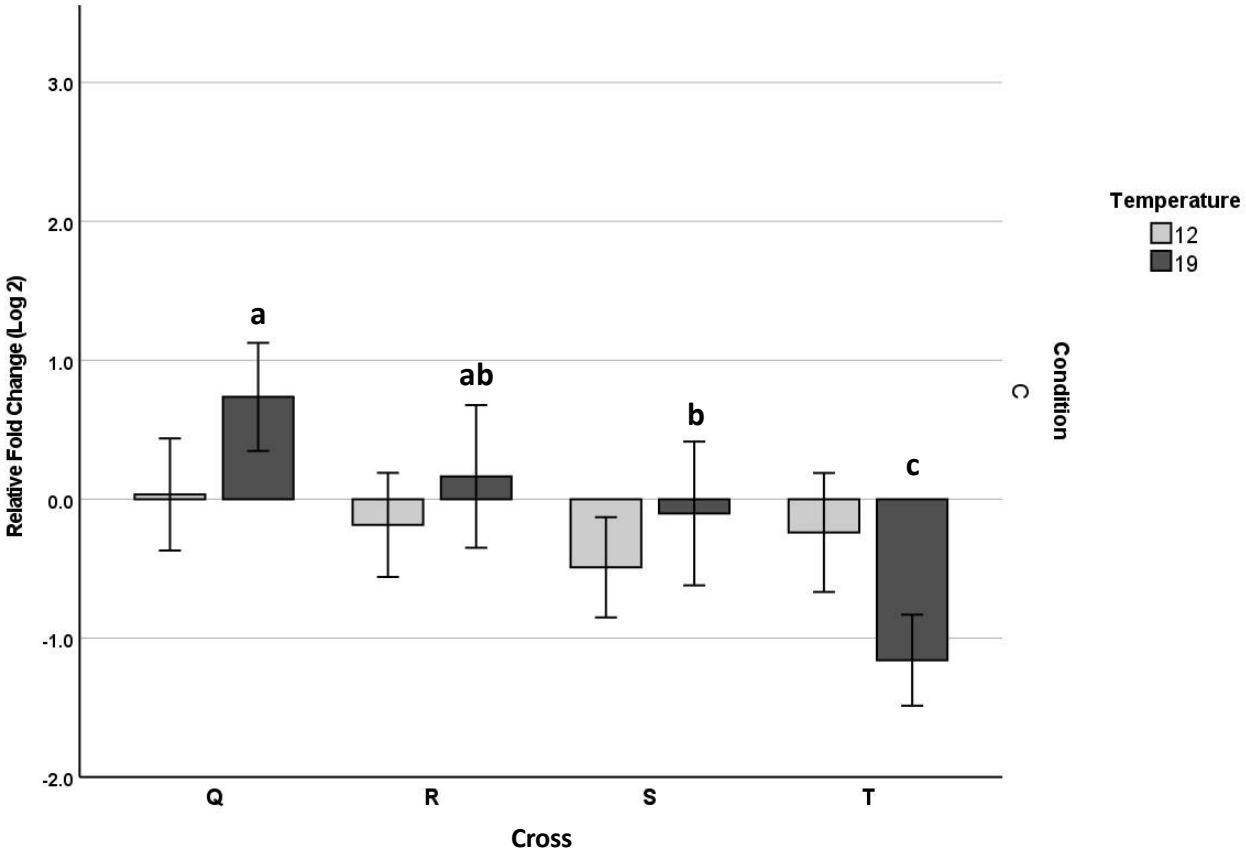

Figure S1. Relative transcript levels of splenic *IFN-γ* comparing four crosses of PBS-injected rainbow trout treated to two different water temperatures. Animals belonging to four crosses (Q, R, S, T) were subjected to either 12ºC or 19ºC water temperatures and injected with either PBS (C). Spleen samples were collected at 0, 6, 24, and 96 hours post injection but were not distinguished by time in this presentation. The presentation of the four crosses was clustered by both experimental temperatures. Cross based differences were only contrasted with other cross cohorts with the same temperature condition. Significant differences are denoted by letters where applicable. (n=24)