Influences of Stress Phenotype, Nutrition and Genetic Background on the Upper Thermal Tolerance of Atlantic Salmon (*Salmo salar*)

by

© Eric Hans Ignatz

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ABSTRACT

Anthropogenic climate change threatens the sustainability of the Atlantic salmon (*Salmo salar*) aquaculture industry. This thesis studied the effects of three factors [i) stress phenotype; ii) supplemental dietary cholesterol; and iii) family/genetic background] on the performance of farmed Atlantic salmon when exposed to an incremental thermal maximum (IT_{Max}, +0.2°C day^{-1}) challenge that mimicked natural summer sea-cage conditions. No differences in the IT_{Max} of male Atlantic salmon characterized as either low (LR) or high stress responders (HR) based on measurements of post-stress cortisol levels at 9°C was found, and interestingly, stress phenotypes were no longer distinguishable from one another when post-stress cortisol levels were analyzed at elevated temperatures. However, some differences in gene expression were found between LR and HR fish in response to bacterial immune stimulation. Notably, HR salmon mounted a greater ($p < 0.05$) innate antibacterial immune response than LR salmon at 20°C, whereas LRs had a greater ($p = 0.057$) response among stress-related transcripts relative to HR fish at 12°C. Supplemental dietary cholesterol did not affect the IT_{Max} of female triploid Atlantic salmon. However, ≤ 5% of the salmon (irrespective of diet) died before temperature reached 22°C, and this suggests that the commercial production of triploids is possible in eastern Canada. In addition, inclusion of +1.30 and 1.76% cholesterol in the diet reduced fillet bleaching above 18°C. Finally, family-based differences in IT_{Max} were detected in diploid, mixed sex, Atlantic salmon, with a 1.7°C separation between the least and most tolerant families. This study also showed that IT_{Max} is a more sensitive and relevant indicator of upper thermal tolerance than the more common/convenient approach of assessing a fish's critical thermal maximum (CT_{Max}). In the final chapter of this thesis, I characterized the four paralogues of *serpinh1* [alias *heat shock protein 47* (*hsp47*)] in Atlantic salmon. This is a well-established biomarker of heat stress

in salmonids, and the reported data provide important insights into the evolutionary history and regulatory functions of this essential gene. Overall, this thesis provides novel information on how the Atlantic salmon aquaculture industry can most effectively assess upper thermal tolerance, and navigate this era of accelerated climate change.

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LIST OF ABBREVIATIONS

°C	Degree centigrade
µL	Microlitre
ω	Omega
ALA	Alpha linolenic acid (18:3n-3)
ANOVA	Analysis of variance
ARA	Arachidonic acid (20:4n-6)
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CNRQ	Calibrated normalized relative quantity
$Cortisol_{ind}$	Individual cortisol value
$Cortisol_{pop}$	Average cortisol value amongst population
CP	Crude protein
C_T	Cycle threshold
CT_{Max}	Critical thermal maximum
d	Day
DHA	Docosahexaenoic acid (22:6n-3)
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid (20:5n-3)
FCR	Feed conversion ratio
g	Gram
gDNA	Genomic deoxyribonucleic acid
GR	Glucocorticoid receptor
GSP	Gene-specific primer
GWAS	Genome-wide association study
h	Hour
HPI	Hypothalamic-pituitary-interrenal
hpi	Hours post-injection
HR	High stress responder
HSI	Hepatosomatic index
IP	Intraperitoneally
IR	Intermediate stress responder
IT_{Max}	Incremental thermal maximum
JBARB	Dr. Joe Brown Aquatic Research Building
K	Fulton's condition factor
kg	Kilogram
L	Litre
LASCCR	Laboratory for Atlantic Salmon and Climate Change Research
LNA	Linoleic acid (18:2n-6)
LR	Low stress responder
m	Metre
mg	Milligram
MICCSA	Mitigating the Impact of Climate-Related Challenges on Salmon Aquaculture
min	Minute

MJ	Megajoule
mL	Millilitre
mRNA	Messenger ribonucleic acid
MSA	Multiple sequence alignment
MUFA	Monounsaturated fatty acid
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometre
NRQ	Normalized relative quantity
NTC	Non-template control
no-RT	No reverse transcriptase
ORF	Open reading frame
p	P-value
PCA	Principal component analysis
PCR	Polymerase chain reaction
PIT	Passive integrated transponder
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
qPCR	Real-time quantitative polymerase chain reaction
QTL	Quantitative trait loci
r^2	Correlation coefficient
RACE	Rapid amplification of cDNA ends
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
RVM	Relative ventricular mass
SD_{pop}	Standard deviation of the population
SE	Standard error
SFA	Saturated fatty acid
SGR	Specific growth rate
SNP	Single nucleotide polymorphism
TGC	Thermal-unit growth coefficient
T_i	Mean daily water temperature
T_m	Melting temperature
TMS	Tricaine methanesulfonate
UTR	Untranslated region
VSI	Viscerosomatic index
W_f	Final fish body weight
WGD	Whole genome duplication
W_i	Initial fish body weight
xg	Times gravitational force
Z_t	Total Z-score

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The research described in this thesis was performed by Eric H. Ignatz, with supervision from Drs. Kurt Gamperl and Matthew Rise. Eric Ignatz was responsible for conducting the experiments, collecting and analysing data, and writing the thesis. However, several others were instrumental in the completion of this research as well as noted below by their CRediT authorship contributions.

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CHAPTER 1: General Introduction

1.1 Introduction

With the stagnation of global fisheries landings, the aquaculture industry currently provides approximately half of the world's seafood (FAO, 2022). Further, global seafood demand is rapidly rising, and the aquaculture sector will need to greatly increase production levels to meet consumer needs (Cai and Leung, 2017). The Atlantic salmon (*Salmo salar*) is the main species produced by the Canadian aquaculture industry, and production was anticipated to increase from ~20,000 – 25,000 metric tonnes (MT) to 60,000 MT in Newfoundland by 2030 (Ignatz, 2019). This was largely due to the significant expansion of existing industry players (Northern Harvest Sea Farms / MOWI Canada East and Cooke Aquaculture Inc.), but also the arrival of Grieg NL Seafarms Ltd. in the province. While this points to a bright future for Atlantic salmon farming in Newfoundland, the salmon aquaculture industry also faces several significant challenges that currently limit production goals.

Global ocean surface temperatures are predicted to increase by 1.5°C above pre-industrial levels by 2052 (IPCC, 2018) and ocean heat waves are increasing in frequency and severity (Frölicher et al., 2018; He et al., 2023; Holbrook et al., 2019; Laufkötter et al., 2020; Oliver et al., 2018). Further, these increases in temperature are often associated with decreases in water oxygen levels (hypoxia) (Rubalcaba et al., 2020; Sampaio et al., 2021). For example, intermittent hypoxic events (i.e., water oxygen levels of 4-6 mg L^{-1} O_2) were reported during the summers of 2008 and 2009 in salmon cage-sites on the south coast of Newfoundland, with some of these events lasting up to two and a half months (Burt et al., 2012). Atlantic salmon in net pens in Tasmania in 2016 experienced surface water temperatures as high as 23°C combined with hypoxia, and this resulted in the salmon actively avoiding hypoxic regions (< 35% O_2 saturation) at the bottom of the cage and selecting water temperatures of ~ 20°C at 5 to 10 m depth (Stehfest

et al., 2017). This led to a considerable contraction of the depth profile used by the fish and severe crowding (Stehfest et al., 2017), and such conditions can have negative consequences on Atlantic salmon welfare and production (Reid et al., 2019). Moreover, recent events in Newfoundland showed that the Atlantic salmon aquaculture industry is particularly vulnerable to climate change-related events (Burke et al., 2020). Prolonged sea surface temperatures > 18°C, coinciding with reduced dissolved oxygen levels (to ~ 70%) and active sea lice (*Lepeophtheirus salmonis*) treatments, led to the mass mortality of 2.6 million Atlantic salmon on the south coast of Newfoundland in 2019 (Burke et al., 2020). These events highlight that the industry needs to take decisive action, and develop new strategies and solutions to mitigate the impacts of climate change on salmon production.

There are several mitigation strategies that may be advantageous for salmon production in the era of climate change, including selective breeding, the use of new genetic strains, changing farm management / fish husbandry practices (i.e., improving site selection and/or using deeper net pens) and altering the nutrition of the salmon to improve their tolerance of elevated temperatures and hypoxia. This thesis examined whether some of these methodologies are feasible options, and can help protect salmon aquaculture production from climate-related challenges.

1.2 Measuring thermal tolerance

There is a general consensus that the optimal rearing temperature for the production of North Atlantic strains of Atlantic salmon is 12-14°C, based on their growth performance and feed conversion efficiency (Handeland et al., 2008; Hevrøy et al., 2013). However, once temperatures exceed the fish's optimum temperature, growth performance decreases, and the

dysregulation of gene expression (including that involved in the stress response) begins (Beemelmanns et al., 2021c, 2021b; Bevelhimer and Bennett, 2000; Gamperl et al., 2020; Ignatz et al., 2022; Jobling, 1997; Shi et al., 2019).

The most common approach to measuring thermal tolerance is to assess the fish's critical thermal maximum (CT_{Max}) (Beitinger et al., 2000; Lutterschmidt and Hutchison, 1997). Studies using this approach expose the fish to quite rapid increases in temperature, which reach lethal limits within hours rather than days to weeks (e.g., Anttila et al., 2013; Atkins and Benfey, 2008; Elliott and Elliott, 1995). Acclimation temperature, oxygen saturation level, fish size and the rate of temperature increase have all been previously shown to directly affect the CT_{Max} of fish (Anttila et al., 2015; Messmer et al., 2017), and thus, these factors must be considered when designing a CT_{Max} experiment.

In the context of aquaculture production, differences in CT_{Max} between Atlantic salmon and cod (*Gadus morhua*) families have been reported (Anttila et al., 2013; Zanuzzo et al., 2019), and there is accumulating evidence that genetics plays a role in determining a fish's tolerance to acute temperature increases. For example, quantitative trait loci (QTL) are associated with CT_{Max} in rainbow trout (*Oncorhynchus mykiss*) and Arctic charr (*Salvelinus alpinus*) (Perry et al., 2001; Somorjai et al., 2003). Furthermore, maximum thermal tolerance was shown to be heritable in mosquitofish (*Gambusia holbrooki*) and killifish (*Heterandria formosa*) (Doyle et al., 2011; Meffe et al., 1995). However, fish in sea-cages are exposed to both acute and seasonal (i.e., gradual) changes in water temperature, and when using a regimen of temperature increase that more accurately reflects the natural progression of temperature increases at cod cage-sites [i.e., using an incremental thermal maximum (IT_{Max}) challenge], no differences were observed in the thermal tolerance (IT_{Max}) between Atlantic cod families (Zanuzzo et al., 2019). Additionally, no

correlation was found between the IT_{Max} and CT_{Max} of cod families (Zanuzzo et al., 2019). These results suggested that it might be difficult to select fish based on IT_{Max} values, and called into question the applicability of selecting broodstock based on CT_{Max} (Zanuzzo et al., 2019).

It has, therefore, been recommended that temperatures be increased at rates that a species would naturally encounter so that information/data on how elevated temperatures affect their biology/physiology are as relevant as possible (Vinagre et al., 2015; Zanuzzo et al., 2019). Based on historical data (Burt et al., 2012; Gamperl et al., 2021; Johansson et al., 2007, 2006), coastal water temperatures in the North Atlantic increase by approximately 1°C per week between late spring (May/June) and late summer (late August/early September). Thus, my thesis used a similar temperature regimen to examine the upper thermal tolerance of farmed salmon sourced from Atlantic Canada.

Temperature and oxygen saturation have an inverse relationship in water, whereby at elevated temperatures, oxygen saturation is reduced. This combined with a temperature-dependent increase in the fish's metabolism, can result in hypoxia in sea-cages during the warm summer months (Gamperl et al., 2021; Stehfest et al., 2017). However, in Newfoundland, salmon rarely experience decreases in oxygen lower than 70% saturation (Gamperl et al., 2021), and it has been shown in a tank-based experiment that moderate hypoxia (i.e., 60-70% O_2 saturation) does not negatively impact the survival of Atlantic salmon at high temperatures (Gamperl et al., 2020). Therefore, while the studies described in this thesis only tested survival of salmon at elevated temperatures under normoxic conditions, it is not expected that the main conclusions of my thesis would be different from those under 'typical' sea-cage conditions.

1.3 Differences between stress coping phenotypes

Individuals of some fish species can be identified as either low responders (LR) or high responders (HR) based on whether they exhibit a proactive or reactive stress coping phenotype, respectively (Andersson et al., 2013; Hori et al., 2012b; Koolhaas et al., 1999). It is also known that LR and HR fish exhibit differences in traits such as growth, condition factor and cardiosomatic index, and in the expression of specific genes of the glucocorticoid stress axis in response to stress (Hori et al., 2012b, 2012c; Johansen et al., 2011). According to the pace-of-life syndrome hypothesis, proactive (LR) responders (e.g., those with bold behaviour coupled with low post-stress cortisol levels) compensate for/offset their higher rates of mortality with increased growth rates and improved fecundity (Damsgård et al., 2019; Réale et al., 2010). Whereas reactive HR fish are generally characterized by decreased mobility, shyer behaviour and slower growth (Damsgård et al., 2019; Réale et al., 2010). These relationships, however, are not always consistent, and Pottinger & Carrick (1999) and Weber & Silverstein (2007) reported that HR rainbow trout grow faster than LR trout. Additionally, there is a paucity of knowledge, or conflicting information, with regard to the immune response and thermal tolerance of these two stress coping phenotypes. For example, while the survival of HR rainbow trout was lower than LR fish exposed to *Aeromonas salmonicida* (the causative agent of furunculosis), the opposite finding was reported in a challenge with *Vibrio anguillarum* which causes vibriosis (Fevolden et al., 1992). In Atlantic salmon, no difference in survival was found between stress coping styles during a *Renibacterium salmoninarum* (causative agent of bacterial kidney disease) challenge (Fevolden et al., 1993). However, HR salmon exhibited higher mortality when exposed to *A. salmonicida* and *Vibrio salmonicida* than LR fish (Fevolden et al., 1993). The results of Fevolden et al. (1993) require further validation though, as in both the *R. salmoninarum* and *V.*

salmonicida challenges, fish showed signs of previous infection with *A. salmonicida*. In terms of thermal tolerance, no difference in CT_{Max} was reported between HR and LR rainbow trout (LeBlanc et al., 2012). Yet, during a heat shock challenge where trout were kept at 25°C for 1 h, HR fish exhibited a more rapid, and greater, heat stress response (as assessed by the expression of HSP70 and other stress proteins) than LR trout, and experienced less protein oxidation (LeBlanc et al., 2012). Finally, while LR fish are more likely to actively avoid hypoxic conditions and seek out normoxic environments (Damsgård et al., 2019; Laursen et al., 2011), Hvas et al. (2017) and Gamperl et al. (2020) suggested that such an avoidance behaviour may contribute to the decreased survival of Atlantic salmon at high temperatures.

In this thesis, stress phenotype is characterized based solely on post-stress cortisol levels. Thus, it is not meant to be conflated with behavioural syndromes in fish. Personality in fish can be defined as individual differences in behaviour that are consistent across time and changes in environment (Réale et al., 2007). Behavioural syndromes can be subdivided into five categories: 1) shyness/boldness; 2) exploration/avoidance; 3) activity; 4) aggressiveness; and 5) sociability (Conrad et al., 2011). As noted above and in numerous other studies (e.g., Höglund et al., 2020; Øverli et al., 2002, 2004), several behavioural characteristics are associated with different stress coping styles in fish. However, it is acknowledged that stress coping behavioural characteristics are not always correlated with stress responsiveness (Basic et al., 2012; Höglund et al., 2020).

1.4 Impact of temperature on fish immune responses and disease resistance

Rearing temperature is known to directly impact the disease resistance and immune responses of teleost fish. For example, recent studies showed that sea lice infestation worsens at elevated temperatures, and that this increases the risk of mortality in Atlantic salmon (Dalvin et

al., 2020; Godwin et al., 2020; Medcalf et al., 2021; Sandvik et al., 2021). This is particularly problematic as farmers in New Brunswick have reported higher water temperatures year-round (by as much as 2°C above historical records), with elevated temperatures maintained for longer periods and lasting until later in the year (ACFFA, 2020). Historically, sea lice levels are generally low from January through to June. However, warmer winter temperatures allow the parasite to survive and infect salmon during these periods (ACFFA, 2020). In contrast, while juvenile rainbow trout exposed to infectious pancreatic necrosis virus (IPNV) at 10°C exhibited a rapid increase in mortality that approached 100%, the initial spike in mortality quickly subsided in fish held at 16°C and mortality in this group did not surpass 40% (Dorson and Torchy, 1981). Jørgensen (1982) showed that the persistence of viral hemorrhagic septicemia virus (VHSV) was inversely proportional to rearing temperature in juvenile rainbow trout, with undetectable viral levels reported at 8 days post-injection (dpi) at 15°C and 20°C, whereas it took 35 dpi for this to occur at 10°C. Somewhat similarly, while the onset of mortality was more acute at 20°C in juvenile salmon exposed to infectious salmon anaemia virus (ISAV), overall mortality was lower in salmon held at 20°C vs. 10°C (Groves et al., 2023). Finally, elevated temperature (16°C compared with 10°C) accelerated the spleen antiviral transcript expression response in Atlantic cod to the synthetic analogue of viral dsRNA, polyriboinosinic polyribocytidylic acid (pIC) (Hori et al., 2012a). In contrast, microarray analysis showed that a much smaller number of genes were differentially expressed at 16°C vs. 10°C when Atlantic cod were intraperitoneally (IP) injected with formalin-killed *A. salmonicida* (Hori et al., 2013). Collectively, these data highlight that temperature's effect on the immune response may be pathogen-dependent, with a larger effect seen in cod stimulated with a viral analogue compared with bacterial antigens (Hori et al., 2013, 2012a). Finally, in post-smolt Atlantic salmon exposed to an incremental thermal

increase up to 20°C, elevated temperature upregulated the constitutive transcript expression of several antibacterial genes and hastened the peak in antibacterial transcript expression by 6-12 h compared to controls at 12°C after vaccine injection (Zanuzzo et al., 2020). Although it did not affect the overall magnitude of immune transcript expression as compared to control fish (Zanuzzo et al., 2020).

However, the effect of temperature on the fish's immune response and disease resistance also appears to be host species-specific. While mortality following furunculosis infection was found to decline progressively with increasing temperature in juvenile coho salmon (*Oncorhynchus kisutch*) and steelhead rainbow trout, this effect was not reported in juvenile sockeye salmon (*Oncorhynchus nerka*) where mortality was almost 100% from 6.7°C to 20.5°C (Sanders et al., 1978). As outlined by Makrinos and Bowden (2016), though, many studies report contrasting or contradictory results on temperature's influence on fish immune systems. There is also the concept of behavioural fever, whereby a fish moves to an area of warmer water to combat disease more effectively (Haddad et al., 2023; Rey et al., 2015; Reynolds et al., 1976). Thus, compared to static environmental conditions in a land-based tank experiment, an Atlantic salmon may be able to behaviourally select warmer waters when provided with access to a temperature gradient in a sea-cage. This will / may enhance its immune function, but also potentially the replication and virulence of the pathogen (e.g., Ishiguro et al., 1981; Sandvik et al., 2021; Tu et al., 1975), and thus, it is difficult to predict whether behavioural fever will improve the fish's resistance to a particular pathogen. Ultimately, the relationship between temperature and the development of disease in fish relies on a complex series of factors.

1.5 Diets tailored for high temperatures

Designing diets specifically for Atlantic salmon reared at high temperatures is vital to the aquaculture industry's growth and sustainability as the threats associated with global climate change worsen. For example, research shows that elevated temperatures affect the nutrient requirements and/or metabolism of phosphorus (Fraser et al., 2019), polyunsaturated fatty acids (PUFAs) (Huguet et al., 2015) and astaxanthin (Grünenwald et al., 2019) in Atlantic salmon. A high temperature diet formulated by Skretting is commercially available in Tasmania and other parts of the world, but no peer-reviewed literature is available on its efficacy (Ruff, 2015) and this diet has not been approved for use in Canada by the Canadian Food Inspection Agency (CFIA). Providing additional dietary cholesterol is one potential way to improve fish upper thermal tolerance, as this strategy can prolong fish survival at high temperatures (Irvine et al., 1957), and cholesterol is important for maintaining cell membrane structure and fluidity (Crockett, 1998). These membrane features are directly impacted by environmental temperature, and increasing temperatures can weaken membrane structure and ultimately result in cell death (Farkas et al., 2001; Fodor et al., 1995; Hazel, 1979; Liu et al., 2019). In addition, there are some studies that show positive effects of increased cholesterol levels. For example, dietary cholesterol supplementation has been shown to improve the rainbow trout's immune response and disease resistance to the bacterial pathogen *Aeromonas hydrophila* (Deng et al., 2013a). Changes in the sterol:phospholipid ratio have also been reported to be a key response to temperature in liver membranes of steelhead trout (Wijekoon et al., 2021). As cardiac performance/function is a key determinant of fish thermal tolerance (Farrell, 2002; Farrell et al., 2009), it is relevant that enriching the cholesterol content of bovine cardiac microsomes improves the conformational stability of proteins and increases their resistance to inactivation at elevated temperature (Ortega

et al., 1996). Lastly, Atlantic salmon aquaculture feeds are formulated with increasing amounts of plant materials that contain significant levels of plant sterols (phytosterols) and lower quantities of cholesterol (Sissener et al., 2017). Dietary phytosterols can negatively impact absorption of cholesterol (Calpe-Berdiel et al., 2009), thus making this essential nutrient less bioavailable which is why supplemental cholesterol can be used to offset this in salmonids (Deng et al., 2013b). Collectively, these results suggested that supplemental dietary cholesterol would benefit Atlantic salmon reared at high temperatures.

1.6 Assessing the vulnerability of triploid salmonids to rising ocean temperatures

Salmonid genomes are pseudotetraploid, as these fish experienced an incomplete restoration of diploidy following the whole genome duplication (WGD) event that occurred ~80 million years ago (Lien et al., 2016). However, to improve the readability of this thesis, the conventional Atlantic salmon genome will be referred to as diploid, and where the second polar body was retained, triploid. Triploid salmon may benefit the aquaculture industry, as they offer an effective, albeit not 100% guaranteed, option for reproductive sterility and genetic containment (Benfey, 2016). Currently, duplicating the female parent's genome via pressure shock is the simplest and most common method for inducing triploidy (Benfey, 2016). Growth performance between ploidies varies in salmonids, but there is a general trend that diploids grow faster during early development, while triploids catch up or surpass diploids in weight gain at harvest as they avoid the diversion of energy toward sexual development (Chiasson et al., 2009; Nuez-Ortín et al., 2017; Weber et al., 2014). However, it has also been suggested that triploid salmonids have a lower thermal optima than diploids (Atkins and Benfey, 2008), and may have a lower tolerance to high temperatures and hypoxia (Hansen et al., 2015; Ojolick et al., 1995;

Sambraus et al., 2018, 2017b; Verhille et al., 2013); although these findings are not always consistent (Benfey et al., 1997; Benfey and Devlin, 2018; Bowden et al., 2018; Ellis et al., 2013). Finally, while rising ocean temperatures can present major challenges to the salmon aquaculture industry, the impacts are not always straightforward. For example, while increasing sea surface temperatures are making it difficult to sustainably produce triploid Atlantic salmon in some regions of Tasmania, more southern locations are benefiting from longer times spent at optimal temperatures for growth (Meng et al., 2022).

Triploid salmonids are also more prone to skeletal deformities and the development of cataracts (Fraser et al., 2020; Jagiełło et al., 2021; Sambraus et al., 2017a). Therefore, it has been proposed that diets should be tailored for triploid Atlantic salmon to help offset these unfavorable abnormalities as they have several different nutrient requirements compared to diploids (Burke et al., 2010; Fjelldal et al., 2016; Ganga et al., 2015; Sambraus et al., 2020, 2017a; Smedley et al., 2016; Taylor et al., 2015; Tibbetts et al., 2013; Vera et al., 2019). While the precise nutritional requirements of triploids have yet to be determined (Benfey, 2011), the information collected in this thesis with regard to dietary cholesterol manipulation may provide valuable information. This is especially relevant as reduced survival, higher levels of emaciation, and lower quality processing scores have been reported in Norway (Madaro et al., 2021), and this raises welfare concerns with regard to the commercial production of triploid Atlantic salmon. Correspondingly, the Norwegian Food Safety Authority has halted the rearing of triploids in the country, requiring that all current stock be harvested before the end of 2023 (Berge, 2021). Therefore, it will be crucial for the Newfoundland industry to better optimize production of triploid Atlantic salmon to avoid a similar outcome.

1.7 Using genomics to study temperature-mediated impacts

Genomics tools and resources can help elucidate the mechanisms behind the impacts of rearing temperature on fish. For example, microarray and multiplex polymerase chain reaction (PCR) data have been used to identify candidate thermal stress biomarkers of Pacific salmon species (Akbarzadeh et al., 2018; Houde et al., 2019; Jeffries et al., 2014, 2012). Suppression subtractive hybridization (SSH) cDNA libraries have been used to identify dysregulated transcripts after heat shock in Atlantic cod (Hori et al., 2010). RNA-sequencing (RNA-seq) has been utilized to enhance our understanding of the biological pathways that are affected by heat stress in rainbow trout (Li et al., 2017) and Atlantic salmon (Ignatz et al., 2022; Olsvik et al., 2013). Furthermore, epigenetic tools and techniques such as microRNA-sequencing (miRNA-seq) and bisulphite sequencing can reveal how non-coding RNAs and DNA methylation are involved in regulating responses to elevated temperatures and/or hypoxia (Bao et al., 2018; Beemelmanns et al., 2021a; Huang et al., 2017; Lai et al., 2016; Zhang et al., 2016). The availability of an assembled Atlantic salmon genome (Lien et al., 2016) is also extremely valuable when assessing features like gene copy number, genome location and the gene structure of specific biomarkers. The above studies have laid the groundwork for using targeted real-time quantitative PCR (qPCR) to analyze specific transcripts of interest that provide pertinent insights into how salmon respond to, and manage, stress at high temperatures.

Genomic and bioinformatics tools can also aid in the structural and functional characterization of genetic biomarkers of interest. Sequencing and transcript expression analyses have been useful in revealing novel information on immune- (e.g., Crossman et al., 2023; Eslamloo et al., 2019; Inkpen et al., 2019) and environmental stress-related (e.g., Bermejo-Nogales et al., 2014; Pan et al., 2004; Roh and Kim, 2022) genes in fish. For example,

characterizing one of most recognized heat stress biomarkers in salmonids, *serpinh1* [alias heat shock protein 47 (*hsp47*)], could improve our understanding of what specific role this gene plays in mitigating the harmful impacts of elevated temperature in Atlantic salmon. This gene/protein is consistently reported to be upregulated at high temperatures in the literature (e.g., Beemelmanns et al., 2021c, 2021b; Ignatz et al., 2022; Jeffries et al., 2014, 2012; Nuez-Ortín et al., 2018; Tomalty et al., 2015). However, little was known regarding its copy number (i.e., the number of *serpinh1* paralogues in the Atlantic salmon genome), or whether the expression of its various paralogues was influenced by sex, differed across tissues or following exposure to environmental stressors. As a pseudotetraploid species, Atlantic salmon can possess four paralogues of a gene if they were retained following the teleost- and salmonid-specific WGDs that occurred ~320 and ~80 million years ago, respectively (Gundappa et al., 2022; Lien et al., 2016). Therefore, gene characterization and transcript expression analyses in this species are not only important for its potential application in aquaculture and fish conservation and management, but are valuable with regard to elucidating the evolutionary fate of genes following multiple WGD events.

1.8 Research objectives

My Ph.D. research used a multi-faceted approach to study the influence of stress coping phenotype, nutrition and family/genetic background on, and the role played by the expression of specific genes in, mediating salmon physiology and survival at high water temperatures. By using functional genomic tools like real-time quantitative polymerase chain reaction (qPCR), alongside measurements of stress biomarkers using enzyme-linked immunosorbent assays (ELISAs), I assessed the role of the above factors in determining how the salmon's performance

(i.e., growth, feed conversion efficiency, stress/immune responses and thermal tolerance) was impacted by the elevated temperatures that salmon are experiencing (or will likely experience in the future) at sea-cage sites. It was originally hypothesized that selection based on stress phenotype and genetic background, as well as providing supplemental dietary cholesterol would all assist in improving the upper thermal tolerance of farmed Atlantic salmon. This research provides key fundamental information that will contribute greatly to our understanding of how this economically important fish species responds to long-term elevations in temperature, and the salmon aquaculture industry with knowledge on which strategies might be effective for mitigating the negative effects of high temperatures and climate change on sea-cage production and survival.

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CHAPTER 2: Phenotypic stress response does not influence the upper thermal tolerance of male Atlantic salmon (*Salmo salar*)

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2.1 Abstract

Fish can be identified as either low responders (LR) or high responders (HR) based on post-stress cortisol levels and whether they exhibit a proactive or reactive stress coping style, respectively. In this study, male Atlantic salmon (*Salmo salar*) from 17 families reared at 9°C were repeatedly exposed to an acute handling stress over a period of four months, with plasma cortisol levels measured at 1 h post-stress. Fish were identified as either LR or HR if the total Z-score calculated from their cortisol responses fell into the lower or upper quartile ranges, respectively; with intermediate responders (IR) classified as the remainder. Salmon characterized as LR, IR or HR were then subjected to an incremental thermal challenge, where temperature was raised at 0.2°C day^{-1} from their acclimation temperature (12°C) to mimic natural sea-cage farming conditions during the summer in Newfoundland. Interestingly, feed intake remained high up to 22°C, while previous studies have shown a decrease in salmon appetite after ~16–18°C. After the first three mortalities were recorded at elevated temperature, a subset of LR and HR salmon were exposed to another acute handling stress event at 23.6°C. Basal and post-stress measurements of plasma cortisol, glucose and lactate did not differ between stress response phenotypes at this temperature. In the end, the average incremental thermal maximum (IT_{Max}) of LR and HR fish was not different (25.1°C). In comparison, the critical thermal maximum (CT_{Max}; temperature increased at 2°C h^{-1}) of the remaining IR fish that had been held at 12°C was 28.5°C. Collectively, these results: 1) show that this population of Atlantic salmon is very thermally tolerant, and further question the relevance of CT_{Max} in assessing responses to real-world temperature changes; and 2) indicate that characterization of stress phenotype at 9°C is not predictive of their stress response or survival at high temperatures. Therefore, selection of fish based on phenotypic stress response at low temperatures may not be beneficial to incorporate

into Atlantic salmon breeding programs, especially if the goal is to improve growth performance and survival at high temperatures in sea-cages.

2.2 Introduction

Global mean ocean temperatures are predicted to increase by 1-3°C above pre-industrial levels by 2052 (IPCC, 2019), ocean heat waves and hypoxic episodes are increasing in frequency and severity (Frölicher et al., 2018; Holbrook et al., 2019; Oliver et al., 2018; Rubalcaba et al., 2020; Sampaio et al., 2021), and such conditions can have negative consequences on fish welfare and production (Reid et al., 2019). For example, the optimal temperature for Atlantic salmon aquaculture production is 12-14°C (Handeland et al., 2008; Hevrøy et al., 2013), and once temperature exceeds 16-18°C growth performance generally decreases and temperature-induced changes in gene (transcript) expression (including the upregulation of genes involved in the stress response) begins (Beemelmanns et al, 2021a, 2021b; Bevelhimer and Bennett, 2000; Gamperl et al., 2020; Jobling, 1997; Shi et al., 2019). Atlantic salmon (*Salmo salar*) in net pens in Tasmania in 2016 experienced temperatures as high as 20°C, and a significant contraction in their vertical distribution (crowding), as surface water temperatures were as high as 23°C and water oxygen levels were inversely related to depth (<35% O_2 saturation at the bottom of the cage) (Stehfest et al., 2017). Wade et al. (2019) reported that summer heat waves not only reduce salmon feed intake and growth, but that these events also led to fillet bleaching (i.e., a decrease fillet pigmentation), and thus, the quality and value of the fish at harvest. Finally, recent events in Newfoundland showed that the Atlantic Canada salmon aquaculture industry is vulnerable to climate change-related events (Burke et al., 2020). Prolonged sea surface temperatures >18°C, coinciding with sea lice (*Lepeophtheirus salmonis*) treatments and moderately reduced dissolved oxygen levels (hypoxia), led to the mass mortality of 2.6 million fish on the south coast of Newfoundland in 2019 (Burke et al., 2020).

These events highlight that the salmon aquaculture industry needs to be proactive with regard to developing new strategies and finding solutions to mitigate the potentially negative impacts of rising seawater temperatures and hypoxia (i.e., climate change) on production and fish welfare. There are several mitigation strategies that may be advantageous for salmon production in the era of accelerating climate change, such as selective breeding, the use of new genetic strains, changing farm management/fish husbandry practices (i.e., improving site selection and/or using deeper net pens) and developing/using specific diets to improve their tolerance of elevated temperatures and hypoxia. For example, Skretting (Stavanger, Norway) has developed an Optiline HT (high temperature) diet for use at salmon sea-cages in Tasmania (Ruff, 2015).

It has been shown in a few species of fish [e.g., Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*)] that individuals can be identified as either low responders (LR) or high responders (HR) based on whether they exhibit a proactive or reactive stress coping phenotype, respectively (Andersson et al., 2013; Damsgård et al., 2019; Hori et al., 2012a; Koolhaas et al., 1999). LR and HR fish exhibit differences in traits such as growth, condition factor and cardiosomatic index, and in the expression of specific genes of the hypothalamic-pituitary-interrenal (HPI) axis in response to handling stress (Hori et al., 2012a, 2012b; Johansen et al., 2011). Further, while LR salmonids are more likely to actively avoid hypoxic conditions and seek out normoxic environments (Damsgård et al., 2019; Laursen et al., 2011), Hvas et al. (2017) and Gamperl et al. (2020) suggested that such an avoidance behaviour may contribute to the death (i.e., decreased survival) of Atlantic salmon at high temperatures.

Given the limited amount of information on the thermal biology of these two stress coping phenotypes, and the potential benefit(s) of using fish of one of the phenotypes at sites where summer conditions are not always favourable, the objective of this study was to determine

if Atlantic salmon characterized as LR or HR based on their cortisol stress response have different temperature-dependent production characteristics (e.g., growth rate, condition factor) and upper thermal tolerance. The most common approach to measuring thermal tolerance is to assess the fish's acute thermal tolerance (critical thermal maximum, CT_{Max}; Beitinger et al., 2000; Lutterschmidt and Hutchinson, 1997). However, this methodology is designed/generally used to assess the temperature limits of specific physiological functions/systems while avoiding any acclimation or 'heat-hardening', and exposes the fish to rapid (as much as 18°C h^{-1}) increases in temperature, and thus, the fish reaches its CT_{Max} within a matter of hours (e.g., see Anttila et al., 2015, 2013; Corey et al., 2017). However, salmon in sea-cages in the North Atlantic are largely exposed to seasonal (i.e., gradual) changes in water temperature, where coastal water temperatures increase by approximately ~1°C per week between late spring (May/June) and late summer (late August/early September) (e.g., see Burt et al., 2012; Johansson et al., 2007, 2006). Thus, I utilized an incremental temperature (IT_{Max}) test in this study (where temperature was increased at ~0.2°C day^{-1}) as first published by Zanuzzo et al. (2019) for Atlantic cod, and recently used by Gamperl et al. (2020) for Atlantic salmon. This protocol results in considerably lower, and more industrially meaningful/relevant estimates of temperature tolerance than does the CT_{Max} test (Zanuzzo et al., 2019). I anticipate that the results of this study will help inform the aquaculture community of how their fish may respond to future climate change-driven shifts in the ocean environment.

2.3 Materials and methods

This study was approved by the Animal Care Committee of Memorial University (protocol #20-01-KG), and salmon husbandry and experimental procedures were performed in

accordance with the Canadian Council on Animal Care Guidelines on the ‘Care and Use of Fish in Research, Teaching and Testing’ (Canadian Council on Animal Care, 2005).

2.3.1 Experimental animals

Previously PIT (passive integrated transponder)- tagged conventional male, diploid Atlantic salmon of 17 families of St. John River origin were obtained from AquaBounty Canada (Prince Edward Island, Canada), and transported to the Dr. Joe Brown Aquatic Research Building (JBARB; Ocean Sciences Centre, Memorial University). The fish were smolted, and initially held in two 3.0 m^3 tanks in a flow-through seawater system at ~15 kg m^{-3} with temperature and oxygen levels maintained at 9.9 ± 0.3°C and >95% air saturation, respectively. Photoperiod was 12 h light: 12 h dark, and the fish were fed a commercial diet (EWOS Adapt Smolt 3.0 mm – minimum 46% crude protein, 26% crude fat; EWOS Canada Ltd, BC, Canada) at a rate of 1.0% body weight day^{-1} using belt feeders. The salmon were allowed 24 days to acclimate to seawater before they were sorted into 0.5 m^3 tanks for experimentation. See Appendix A - Table A-1 for family cross structure. The main goal of AquaBounty’s current breeding program is to select for enhanced growth, and at no time previous to this work were these families tested/selected for thermal tolerance traits.

2.3.2 Experiment #1: determination of stress coping phenotype

2.3.2.1 Experimental design

After acclimation, the salmon were lightly anesthetized using tricaine methanesulfonate (0.1 g L^{-1} AquaLife TMS; Syndel Laboratories Ltd, Nanaimo, BC, Canada), and weighed and measured for fork length, before being distributed equally (36 fish $tank^{-1}$) among eleven 0.5 m^3

tanks. Initial stocking density of each tank was ~12 kg m^{-3}. Only fish initially weighing between 100 and 290 g were used in the study to reduce the effects of size variation; visibly sexually precocious males were also omitted from the experiment. Photoperiod was maintained at 12 h light: 12 h dark, with lux values all at ~100 at the surface of each tank. Tank flows were set at 10 L min^{-1}, with water temperature maintained at 9.0 ± 0.6°C and oxygen at >100% of air saturation. Fish continued to be fed 1.0% body weight day^{-1} for the first two months of the experiment before feeding was transitioned to 1.0% body weight 4× weekly. EWOS Dynamic S 5.0 mm (minimum 46% crude protein, 27% crude fat) was used throughout the experiment, with feed size increasing from 3.0 to 5.0 mm as the fish grew. Vibrational feeders were activated every 2 h for 0.5 s throughout the day to ensure pellets were provided over the course of 12 h. However, the fish were taken off feed 24 h in advance of handling/sampling.

More than 2 weeks were allowed after transfer before sampling was performed to determine basal cortisol concentrations. For this measurement, two fish $tank^{-1}$ were anesthetized (in 0.2 g L^{-1} TMS) and blood (300 μL) was sampled via the caudal vein using 1 mL syringes with 23 gauge 1" needles, and placed into heparinized (400 units mL^{-1}) Eppendorf® tubes on ice. Only samples collected in <3 min were used in subsequent analyses. Within 5 min after collection, the tubes were centrifuged at 1100×g for 1 min at room temperature. Afterwards, the plasma was pipetted in duplicate into new tubes, which were immediately flash-frozen in liquid nitrogen.

One week after the initial sampling, fish were exposed to a standardized stressor to assess post-stress cortisol levels (Hori et al., 2012a) (see Figure 2-1). Approximately 6 fish from an individual tank were netted at a time, and held in air for 1 min. The salmon were then placed into a different 0.5 m^3 tank on the same water supply as their original tank. Groups of ~6 fish were

subsequently exposed to the same stressor, each group 10 min after the other, and moved into their own separate new tank. At 1 h post-stress, each group of fish was quickly anesthetized (in 0.2 g L^{-1} TMS), and blood (300 μL) was collected and plasma obtained as above. This procedure was repeated a total of 4 times for each tank with a month's separation between samplings. Two to four tanks were sampled each day with the entire process lasting three to four days. At each monthly sampling, the tank sampling order was changed. This ensured that tanks located directly next to one another were not sampled on the same day to reduce the potential impact of the presence of the sampling team. One h post-stress was chosen as the sampling point to be consistent with previous research (Hori et al., 2012a), and a recent study from our group (Zanuzzo et al., unpublished) which showed that post-stress cortisol levels peaked at this time point in Atlantic salmon held at 12°C. Water temperature in the holding tanks ranged between 9.3 and 10.2°C across the four months of sampling. At the last sampling, the weights and lengths of all fish were also recorded. After the second month's sampling, some fish were experiencing issues with caudal fin erosion and exophthalmia. Any fish with health concerns were quickly removed from the study and subsequent analyses, and all remaining fish were given a Parasite S (Syndel Canada, Nanaimo, BC, Canada) treatment (250 ppm for 45 min). No further health issues were encountered. In the final month of the experiment, to re-establish desired tank densities (~36 fish $tank^{-1}$), the fish were re-distributed amongst 9 of the original 11 tanks. Tank densities at this stage were ~18 kg m^{-3}.

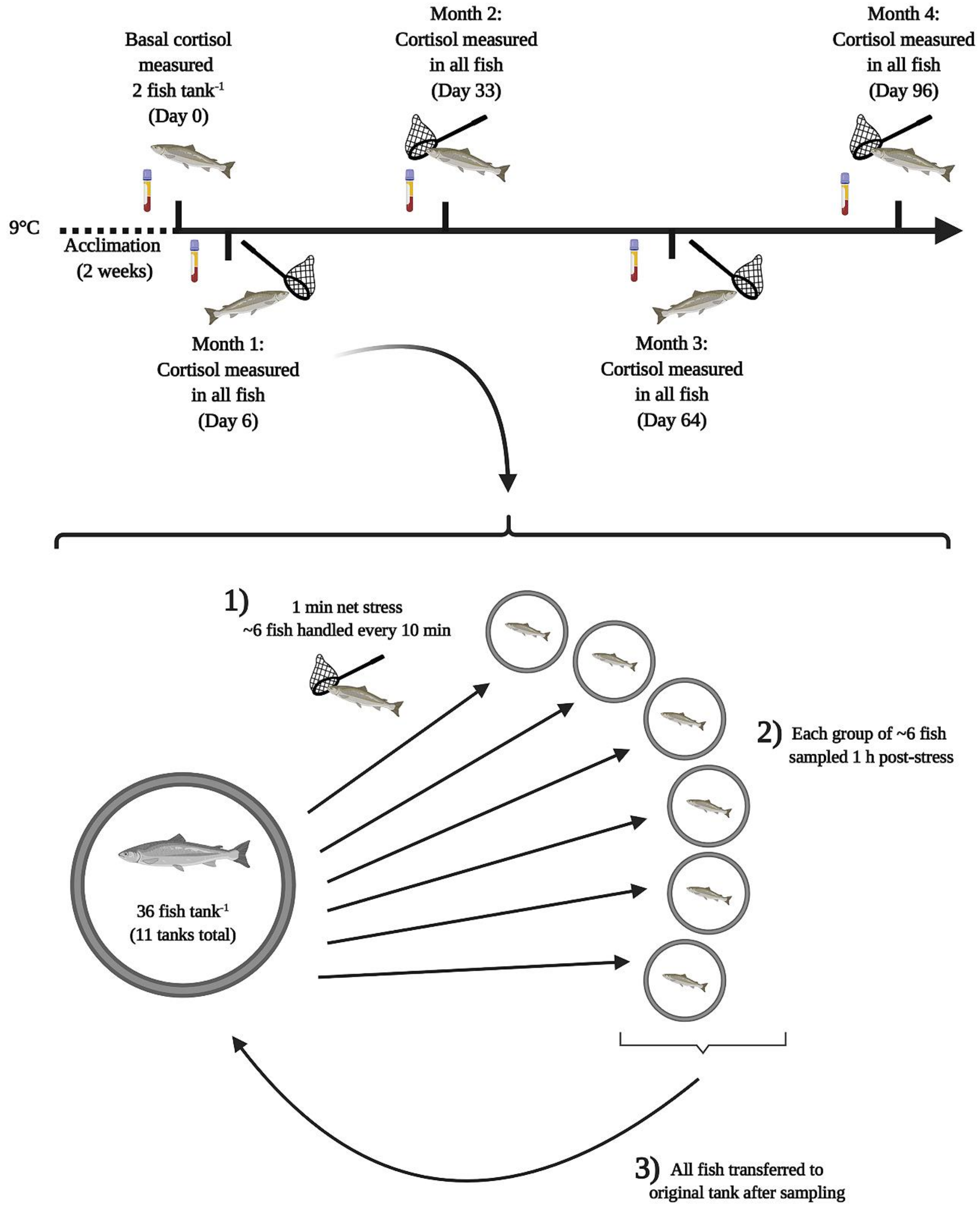

Figure 2-1. Overview of the protocol used in Experiment #1 to characterize male Atlantic salmon based on stress coping phenotype (i.e., LR. vs. IR vs. HR). Created with BioRender.com.

2.3.2.2 Plasma cortisol analyses

Plasma cortisol was measured at 650 nm using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY, USA) following the manufacturer's instructions and a Molecular Devices (San Jose, CA, USA) SpectraMax® M5 microplate reader. Samples were analyzed in duplicate at dilutions of 1:50 and 1:200 at the resting and post-stress time points, respectively. An 8-point standard curve was included in duplicate on each 96-well plate. Performing a 4-parameter logistic fit on the standard curve gave r^2 values of >0.983 for each plate (>85% of standard curves gave $r^2 \geq 0.995$). Samples with coefficients of variance >15% and with a >15 ng mL^{-1} difference in cortisol level between replicates were repeated. Samples were analyzed within a month post-collection. It is important to note that the commercial antibody used in this ELISA can cross-react with other steroids (i.e., cortisone, 11-deoxycortisol and corticosterone), and thus, absolute cortisol concentrations cannot be inferred (Sadoul and Geffroy, 2019). Therefore, all comparisons using the results of this ELISA are relative to one another.

2.3.2.3 Measurement of growth parameters

Weight gain was measured between the initial sampling and that conducted at the end of the experiment. Thermal growth coefficient (TGC) was used to assess growth rate using the following equation (Cho, 1992; Iwama and Tautz, 1981):

$$TGC = \left(\frac{W_f^{1/3} - W_i^{1/3}}{\sum_{i=1}^{n} T_i}\right) \times 1000$$

where W_f and W_i are the final and initial fish body weights (in g), respectively, n is the number of days since W_i, and T_i is mean daily water temperature (in °C).

Specific growth rate (SGR) was calculated using:

$$SGR = \left(\frac{\ln(W_f) - \ln(W_i)}{n}\right) \times 100$$

Fulton's condition factor (K) was calculated as:

$$K = \frac{\text{Fish weight (g)}}{(\text{Fish fork length [cm]})^3} \times 100$$

2.3.2.4 Statistical analyses

Similar to Hori et al. (2012a), a Z_t (total Z-score) was calculated for each fish and used to characterize their stress coping phenotype. For each month's cortisol results, values for a given fish ($Cortisol_{ind}$) were standardized against that month's average cortisol level amongst all fish ($Cortisol_{pop}$) sampled and the overall standard deviation (SD_{pop}) (Weil et al., 2001), and a fish's Z-score was calculated as:

$$Z = \frac{(Cortisol_{ind} - Cortisol_{pop})}{SD_{pop}}$$

Therefore, each fish ended the experiment with 4 individual Z-scores, and these were added together to provide a value for Z_t. Salmon with a Z_t that fell into the first quartile (i.e., lowest total Z-score) were characterized as low cortisol responders (LR), while fish belonging to the fourth quartile (i.e., with the highest total Z-scores) were characterized as high cortisol responders (HR). The remaining fish were all characterized as intermediate cortisol responders (IR). One-way ANOVAs followed by Tukey's HSD post-hoc tests performed using R Studio (R Studio Team, 2015; v.3.6.3) were used to examine differences in parameters between the phenotypes and families. Statistical differences were considered significant at $p < 0.05$.

2.3.3 Experiment #2: assessment of thermal tolerance

2.3.3.1 Experimental design

One month after the fourth month's sampling, and after all fish were characterized based on their Z_t score, the fish were moved to the Laboratory for Atlantic Salmon and Climate Change Research (LASCCR; Ocean Sciences Centre, Memorial University). The salmon were initially held at 10°C, but temperature was slowly increased to 12°C over the course of a week (+0.3°C day^{-1}). Tank flow rates were set to 20 L min^{-1} and photoperiod was maintained on a 14 h light: 10 h dark schedule. Another Parasite S treatment (250 ppm for 45 min) was performed on all fish as a preventative measure before the start of the second experiment. Once fish reached 12°C, the fish were anesthetized (in 0.2 g L^{-1} TMS), weighed and measured for fork length, and the LR and HR fish had colour-coded flag tags (Floy Tag & Manufacturing, Inc., Seattle, WA, USA) inserted into their dorsal muscle to distinguish them. Then, thirteen fish of each stress responder group (i.e., LR, IR and HR) were distributed into each of six 2.2 m^3 tanks, with the exception of the last tank which had 11 LR, 11 HR and 17 IR fish. Initial stocking density was ~5.3 kg m^{-3}. During the first two days of each week, the fish were fed once to satiation in the morning, and then fed 150% of that average value each remaining day of that particular week. On these days, fish were hand fed ~30% of their ration in the AM, and then fed the remaining amount throughout the day using vibrational feeders set to shake for 0.5 s every 20 min. These feeding methods closely followed those performed during Experiment #2 in Gamperl et al. (2020). At this stage of the study, the fish were only fed EWOS 5.0 mm feed. Feed intake was measured daily by dividing the feed provided to each tank by the number of fish in the tank. Feed conversion ratios (FCR) for each tank were then calculated as:

$$\mathrm{FCR} = \frac{\text{Feed intake (g)}}{\text{Fish weight gain (g)}}$$

Figure 2-2 provides an overview of the experimental design. After 2 weeks of acclimation to their new tanks, temperature was increased by 0.2°C day^{-1} in the 4 tanks designated as the 'warm' treatment, while temperature was maintained at 12.0 ± 0.5°C in the remaining 2 'control' tanks. Water temperatures were controlled via large plate heat-exchangers that delivered heated seawater to separate degassers and header tanks for each treatment. Oxygen diffusers were also placed in the 'warm' tanks when they exceeded 20°C to ensure O_2 levels were maintained at >100% air saturation throughout the experiment. Seawater temperatures and dissolved oxygen levels in all tanks were monitored at least once a day manually (YSI, ProODO, OH, USA), and 2 tanks in each group were continuously monitored for these parameters using a YSI 5500D MultiDO Optical Monitoring and Control Instrument.

When fish in the warm treatment reached 20°C, salmon in all tanks (including control) were anesthetized (in 0.2 g L^{-1} TMS), and their weight and fork length were measured. Additional samples were collected at this time point for further analyses conducted through the Mitigating the Impact of Climate-Related Challenges on Salmon Aquaculture (MICCSA) project that will not be described in the current paper. However, the liver and ventricle weights of these fish were measured, and each fish's hepatosomatic index (HSI) and relative ventricular mass (RVM) were calculated using the following equations:

$$\mathrm{HSI} = \left(\frac{\text{Liver weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

$$\mathrm{RVM} = \left(\frac{\text{Ventricle weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

The temperature increase in the experimental tanks was then continued at 0.2°C day^{-1} to determine the IT_{Max} of all remaining fish. In addition, after the first three mortalities were recorded, a subset of LR and HR salmon was exposed to another net stressor (at 23.6°C). The

methodology followed the original design (i.e., as in Figure 2-1) closely, however, only 4 fish (2 LR, 2 HR) were removed from each tank (n = 8 per group in total). These fish were quickly netted and exposed to air for 15 s, anesthetized (in 0.2 g L^{-1} TMS), had 300 μL of blood sampled from their caudal vessels, and were then placed into 0.5 m^3 tanks at 23.6°C. After waiting 1 h, these fish were quickly euthanized (in 0.4 g L^{-1} TMS) and 1 mL of blood was collected for the measurement of post-stress blood parameters. These blood samples were collected in <2 and 3 min, respectively. The initial 300 μL of blood collected were centrifuged at 1100×g for 1 min at room temperature, and triplicate samples of plasma were collected, and then flash-frozen in liquid nitrogen for later analyses of plasma cortisol, glucose and lactate. From the 1 mL of blood sampled at 1 h post-stress, the same process was repeated for collecting plasma. In addition, 50 μL was put into duplicate haematocrit tubes, and 300 μL of blood were placed into duplicate microcentrifuge tubes and quickly flash-frozen for later measurement of haemoglobin levels. All fish that were not sampled continued in the experiment until their IT_{Max} was reached. When each fish became moribund (i.e., lost equilibrium/succumbed, their weight, fork length, liver weight, ventricle weight, and state of sexual maturity were recorded alongside the time and temperature at which they were removed from the experiment.

Lastly, twenty remaining IR salmon from the control treatment were used in a final experiment to assess their acute thermal tolerance. The fish were placed in 0.5 m^3 tanks (5 fish $tank^{-1}$, 4 tanks total) and held at 12°C for 36 h prior to experimentation. On the day of the trial, temperature was raised steadily from 12°C at ~2°C h^{-1}, with temperature and oxygen readings recorded in all 4 tanks every 15 min. Oxygen was maintained at >100% saturation throughout the experiment and tank flows were set to 5 L min^{-1}. Fish were removed once they were unable to maintain equilibrium, and the temperature at this time was recorded as their CT_{Max}. As fish

reached their CT_{Max}, they were euthanized in 0.4 g L^{-1} TMS, and fish weight, fork length, liver weight, ventricle weight, and state of sexual maturity were recorded for all individuals.

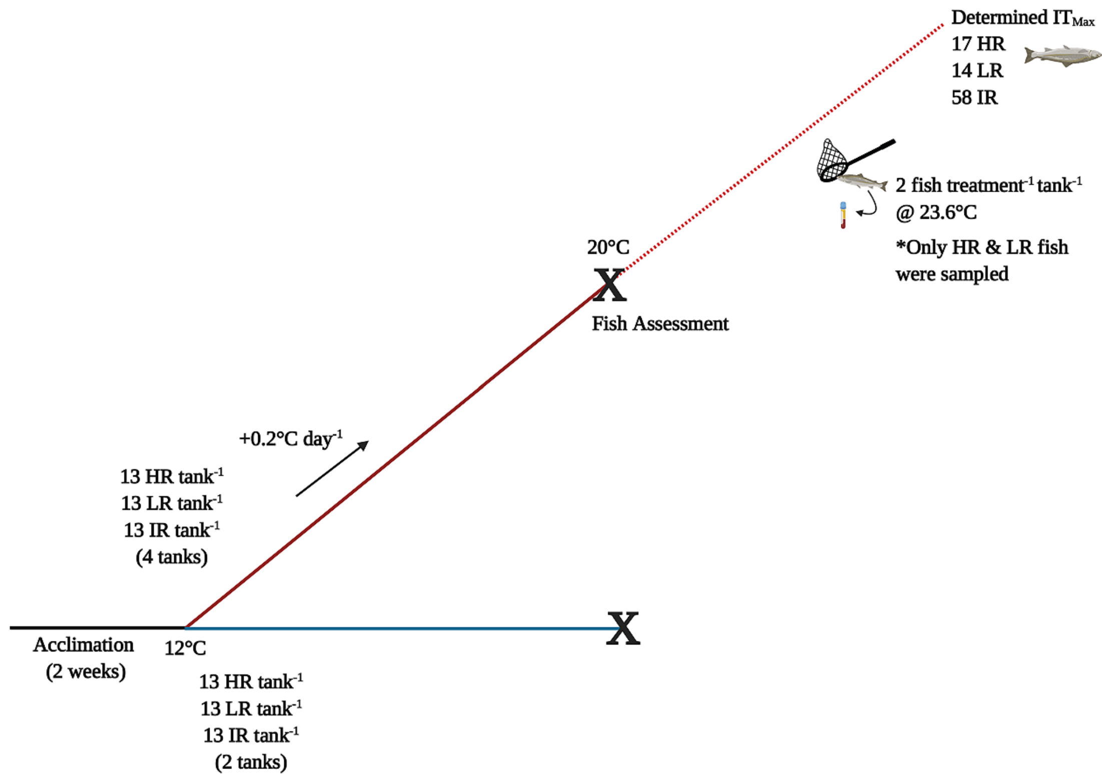

Figure 2-2. Overview of the protocol used in Experiment #2 to assess whether stress coping phenotype affects the thermal tolerance of male Atlantic salmon. Created with BioRender.com.

2.3.3.2 Haematological analyses

To measure haematocrit, the capillary tubes were centrifuged for 2 min at 10,000×g at room temperature. Haemoglobin concentration was determined on triplicate 10 µL blood samples using the cyanomethaemoglobin method (Drabkin's reagent, Sigma D5941) and measuring absorbance at 540 nm using a SpectraMax® M5 microplate reader at 23.6°C. A 5-point standard curve with bovine haemoglobin (Catalog #H-2500; Sigma-Aldrich, Oakville, ON, Canada) was run in triplicate ($r^2 = 0.9999$) on the same plate. Cortisol was measured using the

same ELISA kit and protocol as described above for the initial characterization of fish stress phenotype. Glucose was analyzed using a previously described protocol (Bergmeyer et al., 1974; Clow et al., 2004). Briefly, diluted de-proteinized plasma samples were run in duplicate on the same SpectraMax® M5 microplate reader with absorbance read at 340 nm at 23.6°C. A 7-point standard curve was also run in duplicate ($r^2 = 0.9991$) on the same plate. Lastly, lactate was measured using a method modified from Bergmeyer et al. (1974). The same de-proteinized plasma samples that were analyzed for glucose had their absorbance read at 340 nm at 23.6°C. The 7-point standard curve that was run on the same plate gave an r^2 of 0.9989.

2.3.3.3 Statistical analyses

Statistical analyses were performed as in Experiment #1. Replicate tank means were first compared to confirm the absence of tank effects, with stress phenotype as a fixed factor and tank as a random factor, after which the data from replicate tanks were pooled for further analyses. Data were first assessed via Shapiro-Wilk's normality tests and log_{10}-transformed if necessary to meet testing assumptions. Levene's tests to measure homoscedasticity were also performed to confirm statistical compliance. One-way ANOVAs followed by Tukey's HSD post-hoc tests were used to examine differences ($p < 0.05$) in parameters between the phenotypes. T-tests were used when comparisons were only made between LR and HR fish. Generation of the correlogram, and principal component analysis (PCA) diagrams, using the data collected during the 23.6°C net stress sampling were also performed in R. Results throughout this article are reported as mean ± standard error (SE).

2.4 Results

2.4.1 Characterization of stress coping phenotype

Before the first monthly net stress was performed, average basal cortisol concentrations were 9.1 ± 1.9 ng mL^{-1} based on the assessment of 22 fish. Atlantic salmon were successfully categorized into LR, IR or HR stress phenotypes based on their Z_t scores after four months of assessing individual post-stress cortisol levels. The overall distribution of mean cortisol values for LR, IR and HR fish is shown in Figure 2-3. The average post-stress cortisol levels for the LR, IR and HR fish were 52.6 ± 1.1, 83.9 ± 0.9 and 140.0 ± 3.6 ng mL^{-1}, respectively (all groups significantly different, $p < 0.001$). Weight at the start of Experiment #1 differed between stress responders, with HR fish weighing more than LR fish by 10% (Table 2-1). However, there was no difference in weight, length, growth rate or condition factor at the end of this experiment in which the fish were fed a limited/restricted ration (Table 2-1).

The percentage of LR and HR fish varied considerably among the 17 families, as evidenced by F1 and F11 which contained no LR salmon, and families such as F4, F6 and F15 which had relatively few HR salmon (Figure 2-4A). The highest average post-stress cortisol levels were found in F17 (120.3 ± 9.3 ng mL^{-1}), which significantly differed from F2, F3, F7 and F12 (mean cortisol levels 79.7 ± 7.1, 83.5 ± 6.8, 78.1 ± 6.0 and 74.7 ± 6.0 ng mL^{-1}, respectively). Differences in growth rate between families were also noted (Figure 2-4B). For example, F1 outperformed F10 and F12 in terms of TGC, which is noteworthy as F10 and F12 contained LR salmon (mean cortisol levels 77.2 ± 7.7 and 74.7 ± 6.0 ng mL^{-1}, respectively) and F1 had no LR fish (mean cortisol level 115.9 ± 11.3 ng mL^{-1}). However, there was no overall correlation between individual TGC values and plasma cortisol levels ($p > 0.05$, $r^2 = 0.006$; data not shown).

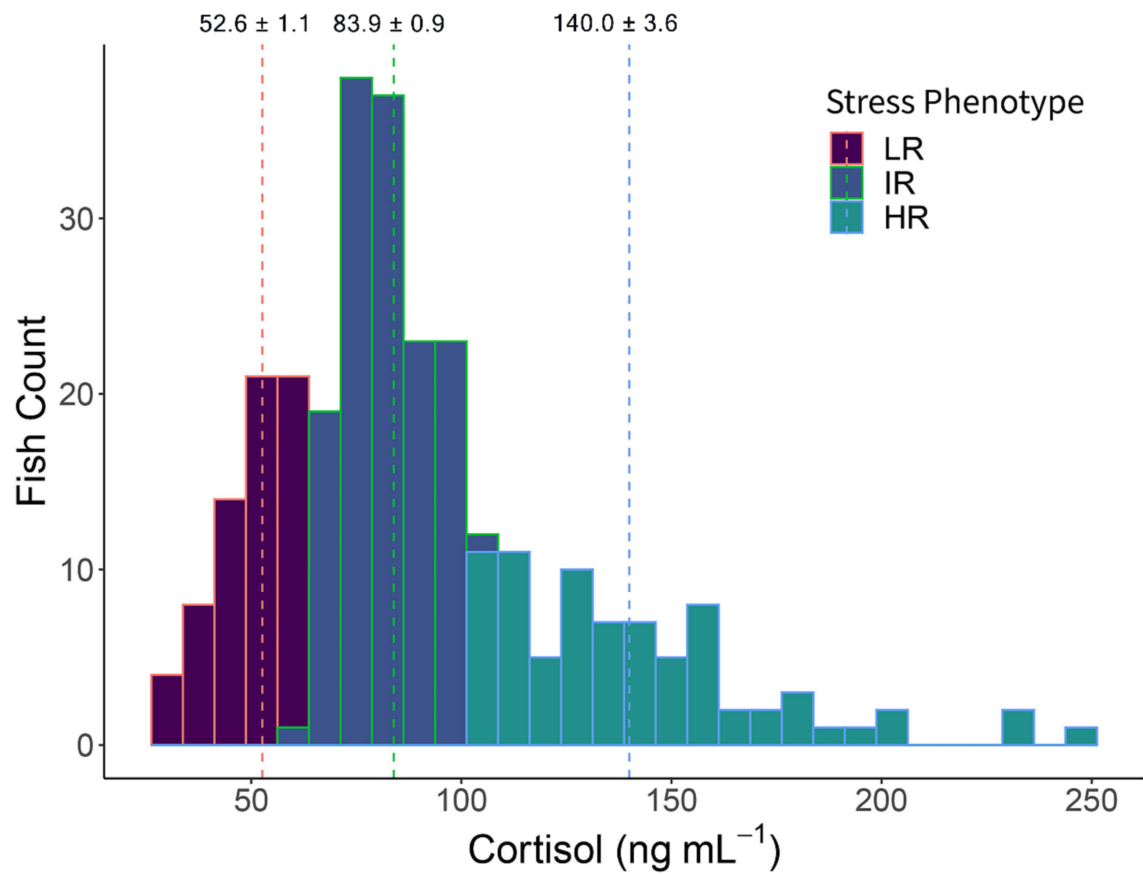

Figure 2-3. Histogram of mean cortisol values for the low (LR), intermediate (IR) and high (HR) stress responders. The dashed lines indicate the average cortisol value for each group.

Table 2-1. Growth performance metrics of LR (n=78), IR (n=153) and HR (n=78) Atlantic salmon during Experiment #1 at 9°C.

	LR	IR	HR
	Mean ± SE	Mean ± SE	Mean ± SE
Initial Weight (g)	154.3 ± 3.6[a]	159.7 ± 2.7[ab]	169.7 ± 4.1[b]
Final Weight (g)	243.0 ± 10.0	253.3 ± 7.2	270.0 ± 9.7
Weight Gain (g)	87.7 ± 8.5	93.6 ± 5.9	100.3 ± 8.3
TGC	0.86 ± 0.10	0.82 ± 0.05	0.85 ± 0.07
SGR (% body weight day^{-1})	0.37 ± 0.03	0.38 ± 0.02	0.39 ± 0.03
Initial Length (cm)	22.7 ± 0.2[a]	23.0 ± 0.2[ab]	23.4 ± 0.2[b]
Final Length (cm)	25.5 ± 0.3	25.9 ± 0.2	26.4 ± 0.3
Initial K	1.31 ± 0.01	1.31 ± 0.01	1.31 ± 0.01
Final K	1.40 ± 0.01	1.40 ± 0.01	1.41 ± 0.01

Lower case letters denote differences ($p < 0.05$) between stress phenotypes. TGC, thermal growth coefficient; SGR, specific growth rate; K, Fulton's condition factor.

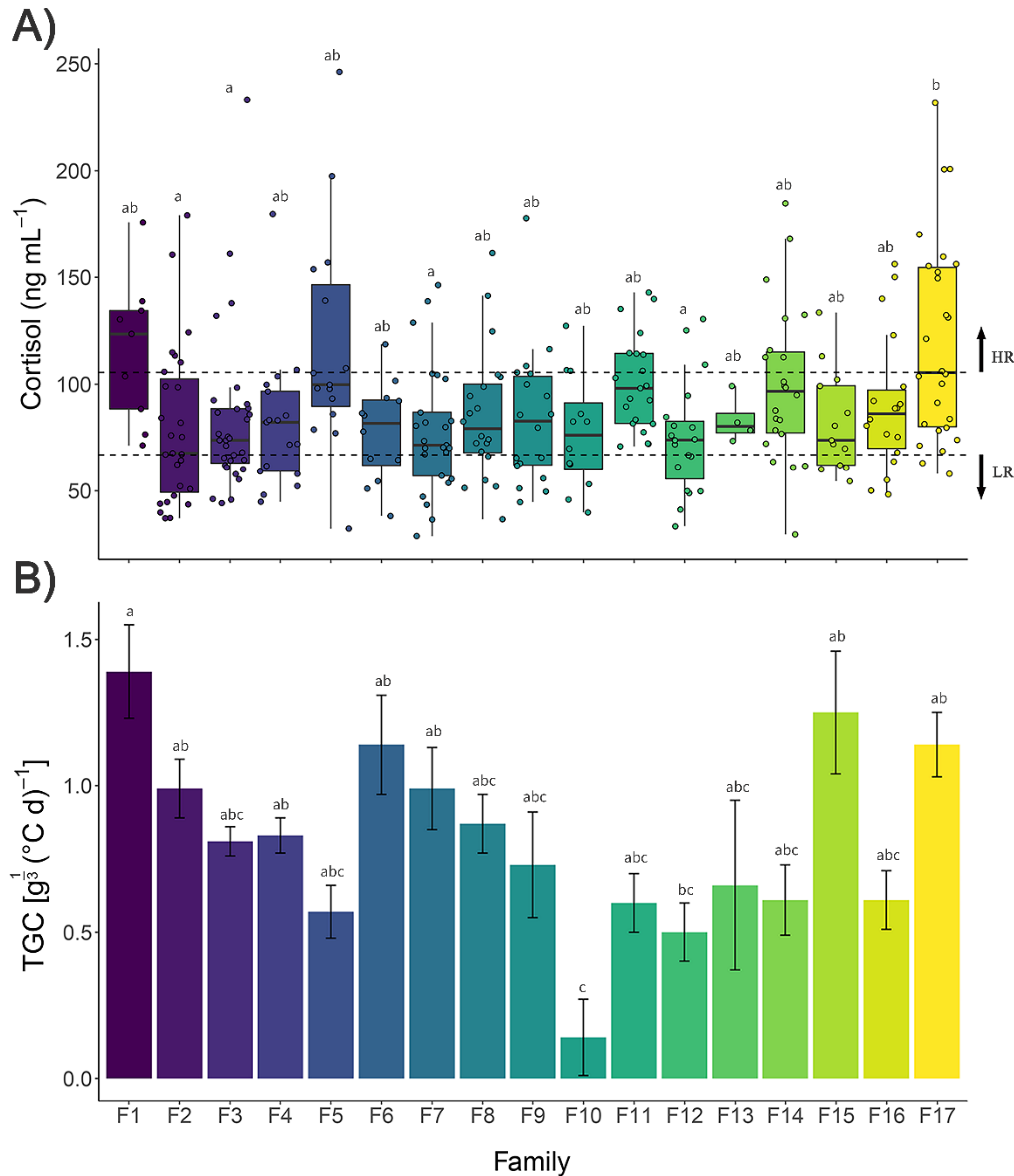

Figure 2-4. A) Average cortisol values for each family based on four months of net-stress sampling. The dashed lines indicate the approximate cut-off values for identifying low (LR) and high (HR) stress responders (i.e., the bottom and top quartiles, respectively). Lower case letters denote significant differences between families ($p < 0.05$). B) Thermal growth coefficients (TGC) (±SE) for each family during the initial characterization of stress phenotype. Families without a letter in common are significantly different ($p < 0.05$).

2.4.2 Performance metrics following an incremental temperature increase to 20°C

Once fish were characterized by their stress phenotype, the impact of an incremental temperature increase (12°C, control vs. 12-20°C, warm) was assessed on their growth performance metrics. When not considering cortisol response phenotype, fish in the 12-20°C treatment grew more (i.e., had greater final weight, gained more weight, and were longer) than fish kept in control tanks at 12°C (Table 2-2). Further, these fish had lower RVM values (by 9.4%) than fish reared at a constant temperature of 12°C. In contrast, there were no significant differences in growth rate, K or HSI between the treatments. Statistical comparisons could not be made of feed intake and conversion between treatments due to lack of replication in the control group (n = 2 tanks). However, the data suggest that feed intake increased with temperature in the 12-20°C treatment (Appendix A – Figure A-1).

Table 2-3 compares the growth performance of the stress phenotypes both between, and within, the treatments. Final weight was higher in HR fish than LR fish in the control group; 517.0 ± 32.5 vs. 425.6 ± 20.7 g, respectively. However, no phenotypic differences were found in weight in the 12-20°C group. Initial K differed between LR and IR salmon in the control group; however, no differences in initial K were found between the stress phenotypes in the 12-20°C treatment, and no differences in final K were observed for either temperature group. In the 12–20°C group, HSI values were 14.6% higher in HR than in LR salmon, and final weight was higher in LR and IR fish in the 12-20°C treatment as compared to the matching phenotypes in the control (12°C) group. Similarly, weight gain, SGR and final length were higher in IR fish from the 12-20°C group as compared to those from the control tanks. Conversely, TGC was significantly (by 24.3%) lower in LR salmon reared at 12-20°C as compared to this phenotype in the control group. Finally, RVM values were also smaller in LR fish exposed to the incremental

temperature increase compared to LR fish reared at 12°C. Rearing temperature did not impact any of the results for HR salmon.

Table 2-2. Growth performance metrics of Atlantic salmon exposed to an incremental temperature increase (12 to 20°C) as compared to the control group (fish maintained at 12°C) in Experiment #2.

	Control		Warm	
	n	Mean ± SE	n	Mean ± SE
Initial Weight (g)	78	295.1 ± 9.4	156	310.5 ± 7.8
Final Weight (g)	78	465.6 ± 15.2[a]	154	517.5 ± 13.9[b]
Weight Gain (g)	78	170.5 ± 9.9[a]	154	204.2 ± 7.9[b]
TGC	78	1.66 ± 0.08	154	1.50 ± 0.04
SGR (% body weight day^{-1})	78	0.85 ± 0.04	154	0.93 ± 0.03
Initial Length (cm)	78	28.3 ± 0.2	156	27.9 ± 0.3
Final Length (cm)	78	31.9 ± 0.3[a]	154	32.6 ± 0.3[b]
Initial K	78	1.33 ± 0.01	156	1.34 ± 0.01
Final K	78	1.41 ± 0.02	154	1.44 ± 0.01
HSI (%)	48	1.04 ± 0.04	48	1.03 ± 0.03
RVM (%)	48	0.070 ± 0.001[b]	48	0.064 ± 0.001[a]
Feed Intake (g fish^{-1} day^{-1})	2	2.85 ± 0.02	4	3.40 ± 0.23
FCR	2	0.90 ± 0.01	4	0.91 ± 0.03

Data represent all fish within a treatment, with no distinction between stress phenotypes. Where significant differences ($p < 0.05$) were detected, lower case letters denote differences between temperature treatments. Statistics were not performed on feed intake or feed conversion data due to limited replication in the control treatment (n = 2 tanks). TGC, thermal growth coefficient; SGR, specific growth rate; K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass; FCR, feed conversion ratio.

Table 2-3. Growth performance metrics of LR, IR and HR Atlantic salmon exposed to an incremental temperature increase (from 12 to 20°C) in Experiment $^{\#}$2 as compared to the control group (fish maintained at 12°C).

	Control						Warm					
	LR		IR		HR		LR		IR		HR	
	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE
Initial Weight (g)	26	270.6 ± 15.6	26	298.2 ± 15.0	26	316.4 ± 17.6	48	304.2 ± 13.9	58	311.6 ± 12.3	50	315.5 ± 14.7
Final Weight (g)	26	425.6 ± 20.7^{Aa}	26	454.2 ± 21.6^{Aab}	26	517.0 ± 32.5^{b}	47	493.6 ± 22.0^{B}	58	532.2 ± 22.7^{B}	49	523.0 ± 27.3
Weight Gain (g)	26	155.0 ± 15.4	26	156.1 ± 13.1^{A}	26	200.6 ± 21.0	47	188.7 ± 11.8	58	215.2 ± 13.1^{B}	49	205.9 ± 15.8
TGC	26	1.79 ± 0.15^{B}	26	1.62 ± 0.11	26	1.59 ± 0.14	47	1.44 ± 0.07^{A}	58	1.55 ± 0.07	49	1.49 ± 0.09
SGR (% body weight day^{-1})	26	0.86 ± 0.07	26	0.79 ± 0.06^{A}	26	0.88 ± 0.07	47	0.91 ± 0.04	58	0.94 ± 0.04^{B}	49	0.92 ± 0.05
Initial Length (cm)	26	27.4 ± 0.5	26	27.7 ± 0.5	26	28.6 ± 0.5	48	28.0 ± 0.4	58	28.5 ± 0.4	50	28.3 ± 0.5
Final Length (cm)	26	31.2 ± 0.5	26	31.7 ± 0.4^{A}	26	32.6 ± 0.6	47	32.3 ± 0.4	58	33.1 ± 0.4^{B}	49	32.5 ± 0.5
Initial K	26	1.29 ± 0.02^{a}	26	1.37 ± 0.02^{b}	26	1.32 ± 0.03^{ab}	48	1.35 ± 0.02	58	1.31 ± 0.02	50	1.34 ± 0.02
Final K	26	1.38 ± 0.04	26	1.41 ± 0.04	26	1.44 ± 0.03	47	1.43 ± 0.02	58	1.42 ± 0.02	49	1.47 ± 0.03
HSI (%)	24	1.01 ± 0.04	NC	NC	24	1.08 ± 0.06	24	0.96 ± 0.04^{a}	NC	NC	24	1.10 ± 0.04^{b}
RVM (%)	24	0.073 ± 0.001^{B}	NC	NC	24	0.067 ± 0.003	24	0.064 ± 0.001^{A}	NC	NC	24	0.064 ± 0.001

Significant ($p < 0.05$) effects of temperature within the same phenotype (e.g., LR) are denoted by dissimilar upper case letters. At the same rearing temperature, differences between phenotypes are identified by dissimilar lower case letters. TGC, thermal growth coefficient; SGR, specific growth rate; K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass; NC, not collected.

2.4.3 Assessment of the stress response at elevated temperatures

After fish morphometrics were assessed at 20°C, temperature continued rising and the stress response of a subset of LR and HR salmon was assessed at 23.6°C after the first 3 mortalities had been recorded. No significant differences in weight, length, K, HSI, RVM, haematocrit or haemoglobin were found between LR and HR fish that were sampled at 23.6°C (Table 2-4). Although it is worth noting that average haemoglobin levels in LR salmon were 14.7% higher than those in HR fish ($p = 0.051$). Average basal levels for glucose, lactate and cortisol were 4.5 ± 0.2 mmol L^{-1}, 2.3 ± 0.2 mmol L^{-1} and 8.3 ± 1.9 ng mL^{-1}, respectively, and not different between the stress phenotypes (Figure 2-5). Exposure to a 15 s net stress induced a physiological stress response in both LR and HR fish (e.g., cortisol increased to 87.4 ± 8.3 ng mL^{-1} at 1 h post-stress). However, all three parameters were indistinguishable between the stress phenotypes (Figure 2-5). Therefore, the initial characterization of a fish's stress responses at lower temperatures (i.e., ~9°C) could not predict the physiological response to the same stressor at elevated temperature (23.6°C). The degree of relatedness between all morphometric and haematological measurements in LR and HR fish is shown in Figure 2-6A. Significant positive correlations were found between weight and length, haematocrit and haemoglobin, K and post-stress glucose, post-stress glucose and post-stress lactate, post-stress glucose and basal glucose, as well as post-stress cortisol and basal lactate. Conversely, significant negative correlations were found between both weight and length vs. post-stress cortisol, length and basal glucose, and between both haematocrit and haemoglobin vs. HSI. The PCA in Figure 2-6B illustrates that the response to net stress at elevated temperature cannot help to differentiate LR and HR fish in multivariate space.

Table 2-4. Morphological metrics, haematocrit and haemoglobin of LR (n=8) and HR (n=8) Atlantic salmon exposed to an incremental temperature increase (from 12.0 to 23.6°C) and a short-term net stress.

	LR	HR
	Mean ± SE	Mean ± SE
Weight (g)	586.7 ± 78.0	490.2 ± 38.4
Length (cm)	34.3 ± 1.6	33.3 ± 0.9
K	1.40 ± 0.04	1.32 ± 0.04
HSI (%)	1.05 ± 0.04	1.17 ± 0.13
RVM (%)	0.068 ± 0.003	0.068 ± 0.003
Haematocrit (%)	38.2 ± 1.1	36.3 ± 2.2
Haemoglobin (mg mL^{-1})	107.7 ± 3.4	93.9 ± 5.4

No significant ($p < 0.05$) differences were found. K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass.

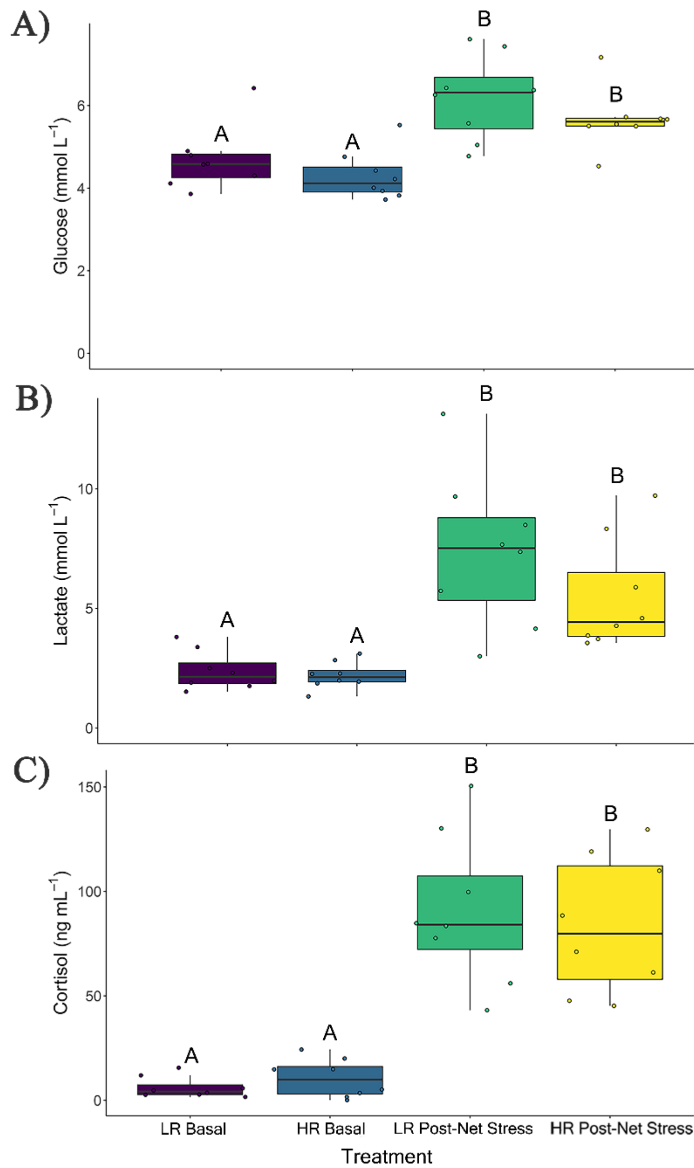

Figure 2-5. Plasma A) glucose, B) lactate, and C) cortisol levels in LR and HR Atlantic salmon (n = 8 each) at 23.6°C. Blood samples were taken immediately following a 15 s net stress event (basal) and 1 h post-net stress. Differences ($p < 0.05$) between time points within a stress phenotype are denoted by upper case letters.

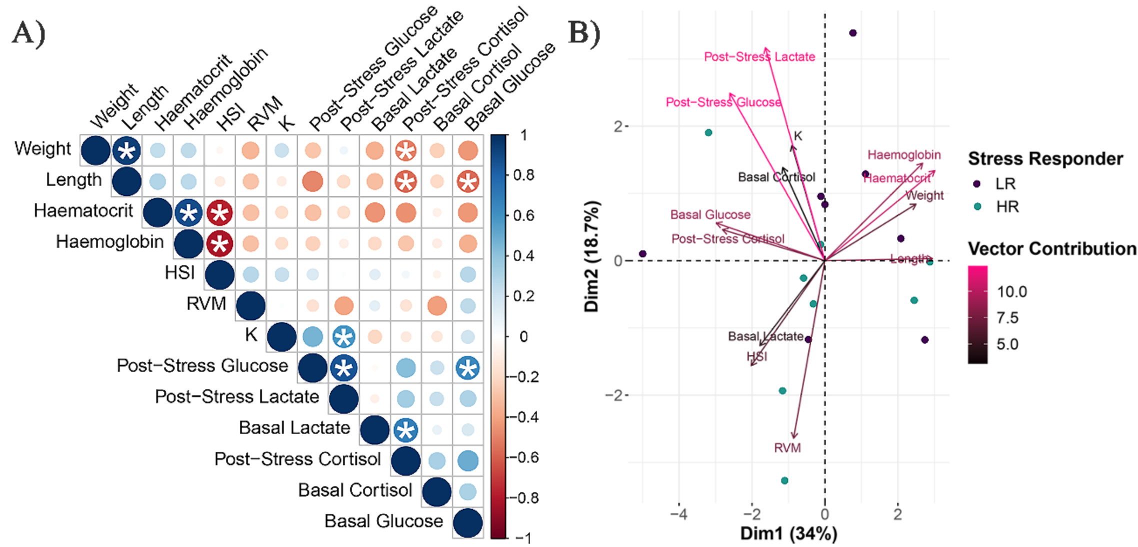

Figure 2-6. A) Correlogram of LR and HR morphometric (weight, length, Fulton's condition factor [K], hepatosomatic index [HSI], relative ventricular mass [RVM]), haematocrit, haemoglobin, and cortisol, glucose and lactate (both basal and post-net stress) measurements collected during the 23.6°C net stress sampling (n = 8). The coloured scale shows the degree of correlation ranging from r = -1.0 (red) to r = 1.0 (blue). The size of the coloured circle indicates its significance level, and circles containing asterisks are significant at $p < 0.05$. B) Principal component analysis (PCA) of the same multivariate dataset. Eigenvectors are coloured based on their contribution to Dimension (Dim) 1 and 2 of the PCA plot.

2.4.4 Thermal tolerance based on phenotypic stress coping response

The remaining salmon in the 12-20°C treatment were exposed to increasing temperatures until they reached their IT_{Max} (~25°C) (Figure 2-2). No differences in upper thermal tolerance were detected between the stress phenotypes (Table 2-5). Statistical comparisons of average IT_{Max} values were also assessed between families with n > 5, but genetics did not have a significant impact on survival at high temperatures. There were no statistically significant differences in weight, weight gain, length, K, HSI or RVM between the stress phenotypes at this sampling point. On average, fish exposed to their IT_{Max} had lost weight since their assessment at 20°C. This is likely to be largely explained by the sharp decrease in feed intake observed after fish reached 22°C (Appendix A – Figure A-2). The acute thermal tolerance (CT_{Max}) of the remaining IR salmon from the 12°C (control) treatment was also determined after Experiment #2 was completed. Exposure to an acute temperature increase (+2°C h^{-1}) led to a CT_{Max} (28.5°C ± 0.1) approximately 3°C higher as compared to the reported measures of IT_{Max} (+0.2°C day^{-1}; IT_{Max} = 25.3°C ± 0.1). Morphometric data of the fish tested in this experiment can be found in Table 2-6.

Table 2-5. Incremental thermal tolerance (IT_{Max}), growth (weight gain) and morphological parameters of LR, IR and HR Atlantic salmon in Experiment #2.

	LR		IR		HR	
	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE
IT_{Max} (°C)	14	25.1 ± 0.2	58	25.3 ± 0.1	17	25.1 ± 0.2
Degree Days at IT_{Max} (°C·d)	14	1392.2 ± 23.6	58	1408.2 ± 13.0	17	1394.1 ± 24.9
Weight (g)	14	399.8 ± 22.9	58	500.2 ± 19.9	17	469.9 ± 40.7
Weight Gain (g)	14	-15.1 ± 7.3	58	-32.0 ± 7.4	17	-38.9 ± 11.3
Length (cm)	13	31.9 ± 0.7	58	33.7 ± 0.4	17	32.8 ± 0.9
K	13	1.25 ± 0.02	58	1.28 ± 0.02	17	1.28 ± 0.03
HSI (%)	13	1.03 ± 0.06	58	1.14 ± 0.04	17	1.20 ± 0.05
RVM (%)	13	0.075 ± 0.002	58	0.077 ± 0.001	17	0.079 ± 0.002

No differences between groups were found. Weight gain was calculated as the weight at IT_{Max} minus the weight assessed at 20°C. K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass.

Table 2-6. Acute thermal tolerance (CT_{Max}) and morphological parameters for IR Atlantic salmon (n=18) from Experiment #2 that were held at a constant temperature of 12°C.

	IR
	Mean ± SE
CT_{Max} (°C)	28.5 ± 0.1
Weight (g)	537.9 ± 20.2
Length (cm)	34.4 ± 0.4
K	1.31 ± 0.03
HSI (%)	1.25 ± 0.05
RVM (%)	0.071 ± 0.002

K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass

2.5 Discussion

The objective of this research was to determine if stress phenotype characterized at 9°C could predict the stress responsiveness and/or thermal tolerance of salmon when exposed to increasing water temperatures following a regimen as is observed at cage-sites in the North Atlantic (Burke et al., 2020; Gamperl et al., 2021; Gollock, 2006; Johansson et al., 2007; Zanuzzo et al., 2019). The hope was to identify a stress phenotype with better growth performance and survival under such conditions, and thus, to provide information to salmon breeding programs that could potentially be used to better prepare the aquaculture industry for challenges related to climate change. Interestingly, the results of this study found that the phenotypic stress response characterized at low temperatures was not indicative of their physiological response to net stress at elevated temperatures or their IT_{Max}.

While differences in weight were found at the start of Experiment #1 and at the assessment point in Experiment #2 in the control group reared at 12°C (HR > LR; Tables 2-1 and 2-3), no differences in weight were found in fish measured at 20°C or in fish when they reached their IT_{Max}. These findings agree with results for stress-characterized pre-adult rainbow trout (Pottinger and Carrick, 1999; Weber and Silverstein, 2007), but even within studies of rainbow trout, opposite growth performance has been reported, wherein LR fish grew more than HR fish (Fevolden et al., 2002; Pottinger, 2006). The direct injection of cortisol, or the use of intraperitoneal cortisol implants, in Atlantic salmon have been shown to reduce growth (Breves et al., 2020; Vargas-Chacoff et al., 2021), therefore it might be expected that LR salmon would have outperformed the HR salmon in the current trial. However, in juvenile masu salmon (*Oncorhynchus masou*), cortisol injections induce the production of plasma insulin-like growth factor binding proteins, and this illustrates that elevated cortisol levels regulate growth in salmonids (Yamaguchi et al., 2021). Perhaps there are other mechanisms underlying of the enhanced growth of the HR stress phenotype, such as individual genetics or social hierarchy that should be studied further.

2.5.1 Upper thermal tolerance

This is the first study to report on the IT_{Max} of Atlantic salmon, following a similar protocol to that used in Atlantic cod (Zanuzzo et al., 2019). While other studies (e.g., Gamperl et al., 2020; Hvas et al., 2017) have reported on the effects of long-term exposure to elevated temperatures on Atlantic salmon, none have previously followed the procedure of raising temperature at a slow incremental level to the point of total mortality within a population of salmon. The results of the current research show that the average IT_{Max} of male Atlantic salmon

from a farmed population of St. John River origin ranged between 25.1 and 25.3°C, with no significant difference in upper thermal tolerance between stress phenotypes. However, the acute thermal tolerance (CT_{Max}; measured at an ecologically-relevant heating rate of 2°C h^{-1}; Rodnick et al., 2004) of IR fish in this study averaged 28.5°C, >3°C higher than their average IT_{Max}. This disparity exceeds the difference found between the two methods in Atlantic cod, which was reported as a ~1°C separation between acute and incremental upper thermal tolerances (Zanuzzo et al., 2019). Interestingly, the CT_{Max} from the current study was generally greater than what has been previously published for hatchery/farmed Atlantic salmon. For example, in juvenile Atlantic salmon, CT_{Max} has been reported to range between 23.9 and 26.1°C (Anttila et al., 2013), ~27.5 and 29.5°C depending on acclimation temperature (Anttila et al., 2015) and ~26-29°C, again with acclimation temperature playing a key role (Bowden et al., 2018). However, all of these studies used smaller-sized juvenile salmon than in the current study, which would likely enhance their CT_{Max} as has been suggested for teleosts in general (Fryxell et al., 2020; Messmer et al., 2017; Peralta-Maraver and Rezende, 2021; Pörtner, 2021). Further, the much faster (and not ecologically relevant) heating regimes used in two of these studies (e.g., Anttila et al., 2015, 2013) likely would also increase their CT_{Max} estimates as has been previously suggested (Mora and Maya, 2006). In addition, the current average CT_{Max} was ≥2°C higher than what was reported in farmed Atlantic salmon at similar life stages tested in respirometers using the same rate of heating (+2°C h^{-1}) and using the same water source as the present experiment (Leeuwis et al., 2019; Penney et al., 2014). Collectively, these results suggest that this population of conventional (i.e., non-transgenic) male Atlantic salmon sourced from AquaBounty Canada may be more thermally tolerant than other farmed populations in Atlantic Canada (i.e., Anttila et al., 2013; Leeuwis et al., 2019; Penney et al., 2014). Genetics likely influenced these results, and

therefore, further investigations of genetic markers of thermal tolerance within this population would be advantageous. In fact, such experiments are currently ongoing. Upper thermal tolerance could have been inadvertently selected as AquaBounty salmon are only reared in land-based, temperature-controlled facilities. While the main goal of their breeding program has historically focused on improving the growth performance of their salmon, it is possible that thermal tolerance traits were linked to this selection. In addition, the findings from this study further question the real-world applicability of measuring the CT_{Max} of various fishes when the question does not have an ecological (e.g., in streams that see large daily fluctuations in temperature or in tidepools in temperate and/or tropical regions) (Caissie et al., 2004; Morash et al., 2018; Richards, 2011) or physiological basis.

2.5.2 Stress responses of characterized fish at elevated temperatures

Atlantic cod, previously characterized as LR or HR using handling stress experiments similar to the current study, were still distinguishable from one another when measuring cortisol levels following a subsequent acute temperature increase (i.e., fish transferred from 10°C to 18°C) (Hori et al., 2012a). These data, thus, suggested that a fish's phenotypic stress response is maintained when fish are exposed to an acute thermal challenge. While the current results showed that chronic exposure to increasing temperatures eliminates phenotypic differences in post-stress cortisol/metabolic stress parameters in this population of salmon (i.e., no differences in post-stress plasma cortisol, glucose or lactate at 23.6°C; Figure 2-5), it is possible that differences in short-term measures of thermal tolerance such as CT_{Max} might be found. However, whether this would be the case is not clear. Whereas no difference in CT_{Max} was reported between adult HR and LR rainbow trout (LeBlanc et al., 2012), a heat shock challenge where

rainbow trout were kept at 25°C for 1 h showed that HR fish exhibited a more rapid and greater heat stress response (as assessed by the expression of HSP70 and other stress proteins) than LR trout, and experienced less oxidative protein damage (LeBlanc et al., 2012). This latter study suggests that differences in thermal tolerance between stress phenotypes might be more subtle than basic assessments of survival can distinguish. Investigations into the transcriptomics and behaviour of the salmon used in these experiments, however, could help in selecting those that are more tolerant of high water temperatures. Initially characterizing fish based on dominance behaviours might be more suitable than assessing cortisol stress response, as subordinate juvenile rainbow trout have been shown to have lower CT_{Max} scores as compared to dominant fish (LeBlanc et al., 2011), and both Hvas et al. (2017) and Gamperl et al. (2020) suggest that behaviour at high temperatures likely influences a salmon's thermal tolerance.

2.5.3 Potential influences of a monosex population

This study used all-male salmon as these were the only fish available from AquaBounty at the time this project was planned. However, using a monosex group of fish could have contributed to the higher thermal tolerance (CT_{Max}) that was observed in these experiments. In Pacific salmonids, sex-specific impacts of temperature and/or stress have been repeatedly reported. For example, in sockeye salmon (*Oncorhynchus nerka*) migrating to their spawning grounds, previous exposure to 18°C increased mortality in general, but appeared to have a greater effect on females than males (Crossin et al., 2008). It has been suggested that in migrating female sockeye salmon mortality is 2.1 times greater on average than in males (Hinch et al., 2021). Higher female mortality is evident in migrating coho (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tshawytscha*), especially if river conditions are more

challenging (e.g., high temperatures) (Hinch et al., 2021). Plasma cortisol levels were higher in migrating female sockeye compared with males, which led to the hypothesis that this compromises their immunity and makes them less resistant to pathogens especially when the rivers are warm (Martins et al., 2012). Finally, it has also been suggested that male coho salmon can better tolerate cardiac hypoxia at high temperature due to higher RVM values, and levels of cardiac lactate dehydrogenase, as compared to females (Little et al., 2020). Altogether, this body of literature suggests that there are likely sex-specific differences among salmonids, and this should be further tested in Atlantic salmon to determine if thermal tolerance differs between males and females. It is often difficult to parse out whether sex influences research results, as most publications do not even mention the sex of study individuals (as reviewed in Edmands, 2021).

2.5.4 Impact of increasing temperature on production relevant traits

Regardless of sex or stress phenotype, temperature influenced a variety of parameters measured in this study. Salmon that were exposed to an increase in temperature from 12 to 20°C grew more than fish in the control treatment that were kept at a constant 12°C. These results agree with past studies that have shown enhanced growth of Atlantic salmon at elevated water temperatures (e.g., Elliott and Elliott, 2010; Imsland et al., 2014; Jensen et al., 2015; Koskela et al., 1997). However, in Gamperl et al. (2020), which tested a similar incremental thermal increase in Atlantic salmon, final weight and growth rate did not differ between warm and control treatments under normoxic conditions, and feed intake was observed to decrease after fish reached 18°C (Gamperl et al., 2020); in contrast to the current study where fish maintained a high level of feeding until 22°C. Although there was an apparent temporary decrease in feed

intake seen in fish after they were handled and assessed at 20°C. Other studies in Atlantic salmon have also reported poorer feed conversion and/or decreased feed intake at 18°C compared to lower rearing temperatures (Kullgren et al., 2013; Sambraus et al., 2017). These results further support the notion that the current study population of farmed Atlantic salmon was able to acclimate to higher temperatures better than previously tested groups, but whether this was related to sex (an all-male population) or the specific genetics of the fish used is not known.

In the current study, RVM was lower in fish warmed to 20°C as compared to fish reared at 12°C (by ~10%). In comparison, Gamperl et al. (2020) found that RVM increased by 21% in fish sampled at 23°C once the population had reached 30% mortality compared to salmon reared at 12°C, but that such a difference was not detectable at the time the fish reached 20°C. In male rainbow trout, RVM of 4°C-acclimated fish was significantly greater than 17°C-acclimated fish, while no difference was reported in females; only at 4°C were RVM values of males greater than that of females (Klaiman et al., 2011). In contrast, in post-smolt Atlantic salmon reared between ~3 and 14°C, females exhibited greater heart weight, width and height with rounder hearts than males (Perry et al., 2020). In the current study, stress phenotype did not influence RVM values at any sampling point. These results contrast with past literature, where the cardiosomatic index was 34% higher in adult HR female rainbow trout compared to LR fish, although the temperature of that study was not reported (Johansen et al., 2011). This result also coincided with differences in transcript expression of selected cardiac markers (i.e., *vmhc*, *smlc2*, *col1a2*, *rcan1*, *vegf*) (Johansen et al., 2011). Collectively, these results suggest that sex influences heart morphology and that ventricle size is only increased when fish are under more severe thermal stress (either at low or high temperatures). However, the effect of stress phenotype is still not clear.

2.6 Conclusions

Contrary to expectations, phenotypic stress response was not a predictive factor of upper thermal tolerance in the male Atlantic salmon tested in this study. Furthermore, while the stress response to net handling was a consistent, and characterizable trait, among fish at 9°C as assessed by circulating cortisol levels, this finding was not repeatable after these fish were exposed to an incremental temperature increase. However, this population of Atlantic salmon was more tolerant of high temperatures than other populations of farmed salmon tested in Atlantic Canada. Furthermore, the fact that there was a large separation between my IT_{Max} and CT_{Max} results (even with CT_{Max} measured at 2°C h^{-1}) highlights the importance of assessing thermal tolerance under environmentally/temporally realistic conditions. For sea-caged salmon, IT_{Max} likely provides a much more accurate estimate of survival at high temperatures. Overall, selection of fish based on phenotypic stress response at low temperatures may not be beneficial to incorporate into Atlantic salmon breeding programs if the goal is to improve growth performance and survival at high temperatures under sea-cage culture conditions. Future exploration into the genomic background of these fish is recommended, not only to help reveal the mechanisms behind their enhanced performance at high temperatures, but also to determine if stronger differences between stress phenotypes can be found at the transcript expression level.

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CHAPTER 3: Impact of stress phenotype, elevated temperature, and bacterin exposure on male Atlantic salmon (*Salmo salar*) growth, stress and immune biomarker gene expression

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3.1 Abstract

In this study, post-smolt male Atlantic salmon, previously identified as low (LR) or high responders (HR) based on post-stress cortisol levels, had their head kidney and liver sampled at 12 and 20°C prior to injection (time 0) and after (i.e., at 12 and 24 hpi, respectively) they were injected with either Forte Micro (a multi-valent vaccine containing bacterin, to capture peak antibacterial responses) or an equal volume of phosphate-buffered saline (PBS). Real-time quantitative polymerase chain reaction (qPCR) was then used to measure the expression of 15 biomarker genes in the head kidney and 12 genes in the liver at each temperature/sampling point. Target transcripts were chosen that were related to growth, stress and innate antibacterial immune responses. Many temperature, phenotype and injection effects were found for individual genes within these 3 broad categories, and multivariate statistical analyses (i.e., PCA and PERMANOVA) were used to look for overall patterns in transcript expression. These analyses revealed that HR salmon at 20°C mounted a more robust response ($p < 0.05$) for the 10 head kidney immune-related transcripts when injected with Forte Micro than LR salmon. In contrast, the 7 liver stress-related transcripts displayed a greater response ($p = 0.057$) in LR vs. HR fish with Forte Micro at 12°C. Overall, while this research did find some differences between LR and HR fish, it does not provide strong (conclusive) evidence that the selection of a particular phenotype would have major implications for the health of salmon over the temperature range examined.

3.2 Introduction

Chronic elevation of plasma cortisol levels through artificial means (i.e., slow-release via intraperitoneal implants or incorporation into the diet) is used to mimic and study natural stress responses, and increases disease susceptibility and mortality in salmonids (Pickering and Duston, 1983; Pickering, 1989; Pickering et al., 1989). It can also lead to negative impacts on growth and feed conversion/digestibility (Pfalzgraff et al., 2021; Vargas-Chacoff et al., 2021), and increase the fish's standard metabolic rate (Pfalzgraff et al., 2022). However, less is known about what effect the stress phenotype of an individual fish has on these traits. It has been shown in species such as Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Atlantic cod (*Gadus morhua*), channel catfish (*Ictalurus punctatus*), zebrafish (*Danio rerio*) and European sea bass (*Dicentrarchus labrax*) that individuals can be characterized as either low responders (LR) or high responders (HR) based on whether they exhibit a proactive or reactive stress coping phenotype, respectively (Chapter 2; Andersson et al., 2013; Chatakondi and Peterson, 2018; Hori et al., 2012a; Koolhaas et al., 1999; Rey et al., 2021). In my previous study, I showed that despite post-net stress plasma cortisol concentrations differing by ~90 ng mL^{-1} between LR and HR post-smolt Atlantic salmon reared at 9°C, this difference in stress phenotype at low temperatures did not predict the robustness of the stress response or survival during an incremental thermal maximum (IT_{Max}) test (Chapter 2). However, much remains to be learned from studying LR and HR fish.

For example, researchers do not have a full grasp of how different stress phenotypes respond to immune challenges. In ~230 g Atlantic salmon and ~250 g rainbow trout that were selectively bred from lines with either a low or a high cortisol stress response, plasma lysozyme activity was significantly higher in HR than LR trout following a low water stress and this

pattern (while not significantly different) was also seen in the salmon (Fevolden et al., 1991). Lysozyme is an important component of the innate immune defense system against bacteria (Saurabh and Sahoo, 2008), and therefore, high levels could be beneficial to HR fish. However, when the next generation of rainbow trout (~210 g) were intraperitoneally (IP) injected with either live *Aeromonas salmonicida* (causative agent of furunculosis) or *Vibrio anguillarum* (causative agent of vibriosis) at 10-12°C, higher mortality was reported for HR trout as compared to LR trout when infected with *A. salmonicida*, while the opposite was true for trout infected with *V. anguillarum* (Fevolden et al., 1992). Further, when the next generation of Atlantic salmon (~40 g) were IP injected with either live *A. salmonicida* at 12°C, or *V. anguillarum* or *Renibacterium salmoninarum* (causative agent of bacterial kidney disease) at 5.5°C, survival was significantly lower in HR vs. LR salmon when infected with *A. salmonicida* or *V. anguillarum*, whereas no difference was detected between stress phenotypes after injection with *R. salmoninarum* (Fevolden et al., 1993). In juvenile Atlantic salmon characterized as being LR or HR after an acute isolation experiment, although HR families were more susceptible to infectious pancreatic necrosis virus (IPNV), there was no relationship between stress phenotype and resistance to furunculosis (Kittilsen et al., 2009). Finally, mortality was >20% higher in juvenile HR channel catfish compared to LR fish 21 days after *in situ* bath immersion with *Edwardsiella ictaluri* (causative agent of enteric septicemia of catfish) (Chatakondi and Peterson, 2018). Ultimately, the above research fails to identify a consistent pattern (i.e., is inconclusive) with respect to the influence of stress phenotype on the capacity of fish to defend against bacterial pathogens that pose significant challenges to the finfish aquaculture industry.

Relatively few studies have examined how gene expression differs between LR and HR fish, particularly within the head kidney or liver (Hori et al., 2012b; Pemmasani et al., 2011;

Samaras et al., 2016; Vargas et al., 2018). The head kidney is a haematopoietic and lymphoid organ that serves as the main mediator of immune responses and stress hormone production in teleost fish (Geven and Klaren, 2017; Press and Evensen, 1999), while the liver regulates a variety of biological processes including nutrient metabolism, endocrine signalling, growth and immunity (Trefts et al., 2017). No previous study has specifically examined what impact temperature has on phenotype-specific constitutive transcript expression or that in response to immune stimulation in these tissues. As stress phenotypes are present within both cultured and wild populations, it is important to understand what impact high temperatures have on how these fish regulate their responses to both temperature and potential pathogens. This is especially pertinent as aquatic ecosystems are warming at drastic rates due to accelerated climate change (IPCC, 2022). Therefore, the objectives of the current study were to determine what effects temperature and bacterial (vaccine) stimulation have on the genomic regulation of growth, stress and the innate antibacterial immune responses of LR vs. HR Atlantic salmon. Based on the literature described above, it was originally hypothesized that HR salmon would mount a greater stress response to vaccine injection at 12°C, and that this enhanced stress response would negatively impact the expression of immune- and growth-related transcripts as compared to LR salmon. However, I anticipated that this pattern may not be present at 20°C given the results of Chapter 2.

3.3 Materials and methods

This study was approved by the Animal Care Committee of Memorial University (protocol #20-01-KG), and salmon husbandry and experimental procedures were performed in

accordance with the Canadian Council on Animal Care Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (Canadian Council on Animal Care, 2005).

3.3.1 Experimental animals

A full description of the rearing conditions and the initial process of characterizing the stress phenotype of the salmon used in this study can be found in Chapter 2. In brief, passive integrated transponder (PIT)-tagged male diploid Atlantic salmon of St. John River origin from AquaBounty Canada (PE, Canada) were sent to the Dr. Joe Brown Aquatic Research Building [JBARB; Ocean Sciences Centre (OSC), Memorial University] where they were smolted. All-male salmon were used in this study because these were the only fish available from AquaBounty at the time of experimentation. After acclimation to 9°C, the fish were exposed to a series of 4 monthly net stress samplings, wherein individual post-stress plasma cortisol was measured 1 h after a 1 min net stress (air exposure). Using the cortisol results, a total Z-score (Z_t; Chapter 2; Hori et al., 2012a; Weil et al., 2001) was assigned to each fish and used to characterize their stress phenotype. Fish were identified as either LR or HR if their Z_t fell into the lower or upper quartile ranges, respectively. Approximately one month later, the salmon were moved to the Laboratory for Atlantic Salmon and Climate Change Research (LASCCR, OSC; Memorial University) into 2.2 m^3 tanks and distributed between two treatment groups. In one group, designated as the 'warm' treatment, fish were exposed to an incremental temperature increase from 12°C at 0.2°C day^{-1} to mimic conditions that these salmon would be expected to face in sea-cage culture during the summer (Burt et al., 2012; Gamperl et al., 2021). The 'control' group was maintained at 12.0 ± 0.5°C for the duration of the experiment. The LR and

HR fish had colour-coded flag tags (Pentair, Apopka, FL, USA) inserted into their dorsal muscle under anesthesia to allow for the identification of stress phenotype.

3.3.2 Experimental design and sampling

Once the warm treatment reached 20°C, all the fish were taken off feed for 24 h, and a subset of fish from both rearing groups was sampled/injected as outlined in Figure 3-1. This was performed approximately three months after the last monthly net stress sampling was completed to characterize the different stress phenotypes. From the tanks (4 total) at 20°C, 2 LR and 2 HR fish were initially sampled, alongside 4 LR and 4 HR from each tank (2 total) at 12°C (n = 8 for each stress phenotype at each temperature). The sampling order of LR and HR salmon was alternated within each tank, with one fish at a time netted out, euthanized in 0.4 g L^{-1} TMS (tricaine methansulfonate; Syncaine TMS, Syndel Laboratories Canada, Nanaimo, BC, Canada) followed by cranial concussion, and sampled. Head kidney (taken from the most cranial portion of this organ) and liver (from the posterior-most lobe) samples were collected in duplicate using standard aseptic techniques, and flash-frozen in liquid nitrogen prior to being stored at -80°C. These fish were assigned as time zero (i.e., unstimulated) controls and used to measure constitutive transcript expression.

Thereafter, 8 fish of each stress phenotype from both treatments (i.e. from the ‘warm’ and ‘control’ groups) were briefly anesthetized (0.2 g L^{-1} TMS), had their weight and fork length recorded, and were intraperitoneally (IP) injected with either 50 µL of phosphate-buffered saline (PBS; Thermo Fisher Scientific, Mississauga, ON, Canada) or 50 µL of a commercial multivalent vaccine (Forte Micro; Elanco Limited, Charlottetown, PE, Canada). This set vaccine injection volume was given at room temperature independent of fish weight based on

manufacturer instructions, and PBS injections of equal volume served as time-matched sham injection controls. Although imperfect, as PBS differs in composition as compared to Forte Micro, PBS-injected fish control for the stress of net capture, air exposure, mild anesthesia and the trauma of injection. Forte Micro contains formalin inactivated cultures of *A. salmonicida*, *V. anguillarum* serotypes I and II, *V. ordalii*, and *V. salmonicida* serotypes I and II in a liquid emulsion with an oil-based adjuvant. PBS injections were given first within each tank, followed by Forte Micro to avoid contamination. However, the injection order of LR and HR fish was alternated between tanks. Once the fish were injected, they were moved into new 0.5 m^3 tanks supplied with 12 or 20°C water at a rate of 5 L min^{-1} (n = 8 fish $tank^{-1}$; 4 tanks $temperature^{-1}$). Fish at 12°C were sampled at 24 h post-injection (hpi) and those at 20°C were sampled at 12 hpi. Zanuzzo et al. (2020) measured innate immune-related transcript expression in response to Forte V II (a vaccine containing formalin inactivated infectious salmon anemia virus in addition to the same bacterial antigens as in Forte Micro) at 6, 12, 24 and 48 hpi in post-smolt Atlantic reared at 12 or 20°C. Based on these results, salmon reared at 12 and 20°C in the current study were sampled at 24 and 12 hpi, respectively, in an attempt to capture peak innate antibacterial immune responses as transcript expression response is accelerated at elevated temperature (Zanuzzo et al., 2020). However, as expression of every target transcript assessed in this study is unlikely to have peaked at these exact times, comparing responses to vaccine injection between temperature groups was not possible. Head kidney and liver samples were collected in the same manner as described above at time zero. Liver (pre-sampling) and ventricle weights were measured in all fish that were sampled. Hepatosomatic index (HSI) and relative ventricular mass (RVM) of these fish were previously calculated and reported (Tables 2-2 & 2-3 in Chapter 2). Blood was not sampled from any fish in this experiment.

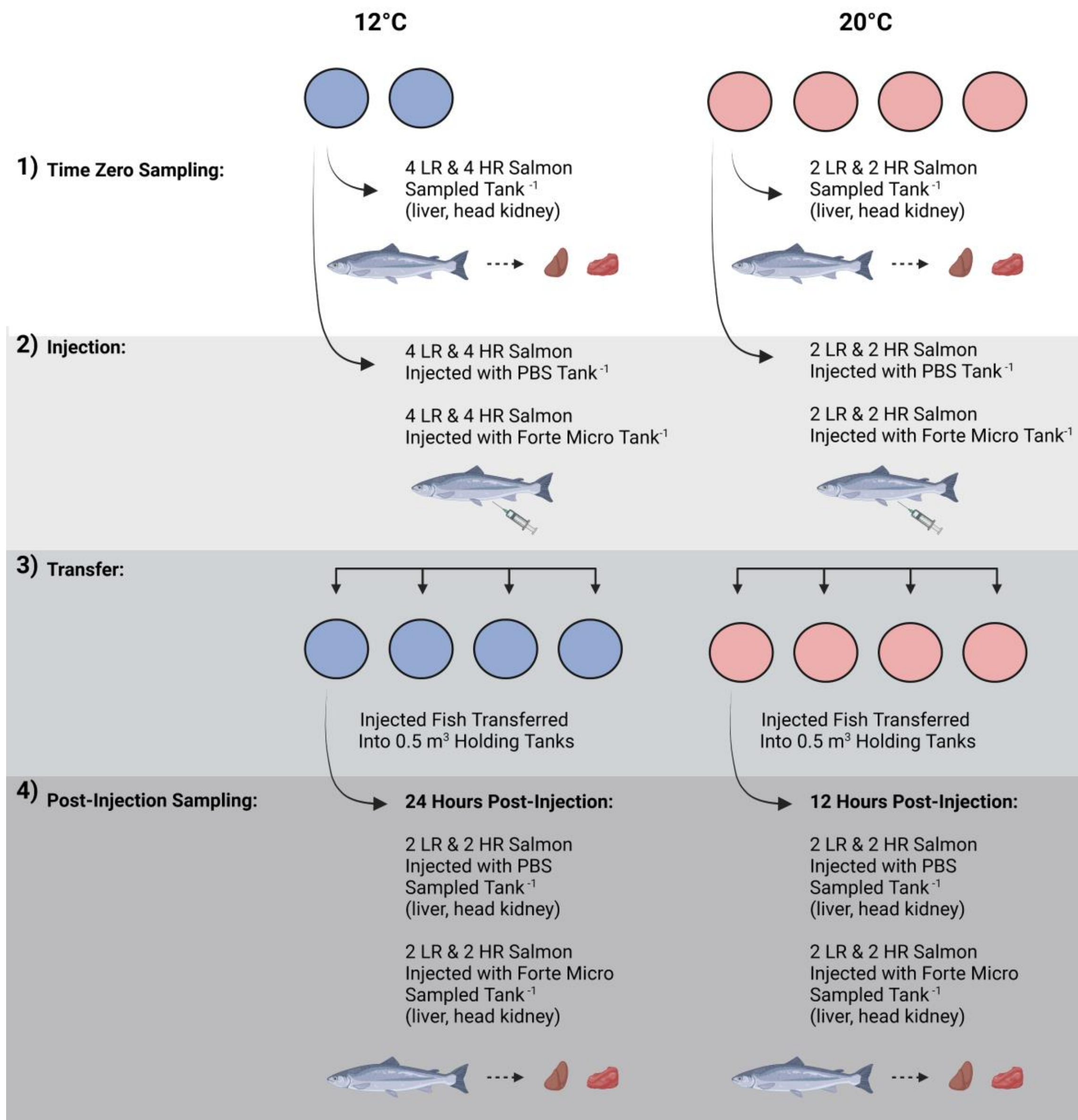

Figure 3-1. Overview of the protocol used to assess whether stress phenotype [i.e., low responders (LR) vs. high responders (HR)] affected the growth, stress and/or antibacterial immune responses of male Atlantic salmon when held at 12°C or exposed to an increasing temperature regimen up to 20°C. Circles represent individual tanks. Created using BioRender.com.

3.3.3 RNA extraction, purification and cDNA synthesis

One LR fish sampled at time zero and one LR fish injected with PBS, both sampled at 20°C, were excluded from the study as they were sexed as females. Head kidney and liver samples from the remaining fish (n = 94 per tissue) were homogenized with stainless steel beads

(5 mm; Qiagen, Mississauga, ON, Canada) and TRIzol® (Invitrogen/Life Technologies, Burlington, ON, Canada). Samples were then centrifuged through Qiagen QIAshredder columns to further aid in homogenization. Subsequent RNA extractions were performed on both tissues according to manufacturer instructions, with the liver samples undergoing a second extraction using the phenol-chloroform phase separation method described in Xu et al. (2013). For both head kidney and liver, 25 μg of total RNA per sample was DNase I-treated (6.8 Kunitz units; Qiagen RNase-Free DNase Set) and column-purified (Qiagen RNeasy Mini Kit) following manufacturer protocols. Using 1.0% agarose gel electrophoresis, RNA integrity was confirmed as evidenced by tight 18S and 28S ribosomal RNA bands. RNA purity was also high, as A260/280 and A260/230 ratios were all ≥ 1.95 and 1.80, respectively, in all samples as determined using NanoDrop® spectrophotometry. A single liver sample (HR salmon at 20°C injected with Forte Micro) was excluded from the study as its RNA failed to meet quality standards after multiple attempts at DNase I-treatment and cleanup. From the remaining samples, cDNA was synthesized from 1 μg of DNase-treated, column-purified, RNA using random primers (250 ng; Invitrogen/Thermo Fisher Scientific), dNTPs (0.5 mM final concentration; Invitrogen/Thermo Fisher Scientific) and M-MLV reverse transcriptase (200 U; Invitrogen/Thermo Fisher Scientific) in 1X first strand buffer and DTT (10 mM final concentration) following the manufacturer's instructions. No reverse transcriptase (no-RT) controls to aid in assessing gDNA contamination were also created from pooling the RNA of randomized samples (n = 23-24 per pool).

3.3.4 Preliminary analysis

To assist in identifying target transcripts of interest for this study, qPCR assays were first conducted on cDNA pools. Eight pools were synthesized, using the same procedure as described above. This involved combining equal amounts of RNA from all available samples of LR or HR fish at 12°C or 20°C injected with PBS, and LR or HR fish at 12°C or 20°C injected with Forte Micro, for each respective pool (n = 7 or 8 per pool). Twenty-six transcripts were initially screened in head kidney and 18 were tested in liver. An additional 3 normalizer transcripts were assessed for each tissue. A narrow list of normalizers was tested as previous research indicated that these targets would likely be the most stable across temperatures and in response to immune stimulation (Chapters 2 & 4; Ignatz et al., 2022). Each qPCR reaction consisted of: 6.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific), 1.46 μL of nuclease-free water (Invitrogen/Life Technologies), 0.52 μL (50 nM) of both the forward and reverse primers and 4 μL of cDNA (diluted 1:20) representing 10 ng of input total RNA. Amplifications were performed using a ViiA7 Real-Time PCR system (Applied Biosystems) and a real-time analysis program consisting of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min, with fluorescence detection at the end of each 60°C step, and was followed by dissociation curve analysis. Pools were run in triplicate alongside no-template controls (NTCs) for each transcript. Multiple transcripts were tested on the same 384-well plate.

Raw cycle threshold (C_T) data were imported into qbase+ (Biogazelle, Ghent, Belgium) (Hellemans et al., 2007). Analysis using geNorm identified *eif3d* and *pabpc1* as the most stable normalizers in head kidney (mean geNorm M value and coefficient of variation of 0.189 and 0.066, respectively) (Vandesompele et al., 2002). In contrast, *ef1a* and *pabpc1* were chosen as

the normalizer genes for the liver (mean geNorm M value and coefficient of variation of 0.324 and 0.113, respectively). Normalized relative quantities (NRQ) were calculated using default amplification efficiencies in qbase+. The NRQ were then used to compare fold-changes in expression between stress phenotypes and the injection treatments. These results were used to select the final list of transcripts for this study, and can be found in Appendix B Tables B-1 (head kidney pools) and B-2 (liver pools). The primers for the transcripts that were not included in the final study design are listed in Appendix B Table B-3, and mostly came from past research (Beemelmanns et al., 2021b; Bower and Johnston, 2010; Caballero-Solares et al., 2017; Emam et al., 2022; Smith et al., 2018; Umasuthan et al., 2020; Xue et al., 2015, 2021).

3.3.5 qPCR analysis

A total of 15 transcripts of interest were ultimately targeted in the head kidney and 12 were assessed in the liver. In head kidney, transcripts coding for glucocorticoid synthesis (i.e., *stara*, *starb*, *cyp11a*, *cyp11b*, *hsd3b*), immune receptors (i.e., *stlr5a*, *cxcr1*), immune effectors (i.e., *campb*, *hampa*, *ch25ha*), a cytokine (i.e., *il1b*), the acute phase response (i.e., *saa5*), complement activation (i.e., *c3a*) and eicosanoid synthesis (i.e., *cox2*, *pgds*) were chosen. In liver, transcripts coding for response to glucocorticoids (i.e., *gra*, *grb*), response to heat stress (i.e., *cirbp*, *hsp70*, *hsp90aa1*, *serpinh1*, *ucp2*), growth (i.e., *ghra*, *ghrb*) and overlapping immune response (i.e., *campb*, *ch25ha*, *pgds*) were selected. Most qPCR primers were taken from previous studies (Beemelmanns et al., 2021b; Caballero-Solares et al., 2017; Eslamloo et al., 2020; Jones et al., 2007; Maugars and Schmitz, 2008; Smith et al., 2018; Soto-Dávila et al., 2020; Xu et al., 2013; Xue et al., 2021); however, *stara*, *starb*, *campb*, *gra*, *grb*, *ghra* and *ghrb* have not been published previously (see Table 3-1 for details on design).

An equimolar pool of cDNA from all samples for a given tissue was used to test amplification efficiencies. Standard curves were generated using four- or five-point serial dilutions (1:2 or 1:3) from 5-10 ng of input RNA. Primer efficiencies met quality standards (Pfaffl, 2001) for linearity ($r^2 \geq 0.992$) and ranged between 90 and 110% (Table 1). Sharp, single, peaks were detected in all melt curves, with the exception of *ef1a* (liver) and *c3a* (head kidney) which had slight shoulders to the left of the primary peak. No evidence of gDNA contamination was found in any of the no-RT controls for either organ. All quality control testing included NTCs for each gene assessed on a plate; these tests never showed signs of contamination.

qPCR reactions were carried out in the same manner as described above for the cDNA pools. Each transcript was run on a single (independent) 384-well plate, which included samples and NTCs in triplicate. Raw expression data for each tissue were imported into qbase+, and technical replicates outside of ± 0.5 C_T from two close replicates were removed. A single head kidney sample (LR salmon at 20°C injected with PBS) was removed from the analysis as it consistently failed to amplify (> 35 C_T) during qPCR. Normalizer genes for head kidney (i.e., *eif3d*, *pabpc1*) were still stable when analyzed amongst all samples (mean geNorm M value and coefficient of variation of 0.369 and 0.131, respectively; with a < 0.5 C_T difference in averages between treatments for *eif3d* and *pabpc1*). The same was noted for *ef1a* and *pabpc1* in liver (mean geNorm M value and coefficient of variation of 0.294 and 0.102, respectively; with a < 1.0 and 0.5 C_T difference in averages between treatments for *ef1a* and *pabpc1*, respectively). In qbase+, NRQ were calculated using amplification efficiencies for each primer pair (Table 1), which were later $\log_2$-transformed in Microsoft Excel.

Table 3-1. qPCR primers used to assess the responses of male Atlantic salmon stress phenotypes when reared at different temperatures and after injection with PBS or the vaccine Forte Micro.

Gene name (GenBank accession number)	Nucleotide sequence (5'-3')	Amplification efficiency (%)	r^2	Amplicon size (bp)	Source
Head Kidney Gene List					
steroidogenic acute regulatory protein a (*stara*) (DY715709)	F: CCTTTATGGGGAGCTGGTG R: TGGGTCCAGACACCTCGTA	90.8	0.996	125	This study[a]
steroidogenic acute regulatory protein b (*starb*) (DQ415678)	F: ATCATTGCTGCGAATGGAG R: AGGGTTCCAATCTCCCATCT	90.8	0.999	150	This study[a]
cholesterol side-chain cleavage enzyme (*cyp11a*, alias *p450scc*) (DQ361039)	F: CTCAAGAATGGGGAGGACTG R: ACTTCATCCAACAGAGGAACAAAG	103.9	0.992	101	Maugars & Schmitz (2008)
cytochrome P450 11B2 (*cyp11b*, alias *p450c11*) (XM_014149090)	F: AAAGAGGGAGGAGAGGAGGAGAG R: GGAGGGAGAGGTGGAGAAGAG	94.9	0.999	135	Maugars & Schmitz (2008)
3β-hydroxysteroid dehydrogenase (*hsd3b*) (XM_014174054)	F: GAGGGGGACATTAGTGATAGTGAG R: GCTGGGTTCCTTTGACGTTG	97.2	0.999	142	Maugars & Schmitz (2008)
toll-like receptor 5a soluble (*stlr5a*) (AY628755)	F: ATCGCCCTGCAGATTTTATG R: GAGCCCTCAGCGAGTTAAAG	97.1	0.999	103	Smith et al. (2018)
C-X-C chemokine receptor type 1-like (*cxcr1*) (CX355704)	F: ATGCTGATTCCCCCTACTCC R: ACACTGCTCAAGCCCAAGAT	96.0	0.991	103	Eslamloo et al. (2020)
cathelicidin b (*campb*) (AY360357)	F: GAGACGCTCTGCAGTAAGGC R: TCAGTGTTGAGGGTGTTGCC	110.0	0.999	131	This study[b]
hepcidin a (*hampa*) (BT125319)	F: ATGAATCTGCCGATGCATTTC R: AATGGCTTTAGTGCTGGCAG	90.1	0.999	134	Eslamloo et al. (2020)
cholesterol 25-hydroxylase-like protein a (*ch25ha*) (BT046542)	F: TAGAGCTGTGATGCTAGTTTAC R: ACCCAGTAGCACTGAGAAGTC	95.0	0.995	106	Eslamloo et al. (2020)
interleukin 1 beta (*il1b*) (AY617117)	F: GTATCCCATCACCCCATCAC R: TTGAGCAGGTCCTTGTCCTT	92.0	0.997	119	Soto-Dávila et al. (2020)
serum amyloid A-5 protein (*saa5*) (BT057477)	F: AGGAGCTGGAAGTTTGTTGC R: TATGCACGCCACATGTCTTT	100.5	0.999	143	Xue et al. (2021)

complement c3a (*c3a*) (XM_014186867)	F: GCTGACAGCATACGTGGTGAA R: ACCCGTCATCTCTGCGTGAA	92.8	0.997	163	Xue et al. (2021)
cyclooxygenase-2 (*cox2*) (AY848944)	F: ACCTTTGTGCGAAACGCTAT R: GAGTAGGCCTCCCAGCTCTT	104.8	0.999	113	Caballero-Solares et al. (2017)
lipocalin-type prostaglandin D synthase (*pgds*) (BT048787)	F: GGTGCTCAACAAGCTCTACA R: GCAGGAAAGCGATGTTGTCA	93.9	0.999	114	Caballero-Solares et al. (2017)
eukaryotic translation initiation factor 3 subunit D (*eif3d*) (GE777139)[c]	F: CTCCTCCTCCTCGTCCTCTT R: GACCCCAACAAGCAAGTGAT	102.9	0.999	105	Caballero-Solares et al. (2017)
polyadenylate-binding protein 1 (*pabpc1*) (EG908498)[c]	F: TGACCGTCTCGGGTTTTTAG R: CCAAGGTGGATGAAGCTGTT	99.5	0.999	108	Xu et al. (2013)
Liver Gene List					
glucocorticoid receptor a (*gra*) (GQ179974)	F: GAGGCTGCAGGTGTCTTATGA R: CTTCCCCAGCTCCTTTATGTA	91.3	0.992	136	This study[a]
glucocorticoid receptor b (*grb*) (EG879323)	F: GAGGTTGCAGGTGTCCTACGA R: CTTACCCAGCTCCTTGATGTA	108.5	0.999	136	This study[a]
cold-inducible RNA-binding protein (*cirbp*) (BT059171)	F: TTGAGTACACAGCGGTGAATT R: ACCAATCTGATGCTATGACGAGA	89.7	0.992	132	Beemelmanns et al. (2021b)
heat shock protein 70 (*hsp70*) (BT045715)	F: AGTGATCAACGACTCGACACG R: CACTGCATTGGTTATAGTCTTG	93.3	0.999	151	Beemelmanns et al. (2021b)
heat shock protein 90-alpha (*hsp90aa1*) (KC150878)	F: CGAGGACATGAAGAAGAGGCAT R: ACACTGTCACCTTCTCCACTTT	92.9	0.996	104	Beemelmanns et al. (2021b)
serpin H1 (*serpinh1*, alias *hsp47*) (XM_014214963)	F: GACCATTCAAAAATCAACCTCA R: CATGGCTCCATCAGCATTCT	90.0	0.994	129	Beemelmanns et al. (2021b)
mitochondrial uncoupling protein 2 (*ucp2*) (XM_014196911)	F: CTGATCTCTGCCGTCACCAT R: AGAAGACTGATGAGGTGAAGACA	96.5	0.999	89	Beemelmanns et al. (2021b)
cathelicidin b (*campb*) (AY360357)	F: GAGACGCTCTGCAGTAAGGC R: TCAGTGTTGAGGGTGTTGCC	94.6	0.999	131	This study[b]
cholesterol 25-hydroxylase-like protein a (*ch25ha*) (BT046542)	F: TAGAGCTGTGATGCTAGTTTAC R: ACCCAGTAGCACTGAGAAGTC	92.7	0.993	106	Eslamloo et al. (2020)

Gene	Primer sequence	Efficiency	R²	Amplicon size	Reference
lipocalin-type prostaglandin D synthase (*pgds*) (BT048787)	F: GGTGCTCAACAAGCTCTACA R: GCAGGAAAGCGATGTTGTCA	93.4	0.999	114	Caballero-Solares et al. (2017)
growth hormone receptor precursor a (*ghra*) (NM_001123576)	F: ACACCTGAGGAGCAGAGGAA R: GTAGCCTCCCACATCAGCAT	99.1	0.998	120	This study[a]
growth hormone receptor precursor b (*ghrb*) (NM_001123594)	F: GAGTGAGGTGAGACCCACG R: AACTCCTTCCTCGGCTTCTC	98.5	0.999	117	This study[a]
elongation factor 1 alpha (*ef1a*) (NM_001141909)[c]	F: GTGGAGACTGGAACCCTGAA R: CTTGACGGACACGTTCTTGA	90.1	0.999	155	Jones et al. (2007)
polyadenylate-binding protein 1 (*pabpc1*) (EG908498)[c]	F: TGACCGTCTCGGGTTTTTAG R: CCAAGGTGGATGAAGCTGTT	97.2	0.999	108	Xu et al. (2013)

[a] Primers were designed as part of two Genomic Applications Partnership Program projects [GAPP #6604, Biomarker Platform for Commercial Aquaculture Feed Development project; and GAPP #6607, Integrated Pathogen Management of Co-infection in Atlantic salmon (IPMC) project] awarded to MLR. These projects were funded by the Government of Canada through Genome Canada and Genome Atlantic, and EWOS Innovation (now part of Cargill, Incorporated). The IPMC project was also funded by the Government of Newfoundland and Labrador's Department of Tourism, Culture, Industry and Innovation (Leverage R&D award #5401-1019-108). These primers were designed by Drs. Jennifer R. Hall and Albert Caballero-Solares.
[b] Primers were designed using Primer3web v.0.4.0 (bioinfo.ut.ee/primer3-0.4.0/).
[c] Normalizer gene

3.3.6 Statistical analyses

The data were first assessed for normality and homoscedasticity via Shapiro-Wilk's and Levene's tests, respectively, and log_{10}-transformed if necessary to meet testing assumptions. Similar to Zanuzzo et al. (2022), I split the statistical analyses into six independent parts/ questions: 1) the effects of stress phenotype and temperature on constitutive expression; 2) the effects of stress phenotype and IP injection of Forte Micro at 12°C; 3) the effects of stress phenotype and IP injection of Forte Micro at 20°C; 4) the effect of IP injection of PBS at 12°C; 5) the effect of IP injection of PBS at 20°C; and 6) the effect of stress phenotype on fold-changes calculated between fish IP injected with PBS and Forte Micro. While the first five analyses listed could have been performed using a four-way ANOVA factorial model [2 stress phenotypes (LR vs. HR) × 2 injections (PBS vs. Forte Micro) × 2 time points (time zero vs. 12 or 24 hpi) × 2 temperatures (12 vs. 20°C)], this would have created many interactions that were not relevant to my main study goals/questions. Thus, to address 1-5 listed above, two-way ANOVAs followed by Tukey's HSD post-hoc tests were used to assess the described variables. Whereas, in part 6, t-tests were used to compare fold-changes between LR and HR salmon at a given sampling point/temperature.

All statistical procedures, including the multivariate analyses [i.e., principal component analysis (PCA) and permutational multivariate analysis of variance (PERMANOVA)] described below, were carried out using R (v.4.1.2) (R Studio Team, 2015). Fold-change values were first calculated by dividing the individual NRQ of Forte Micro-injected salmon by the average NRQ of the temperature and phenotype-matched PBS-injected fish for a given transcript. While biological variability among PBS controls was lost using the fold-change approach, this variability was captured within the ANOVAs used to compare the temperature and phenotype-

matched PBS- and Forte Micro-injected groups. Several studies have calculated fold-change values using the average expression levels of PBS-injected fish relative to fish injected with a vaccine of a different composition (e.g., Barsøe et al., 2021; Braden et al., 2019; Caruffo et al., 2016; Huang et al., 2014; Lund et al., 2019; Rozas-Serri et al., 2019; Veenstra et al., 2017; Yang et al., 2013; Zanuzzo et al., 2020). For multivariate analyses, fold-change values were converted into individual standardized values (z_i) using the following equation:

$$z_i = \frac{x_i - \min(x)}{\max(x) - \min(x)}$$

where x_i is a specific fold-change value for a given comparison (e.g., LR and HR responses to Forte Micro injection at 12°C) and x is the range of fold-change values across that comparison for a given transcript. These standardized fold-change values were then used to conduct the PCAs (factoextra R package) and PERMANOVAs (vegan R package). These tested either all target transcripts assessed in each tissue or subsets of transcripts based on related functions. Statistical differences were considered significant at $p < 0.05$, with trends noted at $0.05 \leq p < 0.10$.

3.4 Results

The complete set of head kidney and liver gene expression statistical results is available in Appendix B Table B-4. The significant results ($p < 0.05$) and some of the key trends ($0.05 \leq p < 0.10$) are detailed below. For ease of interpretation, each multi-panel figure showing qPCR results is individually described in the following order: findings at time zero (i.e., constitutive/basal expression); followed by salmon reared at 12°C that were previously injected with PBS or Forte Micro and sampled at 24 hpi; and lastly, previously injected salmon reared at 20°C and sampled at 12 hpi. Transcripts within each multi-panel figure depicting qPCR results

are ordered based on the functions listed in each figure legend, and then listed alphabetically. In the case of Figure 3-2, these transcripts are listed in descending order by when they are activated in the cortisol synthesis pathway.

3.4.1 Effects of stress phenotype on head kidney transcript expression

The responses of 5 transcripts coding for proteins involved in glucocorticoid synthesis (*stara*, *starb*, *cyp11a*, *cyp11b* and *hsd3b*) within the head kidney of LR and HR salmon exposed to heat stress (20°C) and bacterial immune stimulation are shown in Figure 3-2. At time zero, constitutive expression was unaffected by both temperature and stress phenotype. In fish reared at 12°C, no significant differences between time-matched PBS controls and Forte Micro-injected fish were detected in either stress phenotype (i.e., these transcripts were not significantly responsive to Forte Micro). However, when comparing fold-change values (i.e., comparing time-matched PBS and Forte Micro-injected salmon) between stress phenotypes, a significantly greater absolute fold-change was detected in HR as compared to LR salmon for *stara* (-2.6- vs. -1.1-fold, respectively; Fig. 3-2A). Thus, *stara* expression was lowered by a greater amount by Forte Micro in HR fish. In contrast, in salmon reared at 20°C, while the overall interaction term for injection treatment trended towards significance ($0.05 < p < 0.10$) for *cyp11a*, *cyp11b* and *hsd3b*, no significant differences in fold-change were found between the stress phenotypes (Fig. 3-2C-E).

The expression levels of 2 transcripts coding for immune receptors (*stlr5a* and *cxcr1*) and 3 transcripts coding for immune effectors (*campb*, *hampa* and *ch25ha*) in response to the same treatments are shown in Figure 3-3. Elevated rearing temperature significantly increased the constitutive expression of *stlr5a* in both LR and HR salmon. While not significant in separate

pairwise comparisons, temperature had a highly significant overall effect ($p = 0.005$) on the expression of *campb*, with expression at 20°C being lower than that at 12°C (Fig. 3-3C). PBS injection into 12°C acclimated fish decreased the expression of *cxcr1* and increased the expression of *hampa* in LR salmon as compared to time zero. PBS injection did not have a significant impact on the expression of any other transcripts at 12°C as compared to time zero. Relative to PBS controls, Forte Micro significantly impacted the expression of all 5 transcripts, except for *stlr5a* and *ch25ha* in LR fish at 12°C. A significantly higher fold-change was measured for *stlr5a* in HR fish at this time point as compared to LR fish at 12°C (7.1- vs. 2.2-fold, respectively; Fig. 3-3A). While not significant, a similar pattern of higher fold-changes in HR vs. LR salmon was observed for *campb* (19.2- vs. 10.6-fold, respectively; Fig. 3-3C) and *ch25ha* (5.2- vs. 3.2-fold, respectively; Fig. 3-3E). At 20°C, *campb* expression was significantly higher following PBS-injection relative to time zero in both stress phenotypes (Fig. 3-3D), while the same was true for *stlr5a* expression in LRs (Fig. 3-5A). Forte Micro induced changes in expression as compared to PBS-injected fish in all cases at 20°C except for *ch25ha* in LR fish (although it followed the same pattern). Differences in fold-change between the phenotypes were detected at 20°C for *ch25ha* (3.2-fold HR vs. 1.8-fold LR; $p < 0.01$; Fig. 3-3E). A pattern of higher fold-changes in HR than LR salmon at 20°C was also evident for *stlr5a* (3.4-fold HR vs. 2.3-fold LR; $p = 0.072$; Fig. 3-3A), *campb* (41.9- HR vs. 30.8-fold LR; $p = 0.476$; Fig. 3-3C) and *hampa* (i.e., 8.1- HR vs. 4.7-fold LR; $p = 0.134$; Fig. 3-3D).

The qPCR results from the remaining immune-relevant transcripts (*il1b*, *saa5*, *c3a*, *cox2* and *pgds*) measured in the head kidney are depicted in Figure 3-4. Only the constitutive expression of *c3a* in LR salmon was significantly affected by temperature (with expression higher at 20°C as compared to 12°C by 5.8-fold; Fig. 3-4C); although the same pattern was

evident for HR salmon (a 2.7-fold increase). In fish reared at 12°C, Forte Micro-injected LR and HR fish exhibited higher expression of *saa5* and *cox2* as compared to their respective PBS controls (Fig. 3-4B and 3-4D), and the expression of *il1b* in both stress phenotypes and *c3a* in HR salmon were close to significant (i.e., $0.05 < p < 0.10$; Fig. 3-4A and 3-4C). Injection of Forte Micro did not impact the expression of *pgds* at either time point/temperature (Fig. 3-4E). In addition, a pattern of greater absolute fold-changes (i.e., comparing PBS- and Forte Micro-injected salmon at 12°C) in HR salmon relative to LR salmon was observed for 4 out of the 5 transcripts (i.e., *saa5*, *c3a*, *cox2* and *pgds*; albeit non-significant in all cases). In fish reared at 20°C, PBS injection increased the expression of *il1b* in LR fish relative to time zero (Fig. 3-4A). The injection of PBS, however, did not impact the expression of any other transcript. While the overall interaction term for injection treatment was significant for all 5 transcripts at 20°C, significant differences between time- and stress phenotype-matched PBS- and Forte Micro-injected salmon were only evident for *saa5* and *cox2*, and for *il1b* in HR salmon. Nonetheless, this still indicates that Forte Micro induced changes in the expression of all transcripts relative to PBS controls. Fold-change differences were found when comparing stress phenotype-specific transcript expression responses to Forte Micro vs. temperature/time-matched PBS controls for *il1b* (2.9-fold HR vs. 1.7-fold LR, $p < 0.01$; Fig. 3-4A) and *saa5* (24.9-fold HR vs. 12.3-fold LR, $p = 0.056$; Fig. 3-4B).

Multivariate analyses were conducted to help determine whether some of the trends and patterns described above were significant when the transcript expression data in the head kidney were pooled. While several combinations were tested (Appendix B Table B-5), the strongest results came from analyzing the transcription fold-changes of the 10 immune-related transcripts (*stlr5a*, *cxcr1*, *campb*, *hampa*, *ch25ha*, *il1b*, *saa5*, *c3a*, *cox2* and *pgds*) at 20°C. Figure 3-5A

shows how the individual salmon separate in multivariate space on a PCA plot. LR and HR salmon separated ($p < 0.05$) based on principal component axis 1 (PC1; Fig. 3-5D), and while PC2 helped to separate the eigenvectors of each transcript, it did not differentiate between the stress phenotypes ($p > 0.05$; Fig. 3-5E). As shown in Figure 3-5C, *ch25ha*, *hampa*, *il1b*, *saa5* and *stlr5a* were the highest loading transcripts on PC1, and therefore, contributed the most to separating LR and HR fish on this axis. To further confirm that this difference was real, a PERMANOVA was conducted. This analysis found a significant difference in the fold-changes in transcript expression between the stress phenotypes (Fig. 3-5B). Collectively, these results reveal that HR salmon mounted a more robust immune response when injected with Forte Micro than LR salmon at this elevated temperature.

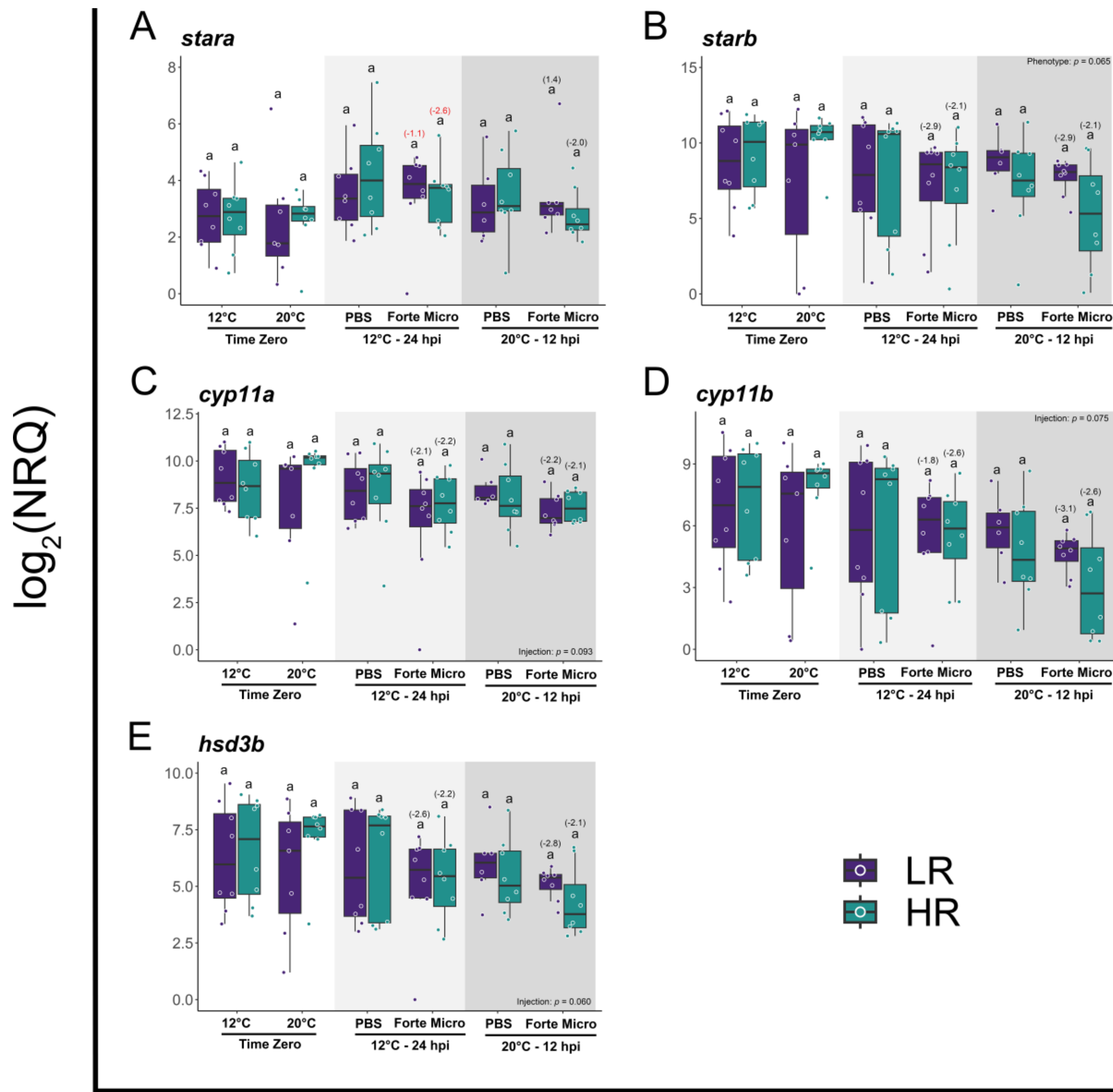

Figure 3-2. Expression levels of 5 transcripts coding for proteins involved in glucocorticoid synthesis [*stara* (A), *starb* (B), *cyp11a* (C), *cyp11b* (D), *hsd3b* (E)] in the head kidney of male Atlantic salmon characterized as being low (LR) or high (HR) stress responders and sampled at 12 or 20°C. Samples were taken prior to injection (time zero; i.e., representative of constitutive expression), and 12 h (20°C) or 24 h (12°C) post-injection (hpi) with PBS or the vaccine Forte Micro. Lower and upper box boundaries indicate the 2nd and 3rd quartiles, respectively, the line inside the box is the median value, and the top and bottom whiskers show the upper and lower 25% of values, respectively. The individual symbols (solid circles) represent individual fish. Normalized relative quantities (NRQs) were compared by two-way ANOVA ($p < 0.05$; n=6-8 per sampling/phenotype). Dissimilar lower case letters denote significant differences between temperatures within a phenotype at time zero, or between injection treatments within a phenotype at 12 or 24 hpi. Numbers in parentheses denote mean fold-change values between time-matched PBS- and Forte Micro-injected fish, with red lettering signifying differences

between phenotypes (t-test; $p < 0.05$). Interactions which did not quite reach significance ($0.05 \leq p < 0.10$) are also indicated at the margin of each panel if significant effects have not already been noted.

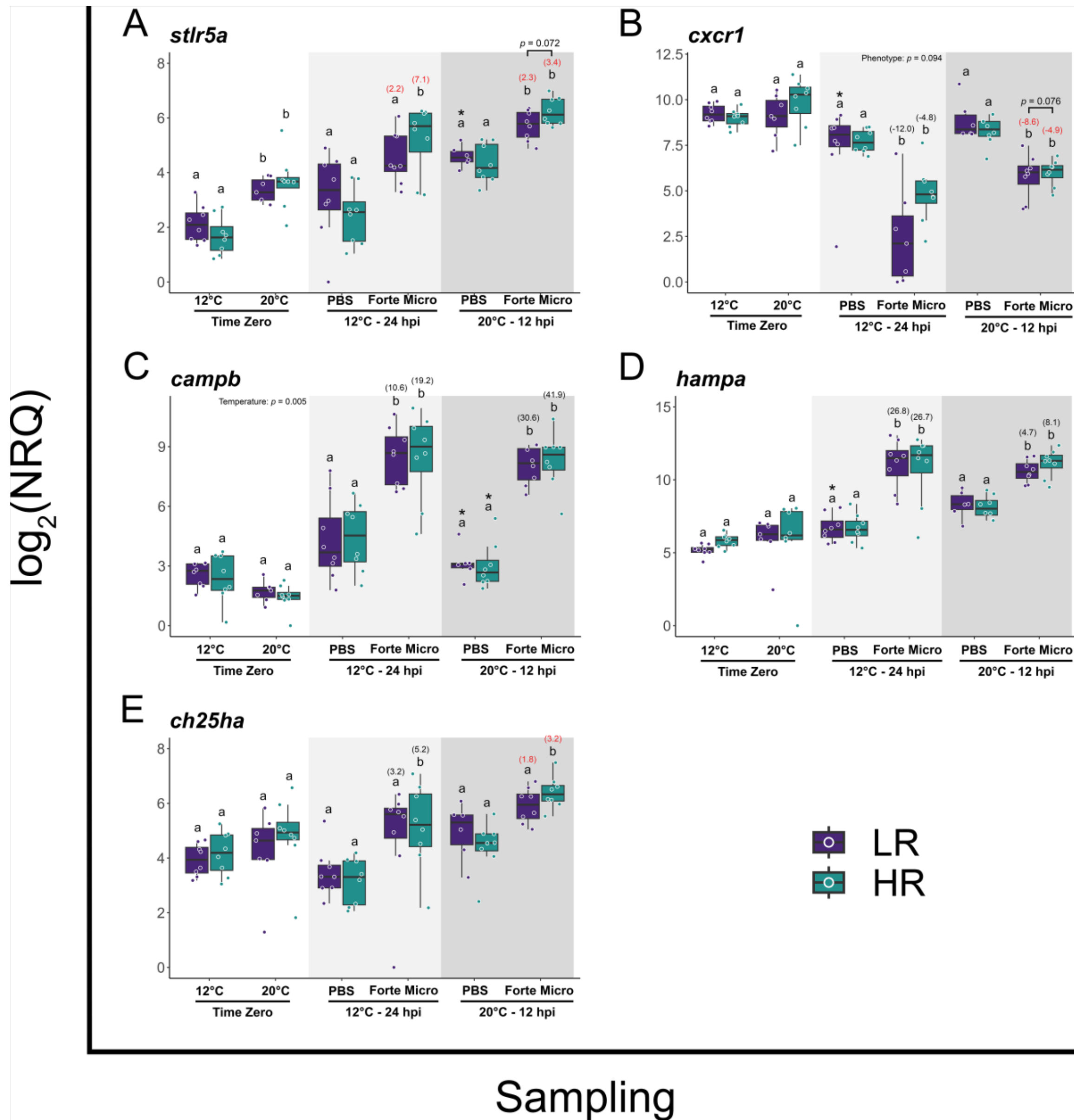

Figure 3-3. Expression levels of 5 transcripts coding for immune receptors [*stlr5a* (A), *cxcr1* (B)] and immune effectors [*campb* (C), *hampa* (D), *ch25ha* (E)] in the head kidney of male Atlantic salmon characterized as being low (LR) or high (HR) stress responders and sampled at 12 or 20°C. Samples were taken prior to injection (time zero; i.e., representative of constitutive expression), and 12 h (20°C) or 24 h (12°C) post-injection (hpi) with PBS or the vaccine Forte Micro. Lower and upper box boundaries indicate the 2nd and 3rd quartiles, respectively, the line inside the box is the median value, and the top and bottom whiskers show the upper and lower 25% of values, respectively. The individual symbols (solid circles) represent individual fish. Normalized relative quantities (NRQs) were compared by two-way ANOVA ($p < 0.05$; n=6-8

per sampling/phenotype). Dissimilar lower case letters denote significant differences between temperatures within a phenotype at time zero, or between injection treatments within a phenotype at 12 or 24 hpi. Numbers in parentheses denote mean fold-change values between time-matched PBS- and Forte Micro-injected fish, with red lettering signifying differences between phenotypes (t-test; $p < 0.05$, unless indicated by brackets above). Asterisks indicate significant differences between temperature and phenotype-matched time zero and PBS-injection measurements (two-way ANOVA; $p < 0.05$). Significant interactions and those which did not quite reach significance ($p < 0.05$, $0.05 \leq p < 0.10$, respectively) are also indicated at the margin of each panel if significant effects have not already been noted.

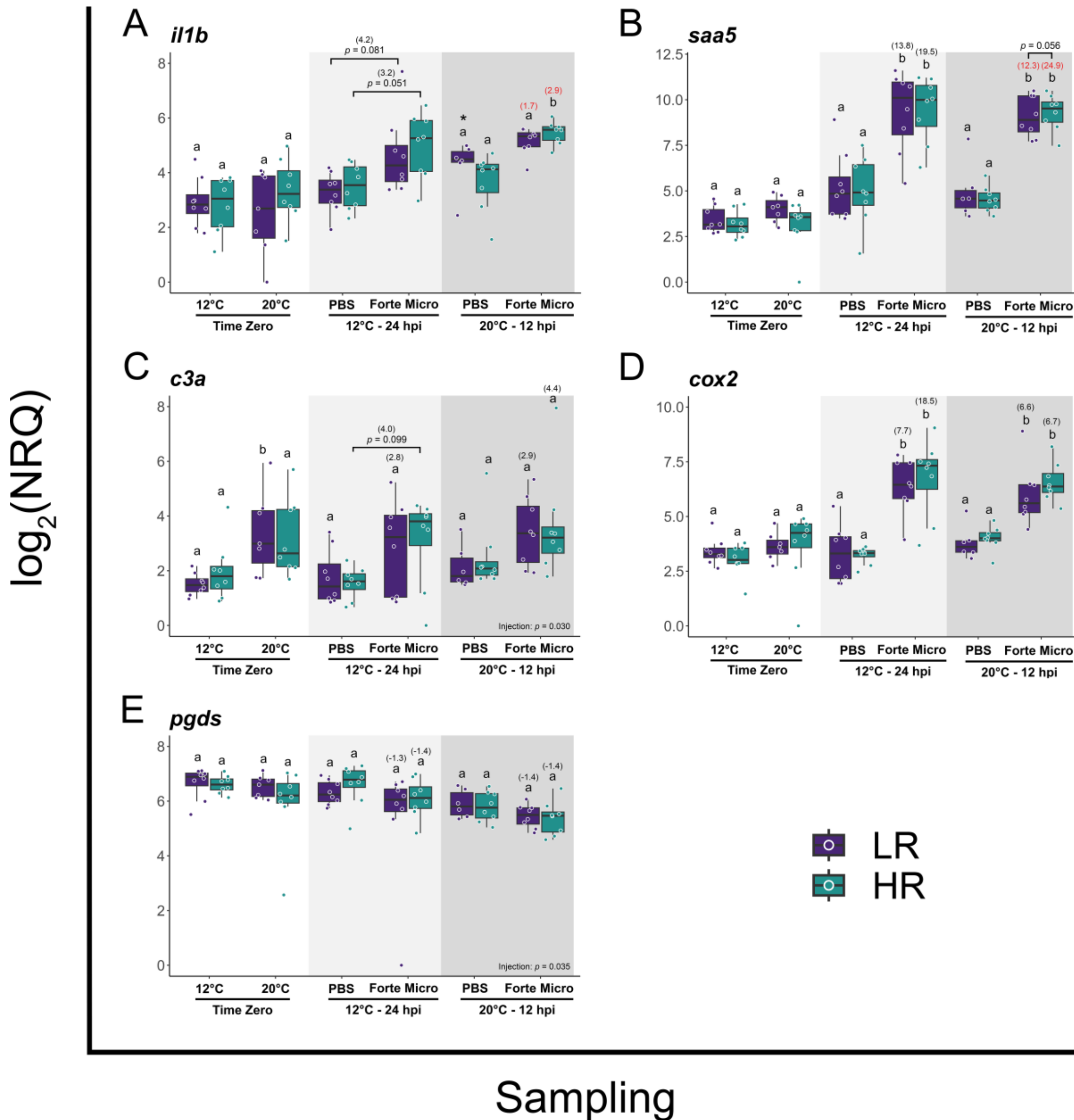

Figure 3-4. Expression levels of 5 transcripts coding for a cytokine [*il1b* (A)] and proteins involved in the acute phase response [*saa5* (B)], complement activation [*c3a* (C)] and eicosanoid synthesis [*cox2* (D), *pgds* (E)] in the head kidney of male Atlantic salmon characterized as being

low (LR) or high (HR) stress responders and sampled at 12 or 20°C. Samples were taken prior to injection (time zero; i.e., representative of constitutive expression), and 12 h (20°C) or 24 h (12°C) post-injection (hpi) with PBS or the vaccine Forte Micro. Lower and upper box boundaries indicate the 2nd and 3rd quartiles, respectively, the line inside the box is the median value, and the top and bottom whiskers show the upper and lower 25% of values, respectively. The individual symbols (solid circles) represent individual fish. Normalized relative quantities (NRQs) were compared by two-way ANOVA ($p < 0.05$; n=6-8 per sampling/phenotype). Dissimilar lower case letters denote significant differences between temperatures within a phenotype at time zero, or between injection treatments within a phenotype at 12 or 24 hpi. Numbers in parentheses denote mean fold-change values between time-matched PBS- and Forte Micro-injected fish, with red lettering signifying differences between phenotypes (t-test; $p < 0.05$, unless indicated by brackets above). Asterisks indicate significant differences between temperature and phenotype-matched time zero and PBS-injection measurements (two-way ANOVA; $p < 0.05$). Significant interactions ($p < 0.05$) are also indicated at the margin of each panel if significant effects have not already been noted.

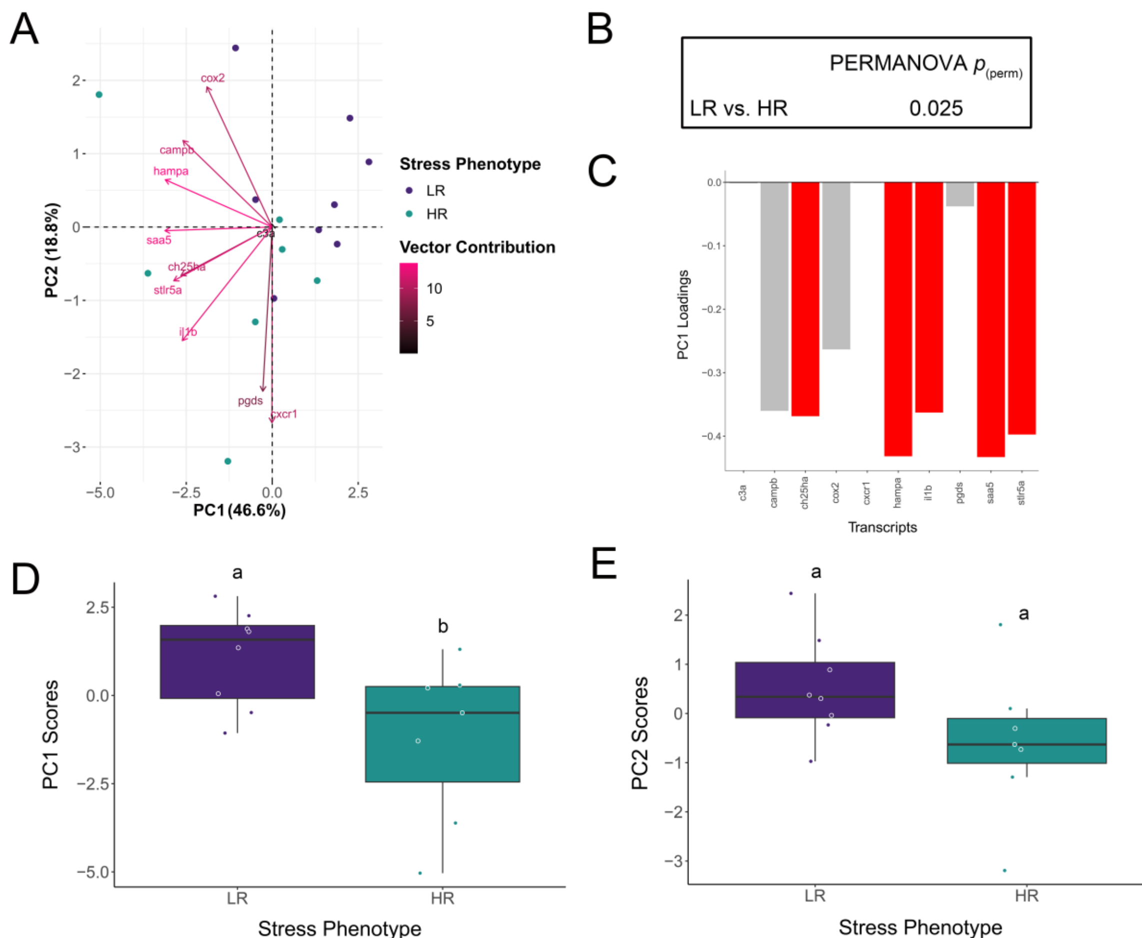

Figure 3-5. Multivariate analyses of the fold-change data measured across 10 immune-related transcripts in the head kidney at 12 hpi (20°C). (A) Principal component analysis (PCA) plot depicting how stress phenotype impacted the distribution of salmon in multivariate space (n = 8 for LR; n = 7 for HR). Eigenvectors are coloured based on their contribution to principal

component axis 1 (PC1) and PC2. (B) Results of the permutational multivariate analysis of variance analysis (PERMANOVA). (C) Loadings of transcripts on PC1 with the top five highest loadings highlighted in red. (D, E) Boxplots of PC1 and PC2 scores, with dots representative of individual salmon from panel A. Dissimilar lower case letters signify differences between stress phenotypes (t-test; $p < 0.05$).

3.4.2 Effects of stress phenotype on liver transcript expression

The responses of selected transcripts in the liver of LR and HR salmon to elevated temperature and bacterial antigen stimulation are shown in Figure 3-6. These include 2 transcripts coding for glucocorticoid receptors (*gra* and *grb*) and 5 transcripts encoding for heat stress responsive proteins (*cirbp*, *hsp70*, *hsp90aa1*, *serpinh1* and *ucp2*). At time zero, constitutive expression of 5 out of the 7 transcripts was influenced by rearing temperature (*gra* and *cirbp* were the exceptions). Expression of *grb* was lower at 20°C as compared to at 12°C in LR salmon (with the same non-significant pattern observed in HR salmon; Fig. 3-6B). All three heat shock proteins [i.e., *hsp70*, *hsp90aa1* and *serpinh1* (alias *hsp47*)] were strongly upregulated (i.e., 3 to 9-fold on average) at 20°C relative to at 12°C in both LR and HR fish. Finally, *ucp2* was downregulated at the higher temperature in both stress phenotypes (Fig. 3-6G).

In fish reared at 12°C the expression of *gra* and *grb* in both stress phenotypes was downregulated following PBS injection as compared to time zero (Fig. 3-6A and 3-6B). Conversely, *hsp70* was upregulated in PBS-injected LR and HR salmon relative to constitutive levels (Fig. 3-6D), and the same was true for *hsp90aa1* in LR salmon (Fig. 6E). In both stress phenotypes, *gra* (↓), *grb* (↓), *hsp70* (↑) and *hsp90aa1* (↑) expression at 12°C, as well as *ucp2* (↑) expression in the LR group, differed significantly between PBS- and Forte Micro-injected fish. Significant ($p < 0.05$) interaction terms for injection treatment were also found for *cirbp* and *serpinh1*, indicating that bacterial stimulation did have an effect on the expression of these transcripts at 12°C (Fig. 3-6C and 3-6F). Significantly higher absolute fold-changes for *gra* (-

2.9- vs. -1.6-fold, respectively; $p < 0.01$; Fig. 3-6A) and *ucp2* (2.3- vs. 1.4-fold, respectively; $p < 0.05$; Fig. 3-6G) were found for 12°C LR fish after injection with PBS and Forte Micro as compared to HR salmon. Further, a similar but non-significant, trend at 12°C was observed for *grb* (-3.4- LR vs. -2.1-fold HR; $p = 0.089$; Fig. 3-6B).

At 20°C, PBS injection significantly increased the expression of *hsp70* relative to time zero in both stress phenotypes (Fig. 3-6D). For *gra* and *grb*, expression was only significantly downregulated following PBS-injection at 20°C as compared to expression at time zero in HR salmon (Fig. 3-6A and 3-6B). Similar to at 12°C, the expression of *gra* (↓), *grb* (↓), *hsp70* (↑), *hsp90aa1* (↑) and *ucp2* (↑) at 20°C was significantly different in both phenotypes between PBS and Forte Micro-injected fish at 20°C. Further, based on the overall interaction term, it appears that PBS vs. Forte Micro injection also impacted the expression responses of *cirbp* (↓, $p = 0.055$) and *serpinh1* (↑, $p = 0.004$). No significant differences in the fold-change of transcript expression were evident between the stress phenotypes at this temperature.

The expression levels of 2 liver transcripts related to growth (*ghra* and *ghrb*), and 3 immune-related transcripts that overlap with the head kidney dataset (*campb*, *ch25ha* and *pgds*), in response to the same temperature and injection treatments are shown in Figure 3-7. The constitutive expression of *ghrb* at time zero in both stress phenotypes, and that of *pgds* in HR fish, were lower at 20°C as compared to at 12°C (Fig. 3-7B and 3-7E). In contrast, for *ghra*, the phenotype interaction term was significant ($p = 0.033$), and appeared to be driven by an increase in expression at 20°C in LR salmon as compared to HR salmon ($p = 0.055$ between stress phenotypes; Fig. 3-7A). PBS injection lowered the expression of *pgds* in LR salmon at 12°C relative to time zero. In salmon reared at 12°C, Forte Micro only increased *campb* and *ch25ha* expression in comparison to the PBS controls at 24 hpi in both stress phenotypes (Fig. 3-7C and

3-7D). Stress phenotype had no effect on the fold-change differences in expression between salmon injected with PBS or Forte Micro. At 20°C, the expression of *ghra*, *campb* and *ch25ha* was significantly higher in Forte Micro-injected LR and HR fish relative to the time-matched PBS controls. Further, the overall term for injection was significant, and this shows that Forte Micro decreased the expression of *ghrb* in comparison to 20°C PBS-injected salmon (Fig. 3-7B). A significant difference in the fold-change of expression between stress phenotypes at 20°C was found for *ch25ha* (14.8-fold HR vs. 7.8-fold LR; $p < 0.01$; Fig. 3-7D).

Multivariate analyses were also used to supplement/enhance the interpretation of the reported findings for the effects of temperature and injection with PBS vs. Forte Micro (Appendix B Table B-5). Analysis of the fold-changes of the 7 stress-related transcripts (*gra*, *grb*, *cirbp*, *hsp70*, *hsp90aa1*, *serpinh1*, *ucp2*) at 12°C was the most informative in this regard (Fig. 3-8A). Although it did not quite reach significance ($p = 0.057$), the PCA analysis suggests that the stress phenotypes could be separated on PC1 (Fig. 3-8D) but not PC2 (Fig. 3-8E). The 3 highest loading transcripts on PC1 were *gra*, *hsp90aa1* and *ucp2* (Fig. 3-8C). A PERMANOVA was used to further investigate this hypothesis (Fig. 3-8B), and while this analysis did not detect a significant effect of phenotype ($p_{(perm)} = 0.051$), overall, these results suggest that LR salmon mounted a more robust stress response to Forte Micro injection at 12°C.

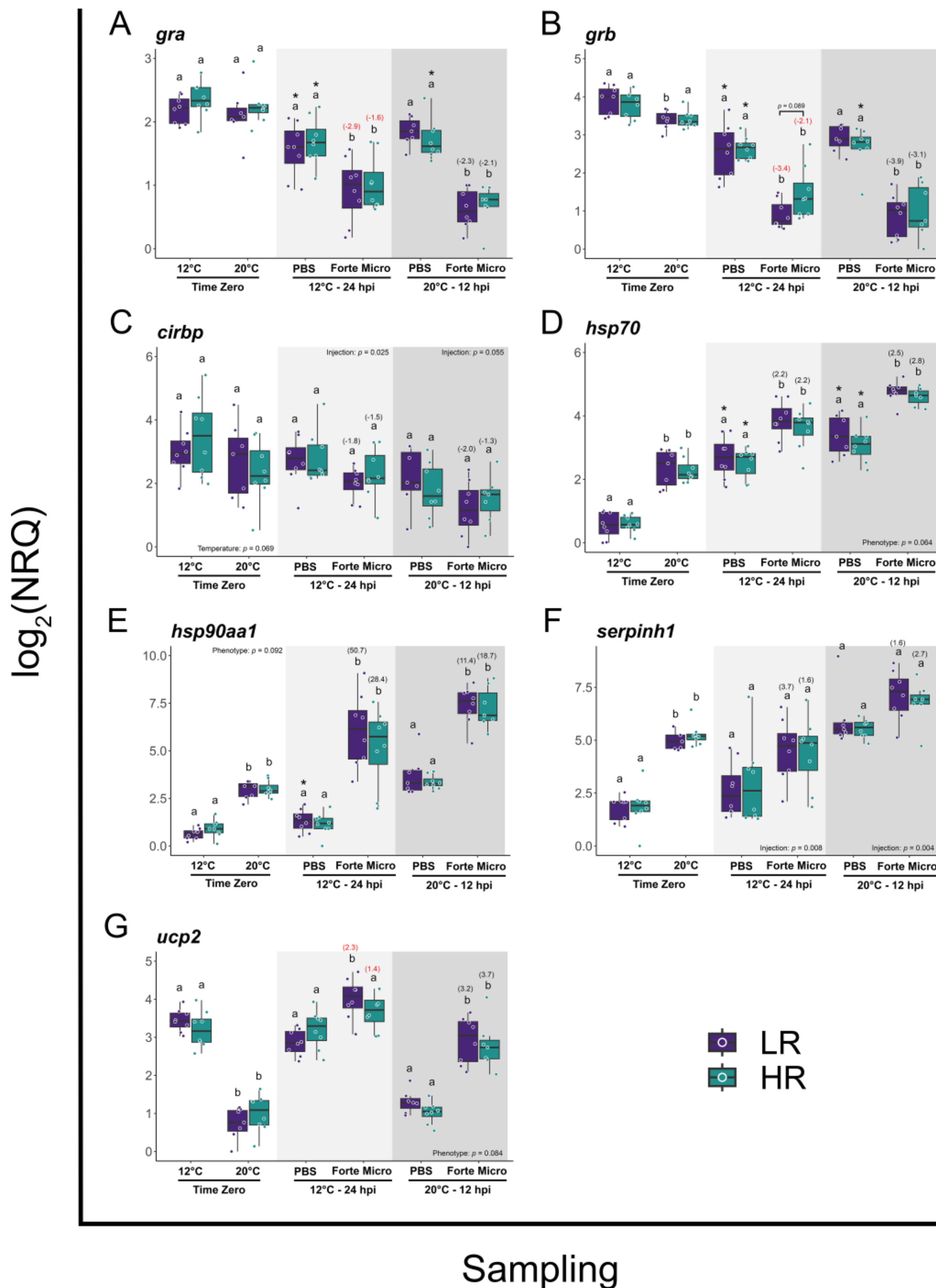

Figure 3-6. Expression levels of 7 transcripts coding for response to glucocorticoids [*gra* (A), *grb* (B)] and heat stress [*cirbp* (C), *hsp70* (D), *hsp90aa1* (E), *serpinh1* (F), *ucp2* (G)] in the liver of male Atlantic salmon characterized as being low (LR) or high (HR) stress responders and sampled at 12 or 20°C. Samples were taken prior to injection (time zero; i.e., representative of constitutive expression), and 12 h (20°C) or 24 h (12°C) post-injection (hpi) with PBS or the vaccine Forte Micro. Lower and upper box boundaries indicate the 2nd and 3rd quartiles, respectively, the line inside the box is the median value, and the top and bottom whiskers show the upper and lower 25% of values, respectively. The individual symbols (solid circles) represent individual fish. Normalized relative quantities (NRQs) were compared by two-way ANOVA (p <

0.05; n=6-8 per sampling/phenotype). Dissimilar lower case letters denote significant differences between temperatures within a phenotype at time zero, or between injection treatments within a phenotype at 12 or 24 hpi. Numbers in parentheses denote mean fold-change values between time-matched PBS- and Forte Micro-injected fish, with red lettering signifying differences between phenotypes (t-test; $p < 0.05$, unless indicated by brackets above). Asterisks indicate significant differences between temperature and phenotype-matched time zero and PBS-injection measurements (two-way ANOVA; $p < 0.05$). Significant interactions and those which did not quite reach significance ($p < 0.05$, $0.05 \leq p < 0.10$, respectively) are also indicated at the margin of each panel if significant effects have not already been noted.

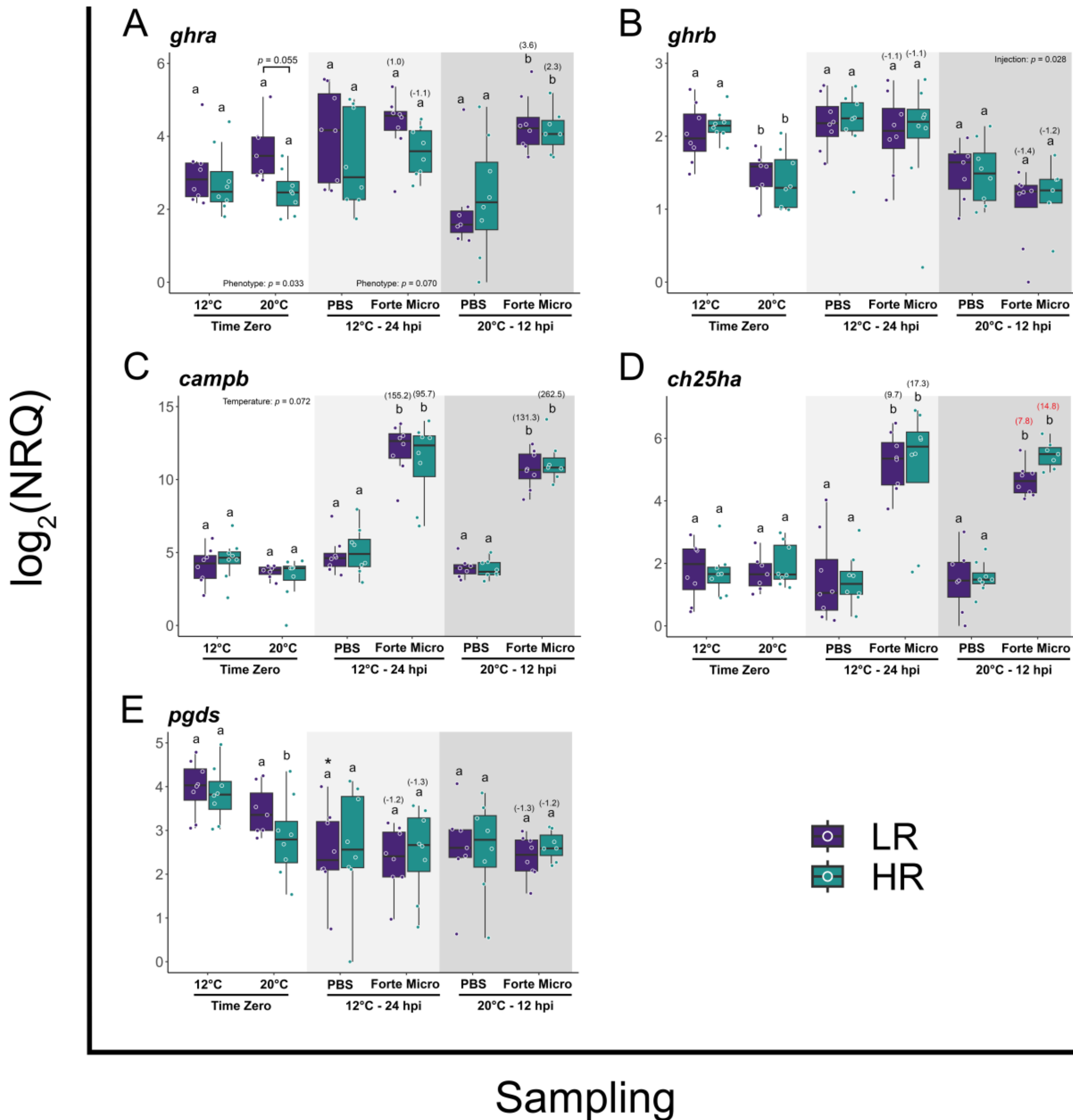

Figure 3-7. Expression levels of 5 transcripts coding for growth [*ghra* (A), *ghrb* (B)] and immune response [*campb* (C), *ch25ha* (D), *pgds* (E)] in the liver of male Atlantic salmon characterized as being low (LR) or high (HR) stress responders and sampled at 12 or 20°C.

Samples were taken prior to injection (time zero; i.e., representative of constitutive expression), and 12 h (20°C) or 24 h (12°C) post-injection (hpi) with PBS or the vaccine Forte Micro. Lower and upper box boundaries indicate the 2nd and 3rd quartiles, respectively, the line inside the box is the median value, and the top and bottom whiskers show the upper and lower 25% of values, respectively. The individual symbols (solid circles) represent individual fish. Normalized relative quantities (NRQs) were compared by two-way ANOVA ($p < 0.05$; n=6-8 per sampling/phenotype). Dissimilar lower case letters denote significant differences between temperatures within a phenotype at time zero, or between injection treatments within a phenotype at 12 or 24 hpi. Numbers in parentheses denote mean fold-change values between time-matched PBS- and Forte Micro-injected fish, with red lettering signifying differences between phenotypes (t-test; $p < 0.05$). Significant interactions and those which did not quite reach significance ($p < 0.05$, $0.05 \leq p < 0.10$, respectively) are also indicated at the margin of each panel if significant effects have not already been noted.

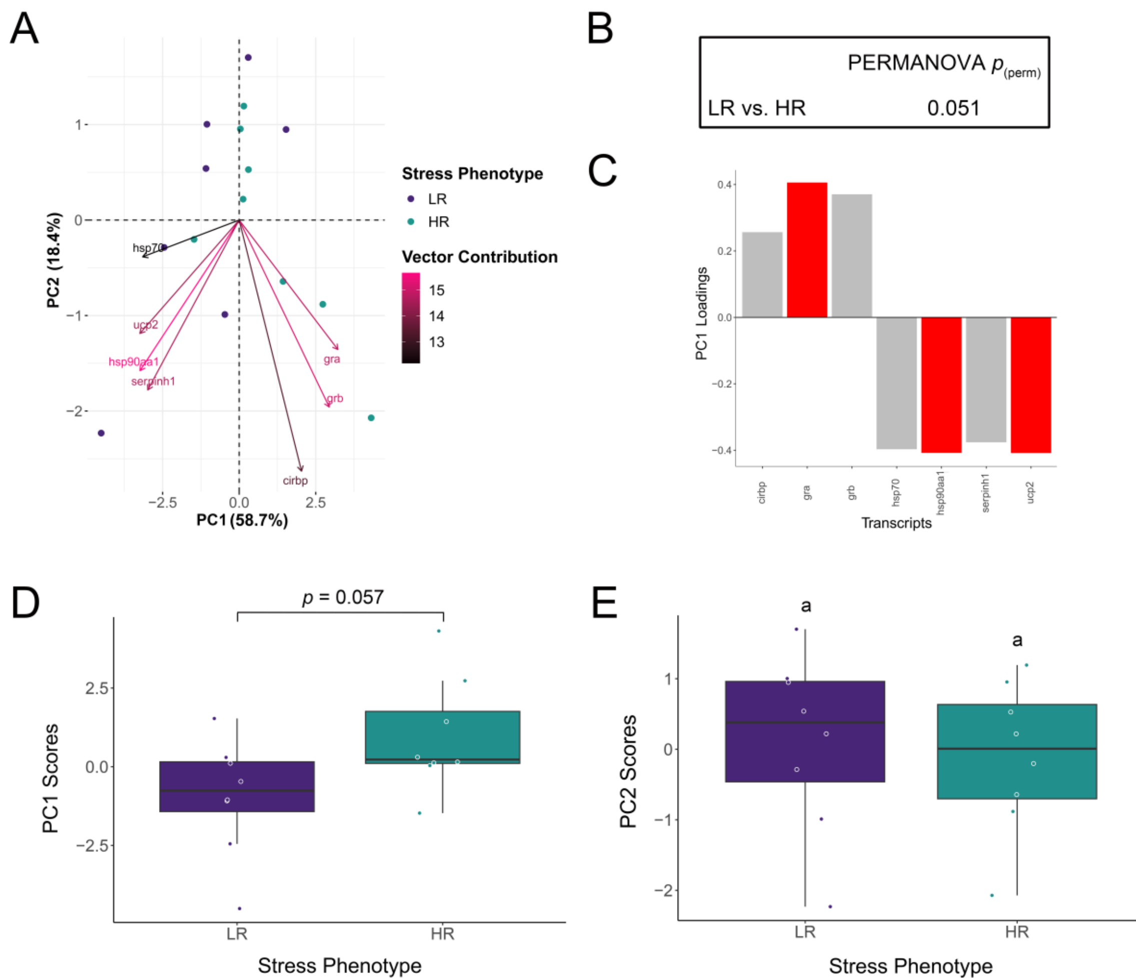

Figure 3-8. Multivariate analyses of fold-change data measured across 7 stress-related transcripts in liver tissue at 24 hpi (12°C). (A) Principal component analysis (PCA) plot depicting how stress phenotype impacted the distribution of salmon in multivariate space (n = 8 for LR & HR). Eigenvectors are coloured based on their contribution to principal component axis 1 (PC1) and PC2. (B) Results of the permutational multivariate analysis of variance analysis

(PERMANOVA). (C) Loadings of transcripts on PC1 with the top three highest loadings highlighted in red. (D, E) Boxplots of PC1 and PC2 scores with dots representative of individual salmon from panel A. The *p*-value is given where a trend was observed (t-test).

3.5 Discussion

Rising sea surface temperatures pose a variety of threats to aquatic species, including Atlantic salmon. However, limited information is available regarding: how suboptimal thermal conditions affect their immune and other biological processes; or how stress phenotype may influence these temperature-dependent responses. In general, genomic studies of immune stimulation primarily focus on the expression of transcripts directly related to immune pathways. However, my study shows that transcripts associated with heat stress and growth, as well as responses to glucocorticoids, may also be useful and important to examine during the response to bacterial stimulation. Interestingly, the expression of *hsp70*, *hsp90aa1* and *ucp2* were significantly higher, while *gra* and *grb* were significantly lower, in the livers of Forte Micro- vs. PBS-injected fish (Fig. 3-6). Similarly, injection of these antigens influenced the expression of *cirbp* (↓), *serpinh1* (↑), *ghra* (↓) and *ghrb* (↓) in at least one temperature/time point combination (Fig. 3-6 & 3-7). A synthesis of the transcripts/pathways affected by Forte Micro injection is shown in Figure 3-9. Notably, transcripts encoding immune effectors in both the head kidney and liver, as well as the acute phase response in the head kidney, were highly responsive to vaccine injection. With regard to the influence of stress phenotype on the transcript expression responses examined, the PCAs and PERMANOVAs: 1) showed that fold-changes in the expression of 10 immune transcripts assessed in head kidney were significantly higher in HR than LR salmon at 20°C (Fig. 3-5); and 2) suggest ($p = 0.057$) that Forte Micro injection resulted in a more robust response of the 7 stress-related transcripts in LR vs. HR fish at 12°C (Fig. 3-8). Collectively, these results show that while differences in transcript expression following bacterial stimulation

between the stress phenotypes are more subtle than expected, the magnitude and direction of differences are influenced by temperature.

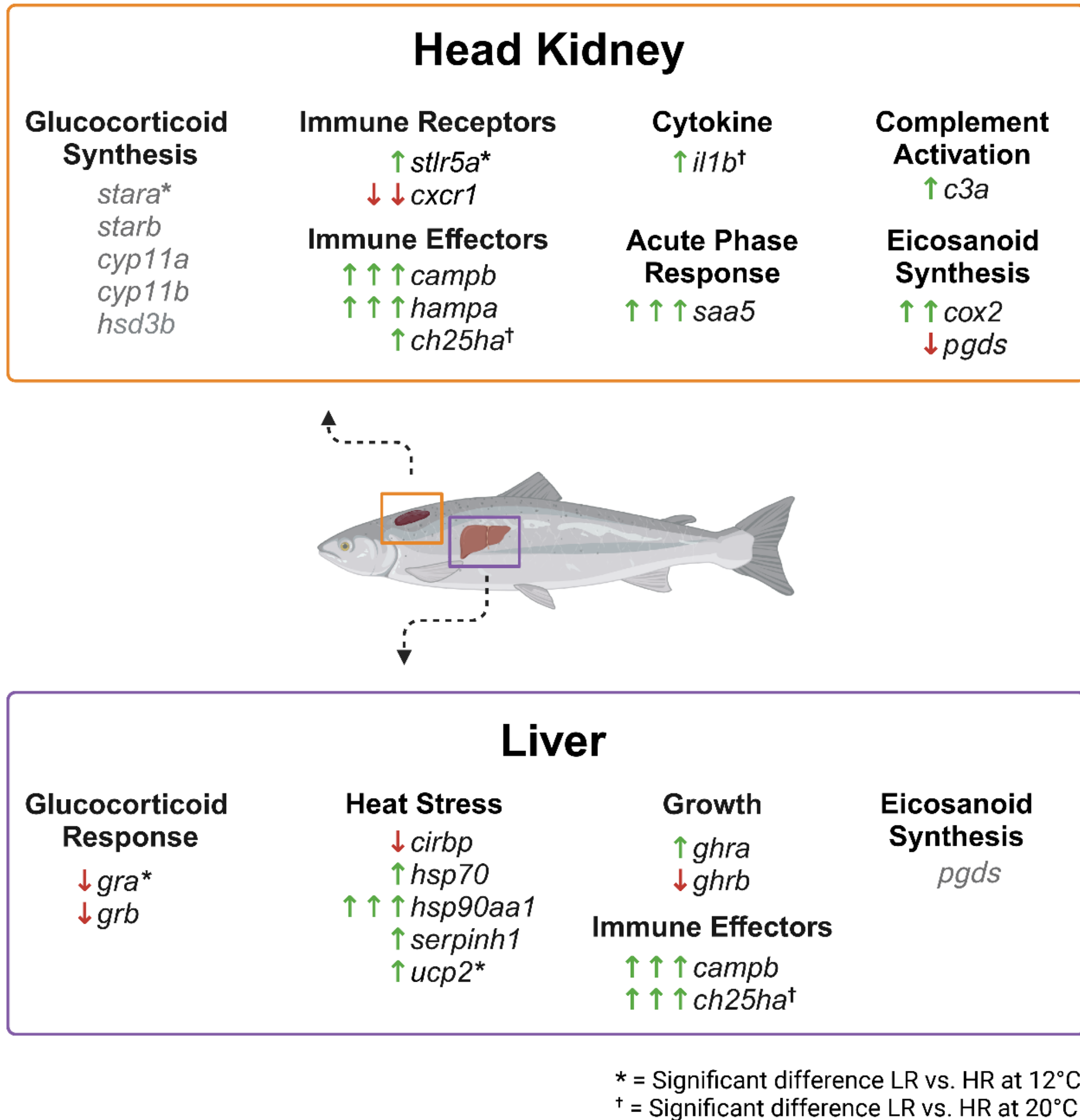

Figure 3-9. Overview of the transcript expression responses of male Atlantic salmon to Forte Micro injection. Black text and arrows indicate whether expression significantly differed ($p < 0.05$) between Forte Micro and PBS-injected fish based on the ANOVA interaction term for injection. The direction of the arrows reflects whether the transcript was upregulated (↑) or downregulated (↓) by Forte Micro relative to PBS injection, with the number of arrows signifying the average fold-change (FC) across treatments (e.g., ↑, FC ≤ 6; ↑↑, 6 < FC ≤ 10; ↑↑↑, 10 < FC). Grey text and the absence of arrows reflects that no significant (p ≥ 0.05) interaction was detected among any comparison. Asterisks (*) and daggers (†) indicate whether fold-

changes significantly differed ($p < 0.05$) between LR and HR salmon at 12 and 20°C, respectively. Created with BioRender.com.

3.5.1 Stress phenotype as a consistent, characterizable trait

Variation in responses to the same stressor, even within Atlantic salmon previously characterized as a particular stress phenotype, has been reported (Fevolden et al., 1991). While I reported consistent responses of LR and HR salmon in the current study to repeated net stresses at 9°C (Chapter 2), there were changes in time, temperature and environment during the course of the current experiment that could have altered the stress responses of individual salmon. It is possible that the fish originally characterized as HR fish adapted to the handling stress and transitioned to being LRs three months later when the current study was conducted. However, I think that this is unlikely as past research has shown that individual stress phenotype remained consistent in rainbow trout 28 months after initial characterization, even with changes in environmental conditions (Pottinger et al., 1992). Therefore, while cortisol levels were not re-measured in this experiment (an admitted limitation of the study), I am confident that the differences I report in transcript expression accurately reflect differences in how Atlantic salmon stress phenotypes respond to elevated temperatures and bacterial immune stimulation.

While it was initially hypothesized that more distinct differences between LR and HR salmon would be detected, I speculate below on why more subtle disparities were observed. For example, no differences were found between stress phenotypes after PBS injection at either temperature. While netting and handling previously invoked a clear separation in post-stress cortisol levels in these fish at 9°C (Chapter 2), these measurements were taken 1 h post-netting/stress. Therefore, with the time points chosen for this study (i.e., 24 hpi at 12°C, 12 hpi at 20°C), I likely missed peak stress (cortisol) responses to handling/injection. Plasma cortisol has been shown to peak by 45 min post-net stress in Atlantic salmon parr at 12°C (Madaro et al.,

2023). Elevated temperatures resulted in earlier peak post-stress cortisol concentrations in post-smolt Atlantic salmon tested between 4-17°C (Madaro et al., 2018). Finally, this pattern has also been observed in post-smolt salmon reared at 12 vs. 20°C, and in addition, it was found that post-stress cortisol levels were only slightly higher than baseline after 24 h at both temperatures (Zanuzzo et al., unpubl.). Thus, at 12 and 24 hpi in the current study, it is likely that both LR and HR salmon had largely recovered from the stress of PBS injection. This may be why no major differences in gene expression were found in PBS-injected fish. However, Forte Micro injection triggered a more robust and longer lasting stress effect in these fish (based on the significant effects observed between Forte Micro- vs. PBS-injected fish), which is likely why I was able to detect some differences between stress phenotypes with antigen stimulation.

3.5.2 Glucocorticoid synthesis/response

Low and high temperatures have previously been shown to affect the regulation of glucocorticoid-related transcripts in teleost fishes. For example, in cunner (*Tautogolabrus adspersus*), *cyp11a* was significantly upregulated at 10°C compared to 0°C in head kidney, while *gr* was downregulated at the higher temperature in both head kidney and liver (Alzaid et al., 2015). In contrast, elevated temperature (37 vs. 27°C) has been shown to negatively impact the expression of transcripts coding for proteins involved in glucocorticoid synthesis in the gonads of adult sheepshead minnows (*Cyprinodon variegatus*), with the expression of *star* and *hsd3b* in the ovary, as well as *cyp11a1* in both ovary and testis, decreased (Bock et al., 2021). Interestingly, however, temperature had no impact on constitutive levels of any of these transcripts in the current study (Fig. 2).

As literature examining how stress phenotypes differ in their regulation of glucocorticoid synthesis/response is limited, I chose to relate my results to how higher cortisol levels in HR salmon may have impacted stress and immune transcript expression in this study. It has been shown that direct injection of cortisol into juvenile gilthead sea bream (*Sparus aurata*) does not affect the expression of *gr1* or *gr2* in liver relative to PBS-injected fish at 72 hpi (Aedo et al., 2021). In contrast, cortisol injection did increase levels of gluconeogenesis-related genes [i.e., *phosphoenolpyruvate carboxykinase* (*pepck*), *glucose 6 phosphatase* (*g6pc*) and *enolase 3* (*eno3*)] compared to sea bream injected with PBS (Aedo et al., 2021). This demonstrates that a sustained high cortisol response may not impact expression of glucocorticoid receptors over relatively long periods of time (as shown in the current study), but that high levels of cortisol can impact other biological functions. GRs are present in the cytosolic and nuclear compartments of rainbow trout hepatocytes, but quickly relocate (within 5 min) to the plasma membrane following cortisol exposure/stress, before returning to their pre-treatment distribution after ~ 60 minutes (Das and Vijayan, 2023). This research highlights the time-dependent nature of GR responses, and it is possible that I missed any change/peak in the expression of *gra* and *grb* following PBS injection. This hypothesis is further supported by Hori et al. (2012b) who observed a 2.4-fold higher induction of *gr1* in the liver of HR vs. LR Atlantic cod 3 h after a stress, but not at 12 h post-stress. With regard to the expression of *star*, *cyp11a* and *hsd3b*, this study (Hori et al., 2012b) also showed that these genes were significantly upregulated in the head kidney of HR vs. LR cod at time zero and 12 h post-handling. This finding is in contrast to the current study, where the only difference in the expression of these transcripts between stress phenotypes was a significantly greater negative fold-change for *stara* in response to Forte Micro injection at 12°C in HR salmon (-2.6-fold HR vs. -1.1-fold LR).

3.5.3 Heat stress

It may seem counterintuitive that absolute fold-change values in stress-related transcripts in the liver appeared to be (p = 0.057) higher in LR salmon at 12°C as compared to HR salmon following Forte Micro injection (Fig. 3-8). However, a greater downregulation of *gra* and *grb* in response to bacterial stimulation in LR fish (Fig. 3-6) may lead to a decreased responsiveness to circulating cortisol in these fish (Alzaid et al., 2015). Further, the greater upregulation of *hsp70*, *hsp90aa1* and *serpinh1* in the LR group at this temperature in response to Forte Micro may reduce cellular stress as these heat shock proteins regulate intracellular (e.g., expression of Toll-like receptors) and extracellular (e.g., autoimmunity) immune activities (Bolhassani and Agi, 2019). HSP70 and HSP90 are also directly linked to GR function, as they are involved in its folding, hormone binding, nuclear transport/retention, transcription activation and degradation (Rose et al., 2010). As LR fish are characterized as having low post-stress cortisol levels, these mechanisms could potentially explain how this key phenotypic characteristic is regulated. In contrast, it might have been expected that no differences among these same transcripts would be found between stress phenotypes at 20°C. Chapter 2 reported that post-stress glucose, lactate and cortisol levels no longer differed between the stress phenotypes at 23.6°C and that LR and HR salmon shared the same incremental thermal maximum. Thus, it appears that the impacts of elevated temperature may supersede at least some of the biological differences that were evident between the stress phenotypes at lower temperatures. Further testing of this hypothesis is warranted to help determine at what temperature differences in post-stress characteristics start to diminish between LR and HR Atlantic salmon.

Regardless of stress phenotype, these heat stress-associated transcripts responded as expected to temperature. At time zero, the heat stock proteins (i.e., *hsp70*, *hsp90aa1* and

serpinh1) were all upregulated ($p < 0.001$) at 20°C relative to 12°C, while the opposite was true for *cirbp* ($p = 0.069$) and *ucp2* ($p < 0.001$). This aligns well with past literature in Atlantic salmon exposed to elevated temperatures (Chapter 4; Beemelmanns et al., 2021b, 2021a; Shi et al., 2019). Interestingly, *hsp70* was downregulated during the early, mid and late stages of vibriosis infection in the head kidney of silver sea bream (*Rhabdosargus sarba*) compared to asymptomatic fish, with no effect observed in liver (Deane and Woo, 2005). However, in the current study, *hsp70*, *hsp90aa1* and *serpinh1* were upregulated in liver at both temperatures in response to Forte Micro injection compared to PBS controls. Based on these disparate results, it is clear that more research is needed to determine what effect(s) live and inactivated bacteria have on the regulation of heat shock proteins.

3.5.4 Innate antibacterial immune responses

Cortisol exposure influences the expression of immune-related transcripts in fish (e.g., Aedo et al., 2023; Carrizo et al., 2021; Martorell Ribera et al., 2020), and the literature in this area aids in the interpretation of the differences I observed between Atlantic salmon stress phenotypes. For example, in rainbow trout myotubes treated with cortisol, the expression of *cxcr2* (3 h post-stimulation) and *c3* (6 h post-stimulation) was higher than that in those treated with PBS (Aedo et al., 2023). In the current study, *cxcr1* appeared to be more strongly downregulated ($p = 0.076$) by Forte Micro injection in LR than HR salmon at 12 hpi (-8.6- vs. -4.9-fold, respectively), whereas that of *c3a* did not differ between the stress phenotypes at either temperature. Thus, the impact that cortisol has on the expression of these transcripts appears to change following bacterial stimulation. Similarly, this was shown in a study where rainbow trout myotubes were treated with either dimethyl sulfoxide (DMSO), cortisol for 3 h, *Piscirickettsia*

salmonis for 8 h or pre-treated with cortisol for 3 h followed by infection by *P. salmonis* for 8 h (Carrizo et al., 2021). Cortisol treatment alone did not impact the expression of *gr1*, *gr2* or *hamp* relative to myotubes treated only with DMSO; however, infection with *P. salmonis* significantly increased the expression of *gr2* and *hamp* after 8 h (Carrizo et al., 2021), and pre-treatment with cortisol followed by bacterial exposure lowered the expression of *gr2* and *hamp* to an intermediate level between baseline and *P. salmonis* infection alone (Carrizo et al., 2021). In the current study, *gra* and *grb* were downregulated in the liver of both stress phenotypes, and *hampa* was upregulated in the head kidney of both LR and HR fish, but there was not an effect of stress phenotype on the expression of either gene. *P. salmonis* is a gram-negative bacterium, like *A. salmonicida* and the species of *Vibrio* found in the Forte Micro vaccine. So the differences observed between the current study and Carrizo et al. (2021) might be explained by the species of fish tested (i.e., Atlantic salmon vs. rainbow trout), the tissue sampled (i.e., head kidney and liver *in vivo* vs. myotubes *in vitro*), the nature of the immune stimulation (i.e., formalin-inactivated vs. live pathogen), or the timing of sampling. In maraena whitefish (*Coregonus maraena*), primary head kidney cells incubated at 18°C and cultured with cortisol were shown to have reduced responsiveness with regard to *il1b*, *saa* and *hsp90aa1* expression following exposure to Toll-like receptor ligands [serving as a pathogen-associated molecular pattern (PAMP)] at 24 h relative to cells exposed to the PAMPs without cortisol present (Martorell Ribera et al., 2020). However, in the current study, the fold-change expression of *il1b* ($p < 0.01$) and *saa5* ($p = 0.056$) was higher at 20°C in HR than LR salmon. Furthermore, my multivariate statistical analyses showed that HR salmon at 20°C mounted an overall ($p < 0.05$) greater immune response than LR salmon. Clearly, further study is warranted to examine what effects acute increases in cortisol have on the antibacterial immune responses of salmon compared to

chronic exposure. However, it will also be important in future work to determine whether differences between LR and HR fish are driven mainly by cortisol, or if other stress hormones also impact transcript expression between stress phenotypes. For example, concentrations of catecholamines, like epinephrine and norepinephrine, could be interesting to study between stress phenotypes as chromaffin cells located in the head kidney secrete large amounts of these hormones when fish are exposed to stressors (Godoy et al., 2018; Perry and Capaldo, 2011; Reid et al., 1998). Ultimately, it is likely that the mechanisms responsible for differentiating LR and HR salmon are complex, and that we do not yet fully understand differences between these phenotypes.

The impact that cortisol has on the expression of immune- and stress-relevant transcripts appears to vary by tissue as well. For example, incubation with cortisol had no impact on the *in vitro* constitutive expression of *il1b*, *cox2*, *hsp70* or *gr1* (or any other gene assessed) in gill, skin or gut tissue of rainbow trout or gilthead sea bream at 2, 4 or 24 h relative to controls (Vallejos-Vidal et al., 2022). Meanwhile, several hundred differentially expressed transcripts were detected in the liver between LR and HR juvenile European sea bass reared at 18°C (Samaras et al., 2016). Gene ontology (GO) analysis found that immune processes like 'antigen processing and presentation of endogenous peptide antigen via MHC class I' were enriched in HR sea bass relative to LRs (Samaras et al., 2016). Interestingly, *c3* expression was greater in the liver of LR vs. HR sea bass (Samaras et al., 2016). Whereas, in the current study, *c3a* expression was measured in head kidney, not liver, and there were no differences in transcript abundance between LR and HR salmon. It would be interesting in future work to examine the regulation of the complement system in the liver between Atlantic salmon stress phenotypes. Additionally, Vargas et al. (2018) compared immune- and oxidative stress-relevant gene expression in the gill

and liver between juvenile LR and HR gilthead sea bream reared at 22°C after 1-, 3- and 7-days post-vaccination with bacterin from *V. anguillarum*. At a minimum of one time point, *il1b*, *tumor necrosis factor alpha* (*tnfa*), *immunoglobulin m* (*igm*), *superoxide dismutase 2* (*sod2*) in gill, and *glutathione peroxidase 1* (*gpx1*) and *catalase* (*cat*) in liver, were more highly expressed in LR vs. HR sea bream (Vargas et al., 2018). Ultimately, it was concluded that LR fish mounted a greater immune response than HR fish (Vargas et al., 2018). The results of Vargas et al. (2018) agree with previous research which reported that HR fish are more susceptible to bacterial pathogens than LR fish (Chatakondi and Peterson, 2018; Fevolden et al., 1993, 1992). However, the current study found that HR salmon mounted a more robust immune response to Forte Micro at 20°C than LR salmon, and that no major differences in the expression of immune-related transcripts were observed between the stress phenotypes at 12°C (aside from a significantly greater fold-change value in *stlr5a* detected in HR vs. LR fish). A live pathogen challenge, therefore, may be more informative in helping to determine whether the differences observed in the present study lead to enhanced survival in HR salmon, particularly at elevated temperatures. To the best of my knowledge, no one has yet investigated how adaptive immune responses may differ between stress phenotypes, which would also be a valuable area of future study.

It is worth noting that the expression of many of the same transcripts assessed in this study (i.e., *stlr5a*, *campb*, *hampa*, *il1b*, *saa5* and *cox2*), regardless of stress phenotype, responded comparably to previous studies on Atlantic salmon challenged with Forte Micro or Forte V II (the latter vaccine containing heatkilled infectious salmon anemia virus in addition to the same bacterial antigens as in Forte Micro) at 12 and 20°C (Zanuzzo et al., 2022, 2020).

3.5.5 Growth

While it is well-established that chronic stress and thus prolonged cortisol production decreases growth in fish (Barton, 2002; Wendelaar Bonga, 1997), only recently has this phenomenon been studied at the molecular level in Atlantic salmon (Breves et al., 2020). Breves et al. (2020) reported that *ghr* expression in the liver and muscle was unaffected by cortisol injection in parr reared at 10°C after 3 and 14 days. In the current study, stress phenotype influenced (p = 0.003 for the stress phenotype interaction term) the expression of *ghra*, with levels lower in HR than LR salmon at time zero and 20°C (p = 0.055). Further, only at 20°C did Forte Micro injection significantly upregulate *ghra* expression relative to PBS controls in both stress phenotypes. In contrast, *ghrb* was downregulated at 20°C following bacterin stimulation, and stress phenotype did not impact the expression of *ghrb* at any temperature/time point. Temperature, however, did have varying effects on these paralogues. Levels of *ghra* did not differ between 12 and 20°C at time zero, while the expression of *ghrb* was lower at the elevated temperature in both stress phenotypes. Thus, there appear to be differences in how the two paralogues of *ghr* in Atlantic salmon are regulated by temperature, bacterial stimulation and stress phenotype. This contrasts with Hevrøy et al. (2015) who reported *ghra* and *ghrb* were both downregulated in post-smolt Atlantic salmon after 15 and 35 days of being reared at 19°C as compared to salmon held at 13°C. Further research is required to determine what impacts gene duplication had on the regulation of these paralogues. In Vargas et al. (2018), *ghr1* was more highly expressed in HR vs. LR gilthead sea bream at 7 days post-vaccination.

Interestingly, in my previous study with these salmon, HRs weighed significantly more than LRs at the same age both initially at 9°C during characterization (i.e., Experiment #1) and in the control group held at 12°C at the final weight assessment (i.e., Experiment #2) (Chapter 2).

However, no differences in weight were detected at warmer temperatures between the stress phenotypes during Experiment #2, and growth rate did not differ regardless of temperature (Chapter 2). In contrast, past research has reported opposite findings, with LR rainbow trout having a greater weight gain compared to HR trout (Weil et al., 2001), even though these trout were at a similar life stage to the salmon in Chapter 2.

3.6 Conclusion

In summary, comparing LR and HR Atlantic salmon at 12 and 20°C, and in response to the vaccine Forte Micro, at the mRNA level revealed a couple of interesting patterns. First, contrary to previous reports, I found that HR salmon at 20°C mounted a more robust innate antibacterial immune response than LR salmon. Second, in LR fish at 12°C it appeared (p = 0.057) that the expression of stress-related transcripts was higher after injection with Forte Micro relative to HR fish, and this may partially explain how the proactive phenotype is able to have lower post-stress cortisol levels. While further testing is necessary to determine whether these findings coincide with benefits to survival following live pathogen challenges, this study provides foundational knowledge that enhances our understanding of these two stress phenotypes. It will be important to build upon this work if we are to better predict how the population structure/composition of wild and farmed Atlantic salmon may be impacted by accelerated climate change and the predicted increase in pathogenic bacterial abundance (Vezzulli et al., 2016). More broadly, it was also determined that vaccine injection impacted the expression levels of transcripts involved in immune responses (i.e., immune receptors, immune effectors and a cytokine) as well as a range of other biological pathways (i.e., acute phase response, complement activation, eicosanoid synthesis, glucocorticoid response, heat stress and

growth; Fig. 3-9). These results identify additional potential antibacterial immune biomarkers that could be used in future studies to enhance our view of how fish will respond to environmental challenges.

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CHAPTER 4: Influence of supplemental dietary cholesterol on growth performance, indices of stress, fillet pigmentation, and upper thermal tolerance of female triploid Atlantic salmon (*Salmo salar*)

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4.1 Abstract

The salmon aquaculture industry must be proactive at developing mitigation tools/strategies to offset the potential negative impacts of climate change. Therefore, this study examined if additional dietary cholesterol could enhance salmon production at elevated temperatures. It was hypothesized that supplemental cholesterol could aid in maintaining cell rigidity, reducing stress and the need to mobilize astaxanthin muscle stores, and improving salmon growth and survival at high rearing temperatures. Accordingly, post-smolt female triploid salmon were exposed to an incremental temperature challenge (+0.2°C day^{-1}) to mimic conditions that they experience in sea-cages in the summer, with temperature held at both 16 and 18°C for several weeks [i.e., 3 weeks at 16°C, followed by an increase at 0.2°C day^{-1} to 18°C (10 days), then 5 weeks at 18°C] to prolong their exposure to elevated temperatures. From 16°C onwards, the fish were fed either a control diet, or one of two nutritionally equivalent experimental diets containing supplemental cholesterol [+1.30%, experimental diet #1 (ED1); or +1.76%, experimental diet #2 (ED2)]. Adding cholesterol to the diet did not affect the salmon's incremental thermal maximum (IT_{Max}), growth, plasma cortisol, or liver stress-related transcript expression. However, ED2 appeared to have a small negative impact on survival, and both ED1 and ED2 reduced fillet "bleaching" above 18°C as measured using SalmoFan™ scores. Although the current results suggest that supplementing salmon diets with cholesterol would have few/minimal benefits for the industry, ≤ 5% of the female triploid Atlantic salmon used in this study irrespective of diet died before temperature reached 22°C. These latter data suggest that it is possible to produce all female populations of reproductively sterile salmon that can withstand summer temperatures in Atlantic Canada.

4.2 Introduction

With the stagnation of the world's capture fisheries, the aquaculture industry now provides almost half of the world's seafood (FAO, 2022). However, with annual global seafood demand expected to increase by 47 million tonnes by the early 2020s, expansion of the aquaculture sector at current rates is only projected to meet 40% of this requirement (Cai and Leung, 2017). Anthropogenic-induced climate change will only exacerbate this situation, as increases in water temperature and acidification, and low oxygen levels (hypoxia), are all anticipated to have long-term impacts on marine aquaculture production (Barange et al., 2018; Maulu et al., 2021; Reid et al., 2019a). For example: the south coast of Newfoundland experienced an unprecedented heat wave in the summer/fall of 2019 that resulted in the loss of more than 2.6 million Atlantic salmon (Burke et al., 2020); and Australian (Tasmanian) cage-sites are already experiencing temperatures as high as 23°C in combination with severe hypoxia (Stehfest et al., 2017), and such conditions are negatively affecting salmon production (Wade et al., 2019). There are several strategies that could potentially mitigate (reduce) the effects of climate change on salmon aquaculture production, such as selective breeding, the use of new genetic strains, changes in farm management / fish husbandry practices (i.e., improving site selection and/or the use of deeper nets) and altering feed formulations (Falconer et al., 2022; Islam et al., 2021b; Maulu et al., 2021; Reid et al., 2019b).

This project addressed the latter of these options by examining if inclusion of higher levels of dietary cholesterol could enhance salmon growth performance and thermal tolerance, as well as reduce stress and the loss of fillet pigmentation (colour) at high temperatures (Grünenwald et al., 2019; Nordgarden et al., 2003; Wade et al., 2019). This approach was taken because, while Irvine et al. (1957) showed that increased dietary cholesterol improved the

survival of goldfish (*Carassius auratus*) at high temperatures, no such research has been conducted with farmed salmonids. Cholesterol is an important component of cellular membranes, and critical for maintaining membrane fluidity/rigidity as environmental temperatures increase (Crockett, 1998; Farkas et al., 2001; Fodor et al., 1995; Hazel, 1979; Liu et al., 2019). Furthermore, in Tasmania, high temperatures at Atlantic salmon sea-cage sites resulted in a decrease in fillet colouration (i.e., 'bleaching': Grünenwald et al., 2019; Nordgarden et al., 2003; Wade et al., 2019). Astaxanthin, a carotenoid typically added to salmon diets to enhance fillet pigmentation, is the most expensive ingredient (per kg) in salmon feed (Solymosi et al., 2015), and therefore, producers need to maximize its deposition and retention in the fish's muscle (fillet). Increasing dietary levels of cholesterol may assist with this, as cholesterol has been shown to increase astaxanthin plasma transport in Atlantic salmon and may enhance its subsequent retention in the fillet (Chimsung et al., 2013, 2014). In addition, there are some studies that report other positive effects of increased dietary cholesterol levels. Dietary cholesterol supplementation has been shown to improve the rainbow trout's (*Oncorhynchus mykiss*) immune response and disease resistance to the bacterial pathogen *Aeromonas hydrophila* (Deng et al., 2013). Changes in the sterol:phospholipid ratio have been reported to be a key response to temperature in salmonid liver membranes (Wijekoon et al., 2021). Further, cardiac performance/function is a key determinant of fish thermal tolerance (Farrell, 2002; Farrell et al., 2009), and enriching the cholesterol content of bovine cardiac microsomes improves the conformational stability of proteins and increases their resistance to inactivation at elevated temperature (Ortega et al., 1996).

While a 'high temperature' diet is available in Tasmania and other parts of the world (OPTILINE HT, Skretting), no peer-reviewed literature is available on its composition or

efficacy, and this diet is not currently approved for use in Canada (Ruff, 2015). Further, as the Atlantic salmon industry is intending to produce 33,000 metric tonnes of all female triploid fish in Newfoundland by 2024 (Ignatz, 2019), producers must be proactive if they are to avoid events similar to that which occurred in 2019 (Burke et al., 2020). This is particularly true, as while the data are equivocal, a number of studies suggest that triploid salmonids are less tolerant of high temperatures (Hansen et al., 2015; Ojolick et al., 1995; Sambraus et al., 2018, 2017; Verhille et al., 2013). Finally, while farming all female triploid Atlantic salmon offers a highly effective strategy to mitigate the risk of genetic introgression by escapees (Benfey, 2016), it is paramount that the industry develops strategies and methodologies to ensure that triploids are able to thrive at higher temperatures.

Therefore, the research conducted was both novel and practical in its design, and provides information that should benefit the Atlantic salmon aquaculture industry in Canada and elsewhere. Increased ocean temperatures can lead to sub-lethal effects that result in loss of production and cause large-scale mortalities (Burke et al., 2020; Wade et al., 2019), and this study provides the industry with vital information regarding the farming of female triploid Atlantic salmon at elevated temperatures.

4.3 Materials and methods

This study was approved by the Animal Care Committee of Memorial University of Newfoundland and Labrador (protocol #20-02-KG), and salmon husbandry and experimental procedures were performed in accordance with the Canadian Council on Animal Care Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (Canadian Council on Animal Care, 2005).

4.3.1 Experimental animals

PIT (Passive Integrated Transponder)-tagged conventional female triploid Atlantic salmon of St. John River origin from AquaBounty Canada (PE, Canada) were shipped to the Laboratory for Atlantic Salmon and Climate Change Research (LASCCR; Ocean Sciences Centre, Memorial University) where they underwent smoltification. These fish were produced from a single reversed-sex neomale (i.e., functionally masculinized genetic female) that had been crossed with 24 females, producing all female offspring. Fertilized eggs were pooled, and then shocked using hydrostatic pressure to induce triploidy (Benfey et al., 1988). Ploidy status of the fish was verified by AquaBounty using flow cytometry (Allen Jr., 1983; AquaBounty Technologies, Inc., 2010) before transfer. Based on a subset of 200 eyed eggs, it was estimated that pressure shocking was ≥ 98% effective in inducing triploidy.

The fish were initially distributed among eight 2.2 m^3 tanks at 16.5 kg m^{-3} in a flow-through seawater system with temperature set to 10°C for the first week before being slowly increased (i.e., at +0.3°C day^{-1}) to 12°C over the course of another week. Tanks were maintained on a 14 h light: 10 h dark photoperiod with flow rates set to 15 L min^{-1}. Oxygen and temperature levels were measured at least once daily in all tanks (YSI, ProODO, Yellow Springs, OH, USA) and 4 tanks were continuously tracked using a YSI 5500D Multi DO Optical Monitoring and Control System. Water oxygen levels were maintained at ≥ 100% air saturation, and the salmon were initially fed a commercial diet (EWOS Dynamic S, 5.0 mm; minimum 46% crude protein, 27% crude fat; EWOS Canada Ltd, Surrey, BC, Canada) to satiation twice daily by hand. Once the fish reached 12°C, they were redistributed amongst nine 2.2 m^3 tanks with 50 fish $tank^{-1}$; initial stocking density ~9 kg m^{-3}. Only fish weighing between 300-500 g were included in the

study to limit the effect(s) of size variation. Salmon were then given another 18 days to acclimate before the initial assessment was performed and the trial started.

4.3.2 Overall experimental design and fish sampling

An overview of the experimental design is shown in Figure 3-1. The initial weights and fork lengths of the fish were recorded after brief anesthesia (0.2 g L^{-1} AquaLife TMS; Syndel Laboratories Ltd., Nanaimo, BC, Canada) at 12°C. The fish were then allowed 2 days of recovery before their commercial diet was switched to the trial's control diet. All diets in this study were formulated to meet or exceed the nutritional requirements for Atlantic salmon (NRC, 2011) and approximate commercial salmon aquafeeds using practical ingredients (Table 4-1). The diets were extruded (5.0 mm pellet size) at the Chute Animal Nutrition Lab, Faculty of Agriculture, Dalhousie University (Truro, NS, Canada). Dry ingredients for both diets were mixed in an industrial 19 L Hobart mixer (Toronto, ON, Canada; Model A-200-T) for five minutes, then extruded into 5 mm pellets using a single screw KAHL extruder with mixing conditioner (Amandus Kahl GmbH & Co. KG, Reinbek, Germany; Type OEE8). The extruded pellets were dried in a shelf oven for eight hours at 60°C and sieved to remove fine particles. Pellets were then vacuum coated with dietary oils (Dinnissen Process Technology, Sevenum, Netherlands), and shipped to Memorial University of Newfoundland and Labrador. In addition to the control diet, 2 experimental diets containing supplemental cholesterol (+1.30% and 1.76% dietary cholesterol, respectively) were also manufactured. The proximate, lipid class and fatty acid composition of all 3 diets can be found in Table 4-2. The amount of supplemental dietary cholesterol included in each experimental diet was selected in an attempt to match the 1.37 and 1.83% total cholesterol values reported in a study on a closely related species (i.e., rainbow

trout; Deng et al., 2013). All diets contained approximately equal levels of protein (51%), lipid (20%), carbohydrate (12%), digestible energy (21 MJ kg^{-1}), phosphorus (1.6%), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (2% total of the diet), and astaxanthin (80 mg kg^{-1}), with all essential amino acids supplied at a minimum of 150% of their known dietary requirement for Atlantic salmon (NRC, 2011). Proximate composition and gross energy density of the test diets were measured following the procedures described in Tibbetts et al. (2020) and their lipid classes and fatty acid profiles were determined following Wei et al. (2022).

The salmon were hand fed the control diet to apparent satiation twice daily (at ~9:00 & 15:00) for 16 days at 12°C, with feeding stopped when a few pellets accumulated on the bottom of each tank. Then, the first sampling was performed. One fish was randomly netted from each tank (n = 9 in total), euthanized (0.4 g L^{-1} TMS), measured for weight and fork length, and sampled. Blood (1 mL) was collected within 2-3 min from each fish via the caudal vein using 1 mL syringes with 23 gauge 1" needles, and placed in heparinized (1000 units mL^{-1}) tubes on ice. The blood was then centrifuged at 1100 × *g* for 1 min at room temperature, then 3 aliquots (100 μL each) of plasma were collected before being flash-frozen in liquid nitrogen. The remaining blood was drained from the fish (via multiple collections using a 3 mL syringe and needle), the viscera was removed, and the liver was weighed. Next, duplicate pieces of liver from the most distal portion of the posterior lobe were sampled using standard aseptic techniques, and quickly flash-frozen. The liver samples were stored at -80°C until one set of samples could be shipped to the Center for Aquaculture Technologies Canada (CATC; Souris, PE, Canada) for processing. The gonads were also examined to confirm that the fish were sterile females. The right side of each fish was then filleted, the bones removed, and the skin-on fillet was placed on plain white

paper inside a white Styrofoam box with a 100 W halogen light bulb hanging 87.5 cm directly above (Ignatz et al., 2020b). The fillets were then scored using the DSM SalmoFan™ colour chart (DSM, 2021), the industry-recognized standard for the visual assessment of the degree of pigmentation in salmon flesh. Two trained technical staff independently scored each fillet to determine its level of colouration, with separate scores assigned to describe the colouration along the lateral line and at the peripheral edges of the fillet. An average of these two values was used as the pigmentation score for each fillet. The fillets were then weighed, and samples of fillet muscle were collected and stored for further analyses conducted through the Mitigating the Impact of Climate-Related Challenges on Salmon Aquaculture (MICCSA) project. These data will be the basis of a separate paper.

The following day, the remaining fish were exposed to an incremental temperature increase from 12°C, where temperature was raised by 0.2°C day^{-1} to mimic conditions that these salmon may experience in sea-cages during the summer in Newfoundland (Burt et al., 2012; Gamperl et al., 2021) or northern Europe (Johansson et al., 2007, 2006). Once the tanks reached 16°C, another sampling was performed following the same procedure as described above (n = 9; 1 fish sampled tank^{-1}) with fish taken off feed 24 h prior to sampling. All remaining fish were also anesthetized (in 0.2 g L^{-1} TMS), and weighed and measured for fork length. A total of 25 fish were removed from the experiment at this point, as these salmon had developed ulcers (~3 cm x 3 cm on average) on their right side. Samples of these fish were taken, and analyzed by the Microbial Pathogenesis and Vaccinology Laboratory (Memorial University), but the results were inconclusive (data not shown). The next day, 3 tanks were switched onto Experimental Diet #1 (ED1; +1.30% cholesterol) and another 3 tanks were provided with Experimental Diet #2 (ED2; +1.76% cholesterol). The remaining 3 tanks were kept on the control diet. The diets were

switched at this stage of the experiment to reflect when salmon farmers would need to consider using a functional feed as temperatures warm. Temperature was maintained at 16°C for another 3 weeks before it was gradually increased (+0.2°C day^{-1}) to 18°C, where temperature was again held for 5 weeks. No fish developed ulcers during the remainder of the experiment.

After exposure to elevated temperatures (i.e., ≥ 16°C) for a total of 65 days, another subset of fish was sampled after being fasted for 24 h. Nine fish per dietary treatment (3 fish $tank^{-1}$) were sampled in the same manner as previously described at 12 and 16°C, with the addition that the viscera was weighed. Only fish that had gained weight were sampled, with 24 out of 27 fish having gained ≥ 10% of their weight since their assessment at 16°C and the remaining 3 sampled fish having gained between 5 and 10%. This was done to ensure that these fish were actively consuming the diets provided. Additional fish were sampled at this time point for separate analyses involved in the MICCSA project that will also not be described here. However, morphometric data collected from these fish are included in the current study.

Once all sampling was completed, temperature was again raised by 0.2°C day^{-1} in all tanks until 50% of the fish in each treatment reached their incremental thermal maximum (IT_{Max}). When each fish lost equilibrium/succumbed, their weight, fork length, liver weight, viscera weight, ventricle weight, and state of sexual maturity (or lack thereof) were recorded, in addition to the time and temperature at which they were removed from the experiment. After the first dietary treatment reached this endpoint, 9 fish per dietary treatment (3 fish $tank^{-1}$) were euthanized (0.4 g L^{-1} TMS), measured for weight and fork length, and sampled. Viscera, liver and ventricle weight were also measured. Fillet weight and colouration were assessed as previously described. In addition, after 50% of the fish within each dietary treatment reached

their IT_{Max}, the remaining fish were euthanized (0.4 g L^{-1} TMS) before weight and fork length were recorded.

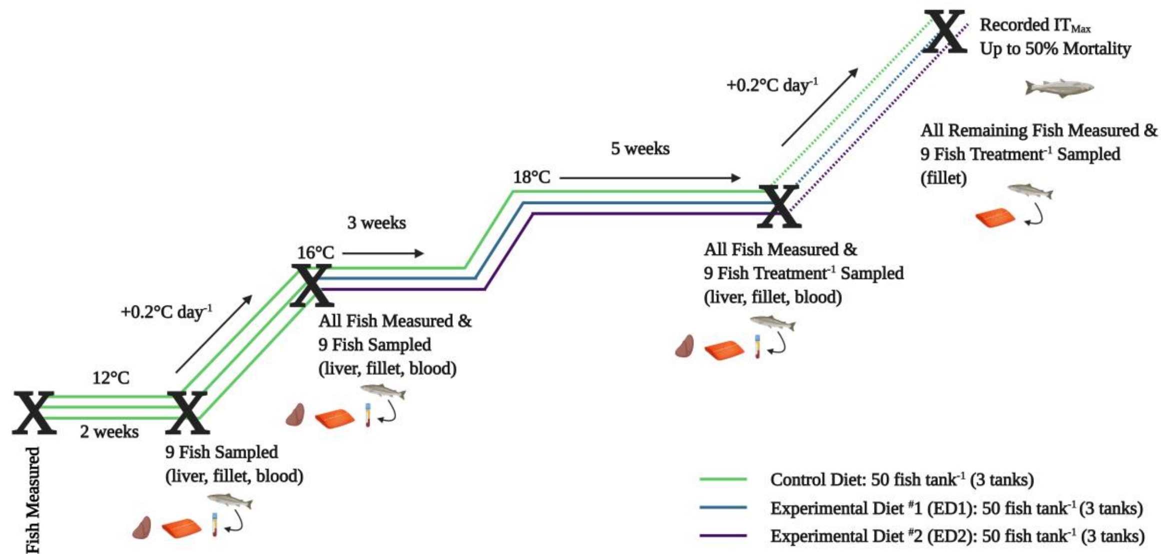

Figure 4-1. Overview of the protocol used to assess whether supplemental dietary cholesterol affected the growth performance, physiology and survival of female triploid Atlantic salmon when exposed to an increasing temperature regimen. 'X's mark sampling time points. Created using https://biorender.com/.

Table 4-1. Formulation of the test diets used to evaluate the effect(s) of supplemental dietary cholesterol on female triploid Atlantic salmon exposed to elevated temperatures.

Ingredient[1] (% of diet)	Control Diet	Experimental Diet #1 (ED1)	Experimental Diet #2 (ED2)
Fish meal (69% CP[2])	18.00	18.00	18.00
Soybean protein concentrate (71% CP)	17.00	17.00	17.00
Soybean meal (48% CP)	12.00	12.00	12.00
Poultry by-product meal (71% CP)	12.00	12.00	12.00
Blood meal (96% CP)	6.72	6.72	6.72
Fish oil	9.00	8.29	8.04
Wheat flour	8.48	8.60	8.64
Poultry fat	4.50	4.15	4.02
Canola oil	4.50	4.15	4.02
Calcium phosphate (monobasic)	4.67	4.67	4.67
Vitamin/mineral premix[3]	0.40	0.40	0.40
Vitamin B4 (choline chloride)	0.40	0.40	0.40
Vitamin C (ascorbic acid; 'Stay-C 35')	0.03	0.03	0.03
Vitamin E (α-tocopherol)	0.03	0.03	0.03
L-lysine	1.68	1.68	1.68
DL-methionine	0.50	0.50	0.50
L-tryptophan	0.01	0.01	0.01
Carophyll pink (10% astaxanthin)	0.08	0.08	0.08
Cholesterol[4]	0.00	1.30	1.76

[1] All ingredients were supplied by Corey Nutrition (Fredericton, NB, Canada), with the exception of the supplemental cholesterol

[2] Crude protein (CP; N × 6.25)

[3] Seawater salmonid mixture

[4] Sigma-Aldrich (Oakville, ON, Canada) CAT# C8503

Table 4-2. Proximate, lipid class and fatty acid composition of the experimental diets fed to the female triploid Atlantic salmon.

	Control Diet No added cholesterol	Experimental Diet #1 1.30% added cholesterol	Experimental Diet #2 1.76% added cholesterol
Proximate composition[1]			
Moisture (%)	5.6	6.3	6.1
Ash (%)	10.1	10.4	10.6
Crude protein (%)	51.4	51.7	51.2
Crude lipid (%)	20.5	19.1	19.3
Carbohydrate[2] (%)	12.4	12.5	12.8
Gross energy (MJ kg^{-1})	21.8	21.5	21.4
Lipid class composition[3]			
Triacylglycerol	115.0	122.1	128.3
Sterol	3.4	18.8	24.5
Phospholipid	23.4	4.9	15.9
Free Fatty Acid	6.8	11.8	10.3
Acetone Mobile Polar Lipid	21.4	8.7	6.4
Total Lipid	171.9	168.8	186.3
Fatty acid composition[3]			
14:0	6.2	5.4	6.0
16:0	22.8	21.5	23.9
16:1ω7	9.6	8.6	9.5
18:0	5.3	5.1	5.6
18:1ω9	37.9	39.1	38.9
18:1ω7	3.8	3.6	3.9
18:2ω6 (LNA)	18.3	18.7	20.4
18:3ω6	0.3	0.3	0.3
18:3ω3 (ALA)	4.0	4.1	4.1
18:4ω3	1.3	1.1	1.3
20:1ω9	2.4	2.5	3.0
20:2ω6	0.2	0.1	0.1
20:3ω6	0.2	0.1	0.2
20:4ω6 (ARA)	0.9	0.8	1.1
20:3ω3	0.1	0.1	0.1
20:4ω3	0.6	0.5	0.6
20:5ω3 (EPA)	8.7	7.4	8.3
22:5ω3	1.3	1.1	1.3
22:6ω3 (DHA)	5.4	4.8	5.5
Σ SFA[4]	37.2	34.6	38.4
Σ MUFA[5]	58.4	58.6	61.0
Σ PUFA[6]	45.0	42.6	46.8
Σ ω3	21.8	19.5	21.5
Σ ω6	20.3	20.6	22.6

ω6/ω3	0.9	1.1	1.1
DHA/EPA	0.6	0.7	0.7
EPA/ARA	9.7	9.3	7.6

[1] Data presented on an as-fed basis
[2] Estimated as: (100% - [Moisture + Ash + Crude protein + Crude lipid])
[3] Data presented on a mg lipid or FAME g^{-1} wet weight basis
[4] Saturated fatty acid
[5] Monounsaturated fatty acid
[6] Polyunsaturated fatty acid

4.3.3 Growth performance

Weight gain was assessed at 16°C, at the end of 5 weeks spent at 18°C, and when fish reached their IT_{Max}. The thermal growth coefficient (TGC) was used to assess growth rate using the following equation (Cho, 1992; Iwama and Tautz, 1981):

$$TGC = \left(\frac{W_f^{1/3} - W_i^{1/3}}{\sum_{i=1}^{n} T_i}\right) \times 1000$$

where W_f and W_i are the final and initial fish body weights (in g), respectively, n is the number of days since W_i, and T_i is the mean daily water temperature (in °C).

Specific growth rate (SGR) was also calculated using:

$$SGR = \left(\frac{\ln(W_f) - \ln(W_i)}{n}\right) \times 100$$

Fulton's condition factor (K) was calculated as:

$$K = \frac{\text{Fish weight (g)}}{(\text{Fish fork length [cm]})^3} \times 100$$

Feed intake was measured daily by dividing the feed provided to each tank by the number of fish in the tank. Average feed intake was also calculated on a percent body weight basis at the assessment points. Liver, viscera, ventricle and fillet weights were used to calculate each fish's hepatosomatic index (HSI), viscerosomatic index (VSI), relative ventricular mass (RVM) and fillet yield, respectively, using the following equations:

$$\text{HSI} = \left(\frac{\text{Liver weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

$$\text{VSI} = \left(\frac{\text{Viscera weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

$$\text{RVM} = \left(\frac{\text{Ventricle weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

$$\text{Fillet Yield} = \left(\frac{\text{Fillet weight (g)} * 2}{\text{Fish weight (g)}}\right) \times 100$$

4.3.4 Basal cortisol levels

Plasma cortisol was measured at 650 nm using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Neogen, Lexington, KY, USA) and a Molecular Devices (San Jose, CA, USA) SpectraMax® M5 microplate reader, following the manufacturers' instructions. Samples were analyzed in duplicate at dilutions of 1:50, with a duplicate 8-point standard curve included on each 96-well plate. A 4-parameter logistic fit of the standard curve gave r^2 values of ≥ 0.994 for each plate. Samples with coefficients of variance >15%, and with a >10 ng mL^{-1} difference in cortisol level between replicates were repeated. It is acknowledged that the commercial antibody used in this ELISA can cross-react with other steroids (i.e., cortisone, 11-deoxycortisol and corticosterone), and thus, absolute cortisol concentrations cannot be inferred (Sadoul and Geffroy, 2019).

4.3.5 Liver RNA extraction, purification and cDNA synthesis

Liver samples (n = 45) were shipped on dry ice to CATC for transcript expression analyses. Isolation of RNA from the liver was completed as per Xue et al. (2020), with the exception of DNase treatment and purification, which was completed with a RNA Clean & Concentrator Kit (Zymo Research, Irvine, CA, USA) as per the manufacturer's instructions. The purity of RNA was determined by spectrophotometry with 260/280 and 260/230 values of ≥ 2.0 and 1.9, respectively, as minimum thresholds for inclusion. RNA integrity was confirmed using 1.0% agarose gel electrophoresis. The RNA samples were standardized to 100 ng/μL, and synthesis of cDNA was completed from 1 μg of RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA. USA) as per the manufacturer's instructions.

4.3.6 RT-qPCR study and analysis

Seven genes of interest were chosen based on past studies that have shown them to be responsive to chronic heat stress in Atlantic salmon liver (Beemelmanns et al., 2021b; Ignatz et al., 2022). Several heat shock proteins [i.e., *hsp70*, *hsp90aa1*, *hsp90ab1*, *serpinh1* (alias *hsp47*)] were selected alongside transcripts associated with oxidative stress (i.e., *cirbp*, *ndufa1*, *ucp2*). All qPCR primers, including the 5 normalizers (see below), were taken from previous studies (Beemelmanns et al., 2021b; Caballero-Solares et al., 2017; Hixson et al., 2017; Ignatz et al., 2022; Jones et al., 2007; Xue et al., 2015). An equimolar pool of cDNA from all samples was used as a calibrator sample between plates, and as source material for standard curve generation. Standard curves for all 12 transcripts were generated using four to six serial dilutions (1:3 or 1:5) from 5-10 ng of input total RNA. Linearity (r^2) ≥ 0.980 and efficiency between 90 – 109% (Table 4-3) met quality standards (Pfaffl, 2001). “No reverse transcriptase” controls, using pools of 22-23 samples, did not reveal signs of gDNA contamination. Melt curves indicated sharp, single peaks with no evidence of primer dimers.

A 1:20 dilution of stock cDNA was used for qPCR analyses. Each reaction was 10 µL, and included 5 µL of SsoAdvance Universal SYBR Green Supermix (BioRad, Saint-Laurent, QC, Canada), 400 nM of each primer, and 3 µL of template (7.5 ng input RNA). Thermal cycling on the 7500 FAST qPCR System (Applied Biosystems) included an initial activation and denaturation step of 95°C for 2 min followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The only exceptions to these parameters were for: 1) *cirbp*, for which 250 nM of each primer and a 62°C annealing step was used; and 2) *hsp90aa1*, for which 500 nM of each primer and a 45 sec annealing step was used. Melt curve analysis included 95°C for 15 sec, 60°C for 1 min, and a

0.5°C/sec ramp to 95°C. Each 384-well plate included triplicate samples, calibrators and non-template controls (NTCs) for each gene present.

Raw cycle threshold (C_T) data were imported into qbase+ (Biogazelle, Gent, Belgium) (Hellemans et al., 2007), where technical replicates outside of ± 0.5 C_T from two close values were removed. While 5 normalizer genes were initially assessed (i.e., *ef1a*, *rpl32*, *eif3d*, *pabpc1*, *polr2*), *ef1a* and *rpl32* were ultimately selected as the study normalizers. This selection was based on low C_T variation among temperature/dietary groups (i.e., < 1 C_T difference in averages between groups) and geNorm recommendations (mean geNorm M value and coefficient of variation of 0.342 and 0.119, respectively) (Vandesompele et al., 2002). Calibrated normalized relative quantities (CNRQs) (Vandesompele et al., 2002) were calculated using C_T values of calibrators across plates and amplification efficiencies for each primer pair (Table 4-3) in qbase+. CNRQs were then $\log_2$-transformed in Microsoft Excel.

Table 4-3. qPCR primers used for assessing heat stress responses in the liver of female triploid Atlantic salmon.

Gene Name (GenBank Accession Number)	Nucleotide sequence (5'-3')	Amplification Efficiency (%)	r^2	Amplicon size (bp)	Source
cold-inducible RNA-binding protein (*cirbp*) (BT059171)	F: TTGAGTACACAGCGGTGAATT R: ACCAATCTGATGCTATGACGAGA	93.4	0.980	132	Beemelmanns et al. (2021)
heat shock protein 70 (*hsp70*) (BT045715)	F: AGTGATCAACGACTCGACACG R: CACTGCATTGGTTATAGTCTTG	91.7	0.988	151	Beemelmanns et al. (2021)
heat shock protein 90-alpha (*hsp90aa1*) (KC150878)	F: CGAGGACATGAAGAAGAGGCAT R: ACACTGTCACCTTCTCCACTTT	104.5	0.987	104	Beemelmanns et al. (2021)
heat shock protein 90 alpha family class B member 1 (*hsp90ab1*) (NM_001123532)	F: AGCCTCACGTTTTTCCAATCG R: TGCGTTGCCCACCATTAACT	91.6	0.994	150	Ignatz et al. (2022)
NADH dehydrogenase 1 alpha subcomplex subunit 1 (*ndufa1*) (BT046880)	F: TGATGGAGAGAGACAGACGAGT R: AGGTGAGATCTGGGATTAGTGGA	96.7	0.988	89	Beemelmanns et al. (2021)
serpin H1 (*serpinh1*) (XM_014214963)	F: GACCATTCAAAAATCAACCTCA R: CATGGCTCCATCAGCATTCT	94.0	0.986	129	Beemelmanns et al. (2021)
mitochondrial uncoupling protein 2 (*ucp2*) (XM_014196911)	F: CTGATCTCTGCCGTCACCAT R: AGAAGACTGATGAGGTGAAGACA	108.7	0.989	89	Beemelmanns et al. (2021)
elongation factor 1 alpha (*ef1a*) (NM_001141909)[a]	F: GTGGAGACTGGAACCCTGAA R: CTTGACGGACACGTTCTTGA	96.7	0.999	155	Jones et al. (2007)
60S ribosomal protein 32 (*rpl32*) (BT043656)[a]	F: AGGCGGTTTAAGGGTCAGAT R: TCGAGCTCCTTGATGTTGTG	98.8	0.998	119	Xue et al. (2015)
eukaryotic translation initiation factor 3 subunit D (*eif3d*) (GE777139)[b]	F: CTCCTCCTCCTCGTCCTCTT R: GACCCCAACAAGCAAGTGAT	101.2	0.998	105	Caballero-Solares et al. (2017)
polyadenylate-binding protein 1 (*pabpc1*) (EG908498)[b]	F: TGACCGTCTCGGGTTTTTAG R: CCAAGGTGGATGAAGCTGTT	94.9	0.998	108	Caballero-Solares et al. (2017)
RNA polymerase II (*polr2*) (CA049789)[b]	F: TTCTGAAAGACCCCCAAGTG R: AGCTCGCTGATGAGGTCAGT	90.0	0.993	145	Hixson et al. (2017)

[a] Normalizer gene chosen for this study.
[b] Normalizer gene tested, but ultimately not chosen for this study.

4.3.7 Statistical analyses

Data were first assessed via Shapiro-Wilk's normality tests and $\log_{10}$-transformed if necessary to meet testing assumptions. Levene's tests to measure homoscedasticity were also performed. To confirm the absence of tank effects, replicate tank means were first compared, with tank as a fixed factor, after which the data from replicate tanks were pooled for further analyses. One-way ANOVAs followed by Tukey's HSD post-hoc tests were used to examine differences ($p < 0.05$) in parameters between: 1) the dietary treatments at a particular temperature; and 2) temperatures within just the control treatment. T-tests were used when comparisons were only made between two variables (e.g., assessments performed at 12°C vs. 16°C). Data are reported as means ± standard error (SE). All statistical procedures were carried out using R (v. 4.1.2) (R Studio Team, 2015).

4.4 Results

4.4.1 Performance metrics following an incremental temperature increase to 18°C

Performance metrics of the fish sampled at 12 and 16°C can be found in Table 4-4. The fish gained almost 100 g, on average, during the 37 day initial period where all fish were fed the control diet as temperature was gradually raised to 16°C from 12°C. This resulted in values of TGC and SGR over this period of 0.85 ± 0.03 [$g^{1/3}$ (°C $d)^{-1}$] and 0.41 ± 0.02 (% body weight day^{-1}), respectively. Although Fulton's condition factor decreased slightly ($p < 0.001$) in salmon at 16°C as compared to those assessed at 12°C, there were no differences in feed intake, HSI or fillet yield between the two assessment points; values for these parameters were approx. 0.75% body weight day^{-1}, 1.26% and 51.2%, respectively. SalmoFan™ colouration values for the fillet increased by one point on average ($p < 0.001$) from 12 to 16°C, indicating that pigmentation of

the fillet intensified as the fish grew (Figure 3-2). Basal cortisol levels (Figure 3-3), and expression of most heat stress biomarkers (i.e., *cirbp*, *hsp70*, *hsp90aa1*, *hsp90ab1*, *ndufa1*; Figure 3-4) were unaffected by this 4°C increase. In contrast, *serpinh1* and *ucp2* were both downregulated at 16°C as compared to at 12°C.

Fish sampled at 18°C in all dietary treatments, on average, gained weight (~50 g; $p < 0.01$) from when they were assessed at 16°C (Table 4-5). However, it is noteworthy that 41.3% of all fish lost weight since the 16°C assessment (-49 g on average), whereas the remaining 58.7% of the fish gained weight (+199 g on average). Feed intake decreased significantly ($p < 0.001$) compared to both previous assessments across all three of the dietary treatments (i.e., by 50.8% and 43.8% on average as compared to those measured at 12 and 16°C, respectively). Similarly, growth rate (i.e., as assessed by TGC and SGR) and condition factor declined by 78.0%, 74.0% and 11.2% ($p < 0.001$), respectively, in salmon reared at 18°C as compared to previous measurements (compare data in Tables 3-4 and 3-5). HSI was also lower ($p < 0.05$) at 18°C as compared to at 12 and 16°C in the control group. SalmoFan™ colour increased significantly (by 1.1 points on average, $p < 0.05$) in the control treatment as compared to when it was assessed at 16°C. No differences were found in fillet yield between any of the three sampling points (temperatures). However, basal plasma cortisol increased ($p < 0.05$) at 18°C compared to at 12°C (average values of 10.9 and 1.8 ng mL^{-1}, respectively). The only transcripts found to be differentially expressed at 18°C were *ucp2* (significantly downregulated from 12 and 16°C), *hsp70* (significantly downregulated from 12 and 16°C) and *serpinh1* (significantly downregulated from 12°C). No differences in any parameter were identified between the three dietary treatments at 18°C (Tables 3-4 and 3-5; Figures 3-2, 3-3 and 3-4).

Table 4-4. Morphological and production metrics for Atlantic salmon first reared at 12°C and fed the control diet for a period of 16 days, and then after they were exposed to an incremental temperature increase up to 16°C (at 0.2°C day^{-1}).

	12°C		**16°C**	
	Mean ± SE	n	Mean ± SE	n
Weight (g)	463.8 ± 3.5[a,1]	450	557.3 ± 5.6[b]	400
Length (cm)	32.6 ± 0.1[a,1]	450	35.1 ± 0.1[b]	400
K	1.33 ± 0.01[b,1]	450	1.28 ± 0.01[a]	400
Weight Gain (g)	-	-	92.0 ± 3.5	400
TGC [$g^{1/3}$ (°C $d)^{-1}$]	-	-	0.85 ± 0.03	400
SGR (% body weight day^{-1})	-	-	0.41 ± 0.02	400
Feed Intake (% body weight day^{-1})	0.80 ± 0.02[1]	9	0.70 ± 0.03	9
HSI (%)	1.33 ± 0.08	9	1.19 ± 0.06	9
Fillet Yield (%)	50.0 ± 1.3	9	52.3 ± 0.9	9

For each parameter, values without a letter in common are significantly different ($p < 0.05$). K, Fulton's condition factor; TGC, thermal growth coefficient; SGR, specific growth rate; HSI, hepatosomatic index. 1Weight, length, K and feed intake at 12°C were measured at the start of the experiment.

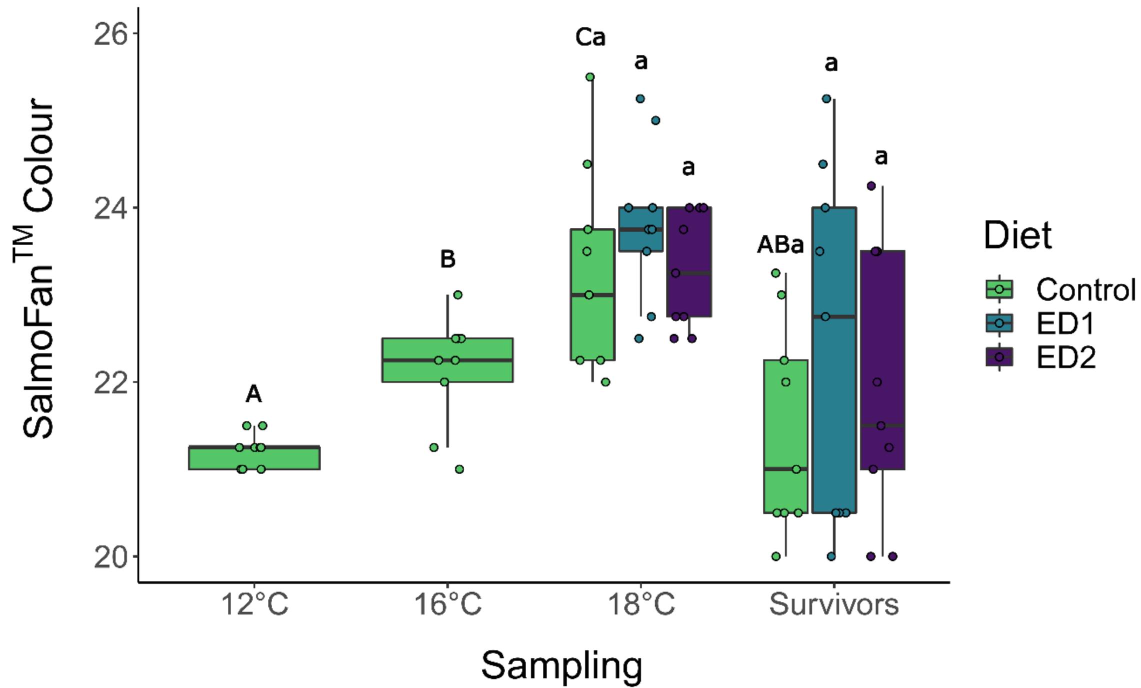

Figure 4-2. SalmoFan™ fillet colour scores over the course of the study (n = 9 per each sampling/dietary treatment). Upper case letters denote significant ($p < 0.05$) differences between samplings within the control treatment. Lower case letters indicate that no differences were detected among dietary treatments within a sampling. There was no difference in the colour scores in fish fed experimental diet #1 (ED1) and #2 (ED2) between samplings.

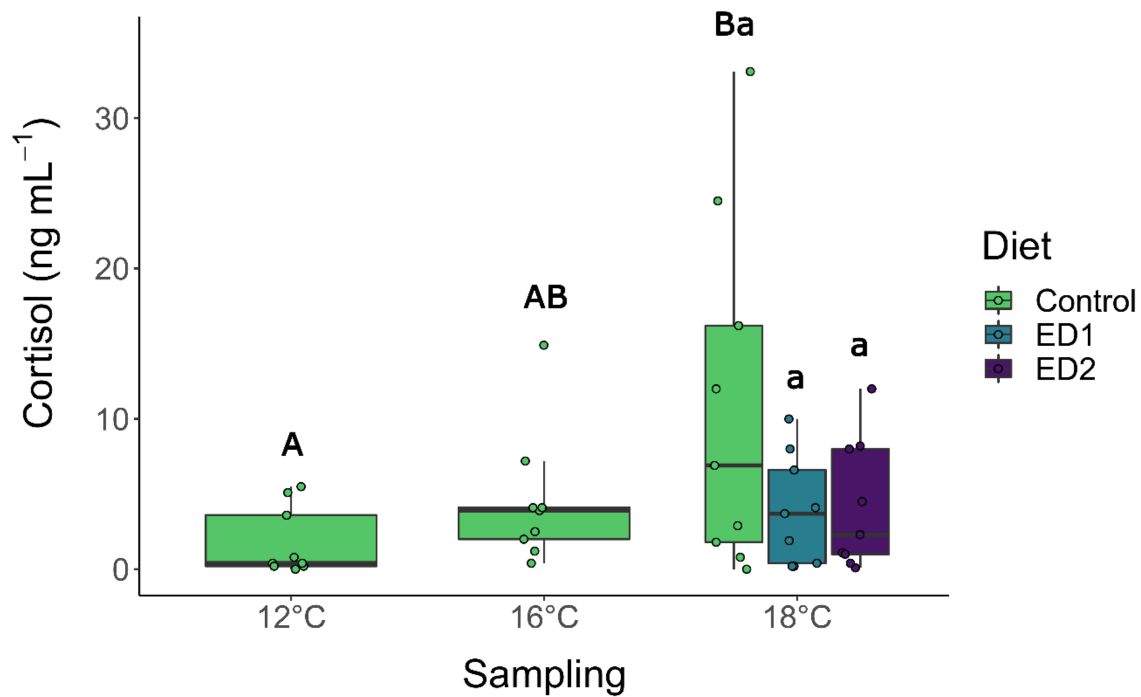

Figure 4-3. Basal (resting) plasma cortisol concentrations over the course of the study (n = 9 per each sampling/dietary treatment). Upper case letters denote significant ($p < 0.05$) differences between samplings within the control treatment. Lower case letters indicate that no differences were detected among dietary treatments at 18°C.

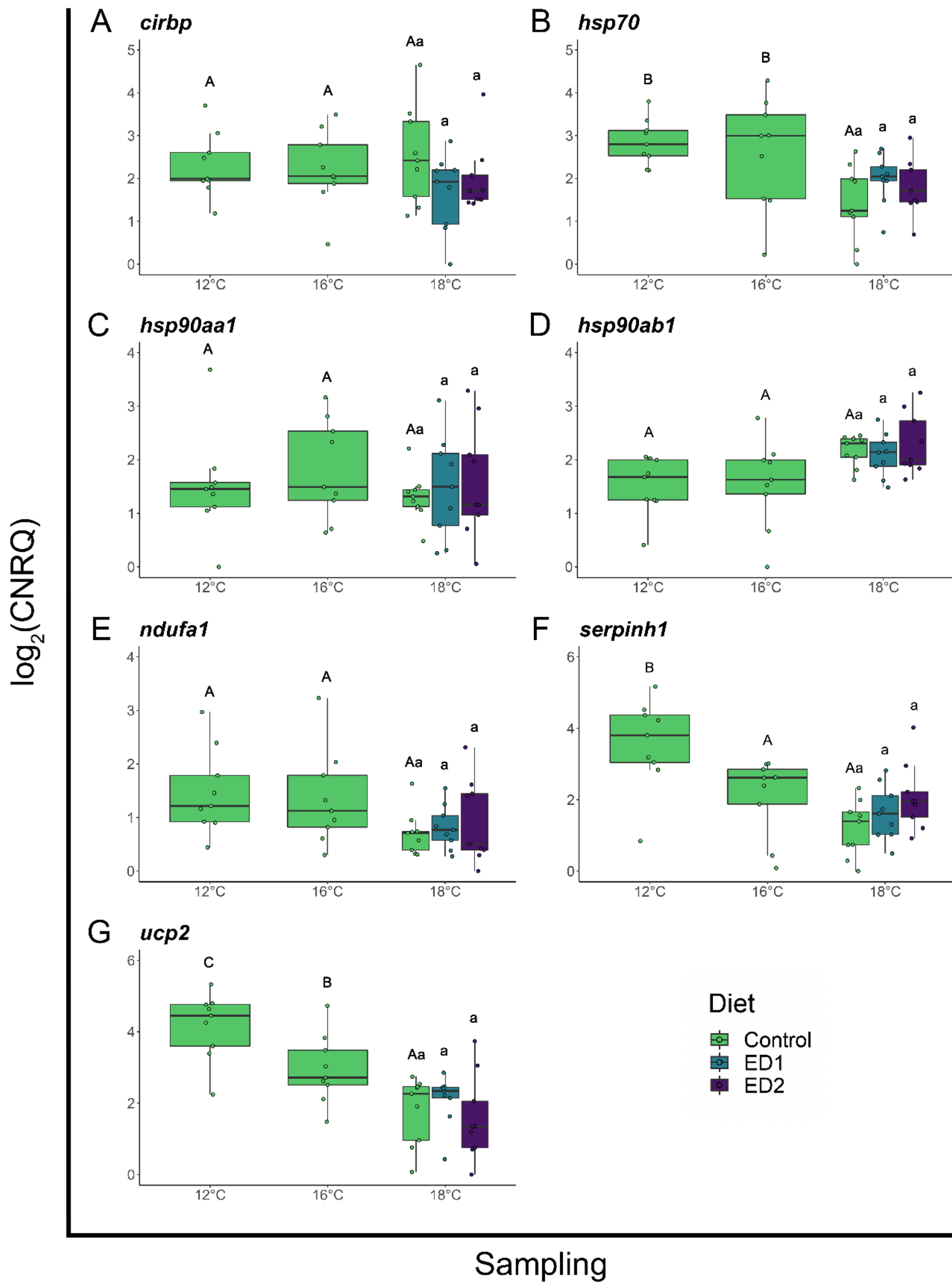

Figure 4-4. Expression levels of 7 transcripts with roles in responding to heat stress [*cirbp* (a), *hsp70* (b), *hsp90aa1* (c), *hsp90ab1* (d), *ndufa1* (e), *serpinh1* (f), and *ucp2* (g)] in the liver of female triploid Atlantic salmon sampled at 12, 16, or 18°C and provided different amounts of dietary cholesterol. Calibrated normalized relative quantities (CNRQs) were compared by one-

way ANOVA ($p < 0.05$; n = 9 per sampling/diet). Upper case letters denote significant differences between samplings within the control treatment. Lower case letters are the same between diets as no significant differences were detected at 18°C. In all cases, the letter "A" signifies the lowest value within a comparison.

Table 4-5. Morphological and production metrics for Atlantic salmon after 65 days fed either the control diet, experimental diet #1 (ED1), or experimental diet #2 (ED2). Fish were fed these diets as temperature was maintained at 16°C for 3 weeks, and then raised to 18°C where temperature was held for 5 weeks.

	Control		**ED1**		**ED2**	
	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n
Weight (g)	604.3 ± 14.5	126	609.9 ± 15.5	129	605.7 ± 13.3	130
Length (cm)	37.3 ± 0.2	126	37.4 ± 0.2	129	37.2 ± 0.2	130
K	1.13 ± 0.01	126	1.13 ± 0.01	129	1.15 ± 0.01	130
Weight Gain (g)	47.4 ± 9.0	126	52.4 ± 10.1	129	51.1 ± 8.1	130
TGC [$g^{1/3}$ (°C $d)^{-1}$]	0.18 ± 0.04	126	0.19 ± 0.04	129	0.19 ± 0.03	130
SGR (% body weight day^{-1})	0.10 ± 0.02	126	0.11 ± 0.03	129	0.11 ± 0.02	130
Feed Intake (% body weight day^{-1})	0.40 ± 0.03	3	0.43 ± 0.07	3	0.35 ± 0.04	3
HSI (%)	0.89 ± 0.03	27	1.04 ± 0.07	27	0.99 ± 0.04	26
VSI (%)	8.41 ± 0.16	26	8.47 ± 0.22	26	8.08 ± 0.21	26
Fillet Yield (%)	52.8 ± 0.9	9	51.2 ± 1.3	9	50.5 ± 0.9	9

No significant differences ($p > 0.05$) were found for any parameter between dietary groups. K, Fulton's condition factor; TGC, thermal growth coefficient; SGR, specific growth rate; HSI, hepatosomatic index; VSI, viscerosomatic index

4.4.2 Performance metrics & survival following an incremental temperature increase until 50% mortality

Feed intake declined steeply after temperature increased above 18°C in all tanks, to a point where hardly any fish were feeding after 19°C (Appendix C - Figure C1). A Kaplan-Meier curve with survival probabilities for each dietary group and a histogram of the IT_{Max} data are shown in Figure 3-5. Overall, there were very few fish that succumbed to the increasing temperature regimen before 22°C (< 5%). However, mortalities increased as the temperature rose further, and the average IT_{Max} at 50% mortality was 22.9 $\pm$ 0.2, 23.3 $\pm$ 0.2 and 23.4 $\pm$ 0.1°C in the ED2, ED1 and control groups, respectively (Figure 3-5B, Table 4-6). Although a logrank test on survival indicated that the Kaplan-Meier curves were not different between the 3 groups (Figure 3-5A; $p = 0.73$), the IT_{Max} values at 50% mortality were higher in control fish as compared to ED2 fish at a p value of 0.068. The study endpoints (i.e., 50% mortality within a particular treatment) were reached at 24.2, 24.4 and 24.0°C in the control, ED1 and ED2 groups, respectively.

Morphological and production metrics of the first 50% of fish within each dietary treatment that reached their IT_{Max} can be found in Table 4-6. Fish in all groups weighed significantly less (by ~29.9%; $p < 0.001$) than when they were assessed at 18°C and their condition factor scores were lower (by ~18.2%, $p < 0.001$), but they had higher HSI values (by ~8.6%, $p < 0.05$) (compare data in Tables 3-5 and 3-6). Interestingly, although no difference was detected within the ED2 dietary group, VSI scores were lower ($p < 0.05$) in both the control and ED1 treatments at 50% mortality (IT_{Max}) compared to at 18°C.

Fish that survived to the endpoint of the experiment (i.e., after 50% of fish in each dietary treatment reached their IT_{Max}), weighed more ($p < 0.01$), lost less weight ($p < 0.01$), were longer

($p < 0.01$) and had higher condition factor values ($p < 0.05$) as compared to fish within their respective dietary treatment that succumbed (compare Tables 3-6 and 3-7). However, these parameters were not different between the groups when survivors were compared (Table 4-7). SalmoFan™ colour scores for the control group were significantly less at this sampling point than when measured at 18°C. In contrast, no such decrease in SalmoFan™ colour score was observed in fish in the ED1 and ED2 groups (Figure 3-2).

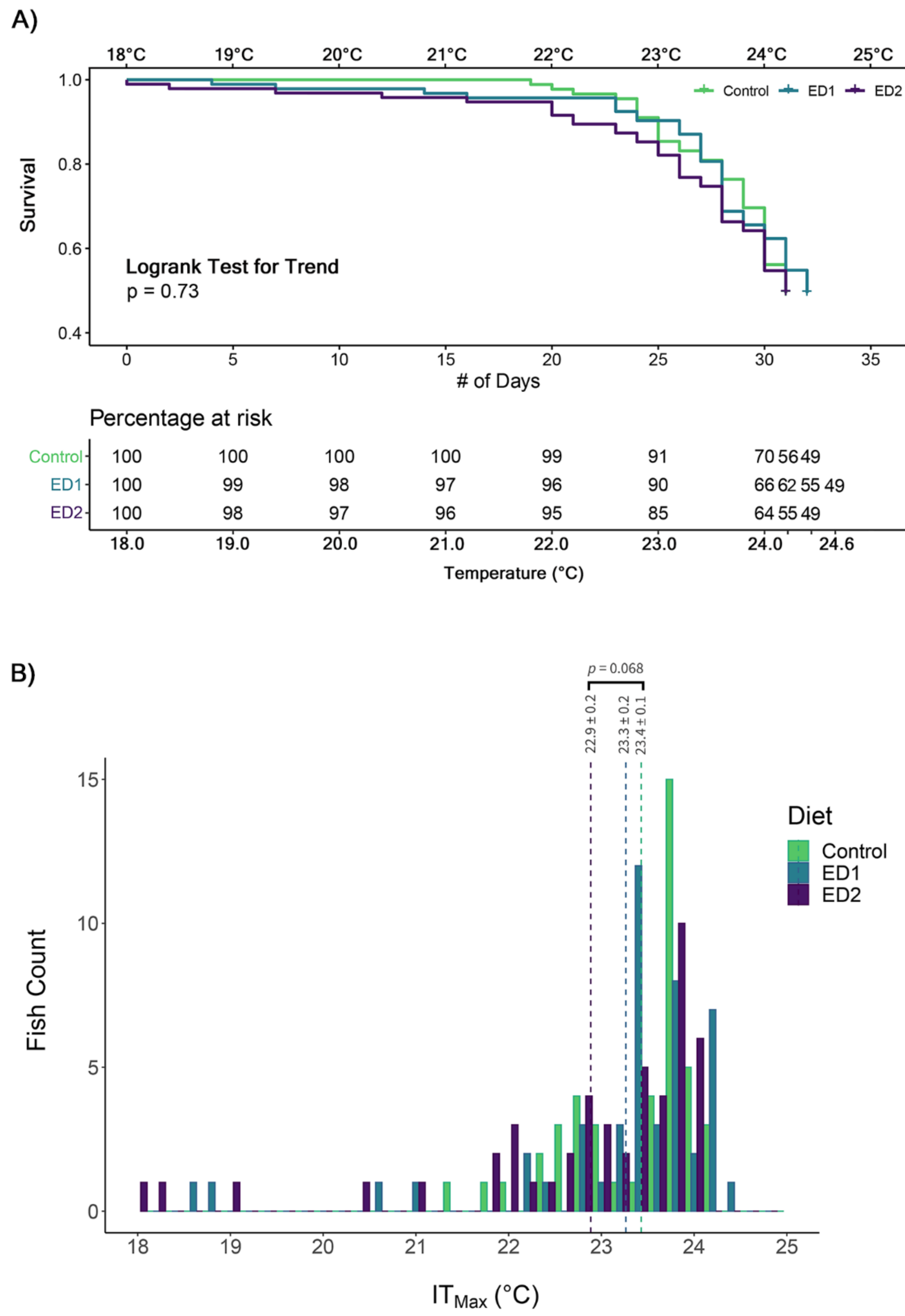

Figure 4-5. The incremental thermal maximum (IT_{Max} at 50% mortality in each dietary treatment) of female triploid Atlantic salmon exposed to a temperature increase of 0.2°C day^{-1} from 18°C after prolonged exposure to elevated temperatures (≥16°C) for 65 days. Fish were fed a control diet, or one of two diets supplemented with cholesterol (ED1 and ED2). (a) Kaplan-Meier survival curves are shown along with the results of the log-rank test to determine significance. (b) Histogram of the same dataset. The dashed lines indicating the average IT_{Max} value for the first 50% of fish that succumbed to high temperature for each dietary treatment.

Table 4-6. Morphological and production metrics, and the incremental thermal maximum (IT_{Max}) at which the first 50% of Atlantic salmon succumbed to the increasing temperature protocol. Fish were fed either the control diet, experimental diet #1 (ED1) or experimental diet #2 (ED2).

	Control		ED1		ED2	
	Mean ± SE	n	Mean ± SE	n	Mean ± SE	n
IT_{Max} (°C)	23.4 ± 0.1	45	23.3 ± 0.2	47	22.9 ± 0.2	48
Weight (g)	416.2 ± 16.9	45	436.4 ± 17.7	47	422.9 ± 17.0	48
Weight Gain / Loss (g)	-76.0 ± 7.7	45	-71.2 ± 8.9	47	-78.9 ± 4.3	48
Length (cm)	35.3 ± 0.4	45	35.7 ± 0.4	47	35.4 ± 0.4	48
K	0.93 ± 0.02	45	0.93 ± 0.02	47	0.93 ± 0.02	48
HSI (%)	1.07 ± 0.03	45	1.05 ± 0.04	47	1.05 ± 0.04	48
VSI (%)	7.44 ± 0.30	45	7.31 ± 0.23	47	7.35 ± 0.24	48
RVM (%)	0.073 ± 0.002	45	0.070 ± 0.002	47	0.079 ± 0.004	48
Fillet Yield (%)	47.2 ± 0.5	9	44.9 ± 1.3	9	44.8 ± 1.7	9

No significant differences between dietary treatments ($p > 0.05$) were found for any parameter. Weight gain / loss is in comparison to the assessment that was performed at 18°C. K, Fulton's condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index; RVM, relative ventricular mass.

Table 4-7. Morphological parameters for Atlantic salmon that survived to the endpoint of the experiment (i.e., when 50% of each population reached their incremental thermal maximum [IT_{Max}]); control (n = 44), experimental diet #1 (ED1; n = 46), experimental diet #2 (ED2; n = 47).

	Control	ED1	ED2
	Mean ± SE	Mean ± SE	Mean ± SE
Weight (g)	541.7 ± 19.1	535.1 ± 23.5	555.6 ± 18.9
Weight Gain / Loss (g)	-38.8 ± 7.6	-35.0 ± 5.2	-56.2 ± 5.7
Length (cm)	37.6 ± 0.3	37.5 ± 0.4	37.6 ± 0.4
K	1.00 ± 0.02	0.98 ± 0.02	1.02 ± 0.02

No significant ($p > 0.05$) differences between dietary treatments were found. Weight gain is in comparison to the assessment that was performed at 18°C. K, Fulton's condition factor.

4.5 Discussion

The objective of this research was to determine if supplementary dietary cholesterol could improve the upper thermal tolerance and growth performance of female triploid Atlantic salmon, a commercially important farmed fish in Newfoundland, Canada. Further, this study examined if adding cholesterol to salmon diets could preserve / protect fillet pigmentation in fish exposed to rising temperatures or alter the temperature-induced stress response. Supplemental dietary cholesterol did not improve survival or enhance growth at elevated temperatures, or alter the hepatic expression of heat stress-associated transcripts, and may have negatively affected survival at the highest inclusion level (+1.76% cholesterol). However, adding cholesterol to the diet: did limit fillet bleaching (i.e., loss of pigmentation) from occurring (at least in some salmon), as only the control treatment saw a significant decrease in SalmoFan™ scores after 50% of fish reached their IT_{Max}; and potentially reduced basal cortisol (i.e., physiological stress) levels at elevated temperatures.

4.5.1 Growth performance

Interestingly, a high proportion (i.e., >40%) of salmon lost weight when reared at temperatures between 16 and 18°C. It is possible that this was directly related to triploidy or the particular stock used, and that they did not increase feed intake as temperature rose and feed intake fell noticeably at 19°C. For example, it has been previously reported that triploid Atlantic salmon exhibit reduced feed intake at elevated temperatures (i.e., 18°C) as compared to diploids (Sambraus et al., 2018, 2017). AquAdvantage Salmon (growth hormone transgenic female triploid Atlantic salmon produced by AquaBounty) have reduced values for TGC at temperatures

above 16°C compared to 10.5 and 13.5°C (Ignatz et al., 2020b). Further, Chapter 2 showed that conventional male diploid Atlantic salmon sourced from AquaBounty Canada, and exposed to a similar incremental thermal challenge, increased their feed intake until 22°C. However, this finding may have also been influenced by other factors. First, a small proportion of fish in this study developed dermal sores before the assessment at 16°C, so it is possible that some fish were combatting an unknown infection despite the absence of clinical signs (i.e., did not develop dermal sores like other fish). Second, while genetic variation in this study was low (i.e., all fish were full- or half-siblings), there is still potential that female parentage influenced growth performance at elevated temperatures. Finally, feed intake in the current experiment regardless of rearing temperature was lower than anticipated. At the start of the experiment, when fish were abruptly switched from a commercial feed to the control diet, feed intake dropped by ~39.3% over the next 2 weeks before increasing again (Appendix C Figure C-1). A more gradual transition between the commercial feed and the test control diet may have better maintained feed intake during the early portion of the study (Dessen et al., 2020).

4.5.2 Fillet pigmentation

It is paramount that salmon aquaculture producers maximize fillet pigmentation / astaxanthin levels. High astaxanthin retention is associated with higher total antioxidant capacity in juvenile rainbow trout (Rahman et al., 2016); a potentially useful trait in fish exposed to elevated temperatures. In addition, darker fillet pigmentation is more attractive to consumers (Lerfall et al., 2017). Yet astaxanthin is both an expensive (Solymosi et al., 2015) and a restricted additive (i.e., regulations limit dietary carotenoid inclusion at ≤ 80 mg kg^{-1} feed) (Wrolstad and Culver, 2012). Several studies have reported a decrease in fillet astaxanthin concentration /

pigmentation in adult Atlantic salmon during the summer months (i.e., at high water temperatures), which is likely associated with increased oxidative stress and the mobilization of astaxanthin to maintain redox homeostasis (Grünenwald et al., 2019; Nordgarden et al., 2003; Wade et al., 2019). A significant decrease in SalmoFan™ colouration at 50% mortality as compared to at 18°C was only observed in the control group in the current study, and this suggests that supplemental cholesterol reduced astaxanthin mobilization or increased absorption/retention (Chimsung et al., 2014, 2013).

While SalmoFan™ scores increased between 16 and 18°C, it is noteworthy that values only increased by ~1 point, the same amount that they increased during the short-term transition from 12 to 16°C (20 days). These data suggest that astaxanthin retention was difficult for triploid salmon at temperatures ≥ 16°C, and the results of Ignatz et al. (2020b) support this hypothesis. SalmoFan™ scores were lower in 1.5 kg AquAdvantage Salmon reared at 16.5°C compared to at 13.5°C (Ignatz et al., 2020b). Single nucleotide polymorphisms (SNPs) for *dual oxidase 2* (*duox2*) and *dual oxidase maturation factor 1* (*duoxa1*) have been identified in Atlantic salmon hindgut samples via RNA-sequencing (RNA-seq) that are associated with high (i.e., SalmoFan™ scores ≥ 25 following summer rearing) and low fillet colouration (i.e., SalmoFan™ scores ≤ 24), respectively (Vo et al., 2021). It would be valuable in future studies to examine if these types of markers can be validated in salmon exposed to IT_{Max} protocols using either RNA-seq-based identification of trait-associated SNPs or SNP genotyping using gDNA.

4.5.3 Stress indices

Overall, the cortisol and hepatic transcript expression data suggest that female AquaBounty triploid salmon that were still feeding were not experiencing stress at temperatures

up to 18°C. However, at 18°C, basal cortisol levels increased slightly in the control treatment compared to measurements at 12°C (Figure 3-3). While no significant differences were detected between dietary treatments at 18°C, average cortisol levels were 64.2% and 61.5% lower in the ED1 ($p = 0.14$) and ED2 ($p = 0.16$) groups, respectively, as compared to the controls. Therefore, supplemental cholesterol may potentially lower resting cortisol concentrations. The current results agree with past findings on ~ 800 g AquAdvantage Salmon, where no differences in basal cortisol levels were detected between fish reared at 10.5, 13.5 or 16.5°C (Ignatz et al., 2020a). While it could be hypothesized that supplemental dietary cholesterol might increase basal cortisol concentrations, as cholesterol is a precursor in cortisol synthesis (Alsop and Vijayan, 2009), this was not observed in the current study. It is possible that the additional cholesterol assisted in maintaining cell membrane rigidity (Crockett, 1998), and ultimately reduced cellular and overall stress. Differences in cortisol regulation could have also altered gluconeogenesis in the liver and modulated energy (e.g., lipid) stores within the fish (Kuo et al., 2015). However, these hypotheses would need to be tested.

In contrast, the transcript expression of heat stress biomarkers measured in the liver does not align well (with the exception of *ucp2*) with past investigations of chronic/prolonged heat stress in Atlantic salmon (Beemelmanns et al., 2021b, 2021a; Ignatz et al., 2022). It was anticipated that the expression of heat shock proteins (i.e., *hsp70*, *hsp90aa1*, *hsp90ab1*, *serpinh1*) would be upregulated, and that expression of transcripts associated with oxidative stress (i.e., *cirbp*, *ndufa1*, *ucp2*) would be downregulated at 16 and/or 18°C as compared to at 12°C (Beemelmanns et al., 2021b, 2021a; Ignatz et al., 2022). However, in this study, I report that *hsp70* and *serpinh1* were downregulated at 16 and/or 18°C compared to at 12°C, and that temperature had no effect on the expression of *cirbp*, *hsp90aa1*, *hsp90ab1* and *ndufa1* (Figure 3-

4). While it is not surprising that some heat shock proteins were not yet upregulated at 16°C, an incremental temperature increase (+1°C week^{-1}) up to 18°C from 12°C previously increased the expression of *hsp70*, *hsp90aa1* and *serpinh1* in diploid post-smolt Atlantic salmon (Beemelmanns et al., 2021a). The fact that fish in the current study spent 65 days at temperatures ≥ 16°C before sampling at 18°C could potentially explain the differences observed. Prolonged exposure to elevated temperatures could lead to acclimatory responses, thus limiting the requirement for heat shock proteins to prevent protein damage or denaturation. Although, 800 g AquAdvantage Salmon reared in freshwater at 16.5°C for several months still exhibited higher expression of *hsp90ab1* and *serpinh1* and lower expression of *cirbp* compared to salmon reared at 10.5°C (Ignatz et al., 2022). The differences in time spent at elevated temperatures, genetic background, water salinity, ploidy and/or transgenesis make it difficult to directly compare with past results (Beemelmanns et al., 2021a; Ignatz et al., 2022).

It is also possible that this discrepancy was due to the fact that the fish were potentially exposed to an unknown pathogen from 12 to 16°C. However, this hypothesis is quite speculative. Eslamloo et al. (2022) showed that: *hsp70*, *hsp90aa1* and *cirbp* transcript expression were upregulated, while that of *serpinh1* and *ucp2* was downregulated, in the skin of ~200 g (8°C acclimated) diploid Atlantic salmon exposed to *Moritella viscosa*; whereas, liver *hsp70*, *hsp90aa1, serpinh1* and *ucp2* transcription were upregulated, and *cirbp* transcription downregulated, in ~500 g male diploid Atlantic salmon reared at 12 or 20°C and injected with a commercial multivalent vaccine (i.e., Forte Micro; containing formalin-inactivated cultures of several bacterial pathogens) (Chapter 3).

Collectively, this body of research/data, indicates that more research needs to be conducted to elucidate the impact that chronic exposure to elevated temperatures has on the

hepatic transcript expression of female triploid Atlantic salmon. Such research could also help to identify potential paralogue-specific differences in transcript expression.

4.5.4 Survival at elevated temperatures

It has been suggested that triploid salmonids have reduced survivorship at elevated temperatures compared to diploids (Hyndman et al., 2003; Ojolick et al., 1995), although this phenomenon is not observed consistently (Benfey et al., 1997; Bowden et al., 2018; Ellis et al., 2013). This is the first study to expose triploid Atlantic salmon to an incremental temperature increase (i.e., an IT_{Max} test) that mimics the natural conditions that these fish are expected to encounter in the North Atlantic as ocean temperatures rise. In the current study, my triploid all-female salmon did not experience large numbers of mortalities until after 22°C, which is similar to what has been reported for diploid mixed-sex Atlantic salmon exposed to comparable gradual increases in temperature (Bartlett et al., 2022; Gamperl et al., 2020). However, it must be noted that: 1) these fish are from a commercial farm that has held salmon for 25 years (~ 8 generations) in land-based systems where the lowest temperature they experience is 6°C; 2) that long-term rearing conditions can have a significant effect on a fish's response to environmental conditions (e.g., Adams et al., 2022; Faust et al., 2004); and 3) that diploid male salmon from AquaBounty have an even higher IT_{Max} (by > 1°C) at which 50% mortality occurred than the fish used in the current study (Chapter 2). Thus, the high IT_{Max} values reported for all-female triploid salmon may be specific to AquaBounty's stock. It is also possible, that in comparison to previous IT_{Max} tests on diploid Atlantic salmon where fish did not spend an extended period at ≥ 16°C (Chapter 2; Bartlett et al., 2022; Gamperl et al., 2020), that the IT_{Max} of the current triploids may be underestimated. Nonetheless, the most important finding here is that triploid all-female salmon

can be developed that are able to withstand the highest summer temperatures that are reported in Atlantic Canada and in Europe (Burke et al., 2020; Gamperl et al., 2021; Johansson et al., 2007, 2006). Advances in genotyping methods could lead to the selection of Atlantic salmon (stocks) with even higher values for upper thermal tolerance, as this trait has been shown to be heritable in other salmonids (Gallardo-Hidalgo et al., 2021; Ihssen, 1986; Perry et al., 2005). Indeed, analyses of AquaBounty's genetics in relation to IT_{Max} are ongoing.

4.6 Conclusions

The results of this study are particularly relevant to the production of female triploid Atlantic salmon. As the triploid salmon used in this study did not experience high mortalities until after 22°C, this suggests that their production is potentially suitable for the North Atlantic. However, further study is required to verify if this is true across all farmed triploid populations, or specific to those reared in the land-based facilities at AquaBounty Canada.

Additional research is also recommended into the use of dietary manipulation to enhance the production of farmed all-female triploid Atlantic salmon. While the current results suggest that supplemental cholesterol may not be beneficial to Atlantic salmon reared at elevated temperatures, other ingredients/additives (e.g., prebiotics, vitamins C and E, selenium) show promise for improving thermal tolerance in, and/or offsetting the negative impacts of heat stress on, other fish species (Dong et al., 2020; Gupta et al., 2010; Islam et al., 2021a; Khosravi-Katuli et al., 2021). It also remains to be determined/reported what effect additional dietary cholesterol has on other important characteristics (e.g., the innate antibacterial and antiviral immune responses, or tissue lipid composition) in the salmon used in the current study (although these

data are currently being analyzed; Ignatz et al., in prep.). Therefore, it is still possible that increased dietary cholesterol may have benefited these salmon in other ways.

Finally, it may also be worthwhile to test lower inclusion levels of supplemental cholesterol. In this study, I attempted to match the 1.37 and 1.83% total dietary cholesterol values reported in Deng et al. (2013), where at the lower inclusion level, supplemental cholesterol enhanced antioxidant activity and survival of rainbow trout following injection with *A. hydrophila*. However, total sterols (Table 4-2) in ED1 and ED2 were both higher, at 2.13 and 2.54%, respectively. Dietary non-cholesterol sterols (i.e., phytosterols) are included in the total sterol amounts and possible positive effects of supplemental cholesterol may have been negated by them. However, in this regard the data are again ambiguous. Whereas Deng et al. (2013) suggest that positive effects of supplemental cholesterol may not be seen at such high inclusion levels, the addition of 2.0 - 2.69% cholesterol improved astaxanthin absorption in pre-adult Atlantic salmon reared at 10 and 12°C without any mention of negative effects (Chimsung et al., 2014, 2013). Overall, the current study results provide novel insight into the roles that both rearing temperature and dietary cholesterol may have on the production of female triploid Atlantic salmon. However, further research will help optimize strategies to improve performance in these farmed populations.

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CHAPTER 5: The Atlantic salmon's (*Salmo salar*) incremental thermal maximum is a better indicator of family-based differences in upper temperature tolerance than its critical thermal maximum

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5.1 Abstract

Rising sea surface temperatures and heat waves are a challenge to salmon aquaculture, and the industry must endeavour to mitigate their impacts. To investigate genetic-based differences in upper thermal tolerance, 20 salmon families were exposed to an incremental temperature increase (+0.2°C per day from 12°C) to mimic the rise in temperatures experienced at sea-cages in Atlantic Canada during the summer, or held at 10°C. Post-smolt Atlantic salmon exposed to the incremental temperature increase up to 20°C gained more weight than fish reared at 10°C over the same period, and there were family-specific differences ($p < 0.05$) in growth and body morphometrics. Significant differences were also detected between the families' incremental thermal maximum (IT_{Max}), with average family IT_{Max} values ranging from 23.3 to 25.0°C. Fish weight and growth rate were not significantly related to IT_{Max}, but a negative correlation ($p < 0.01$) was found between hepatosomatic index (HSI) and IT_{Max}. In contrast, the critical thermal maximum (CT_{Max}, measured using a temperature increase of 2°C h^{-1} from 10°C) of the bottom and top four temperature tolerant families (as determined by IT_{Max}) did not differ (~28.0°C). A negative correlation ($p < 0.01$) between HSI and CT_{Max} was also found. However, no relationship was evident between relative ventricular mass (RVM) and IT_{Max} or CT_{Max}. My data show that IT_{Max} is a more sensitive metric of a salmon's upper temperature tolerance than even an 'environmentally relevant/realistic' CT_{Max} test, and suggest that it could be used in breeding programs that aim to enhance the salmon's tolerance to withstand rising ocean temperatures.

5.2 Introduction

Water temperature plays a dominant role in regulating the physiology, metabolism and behaviour of aquatic ectotherms (Clarke and Johnston, 1999; Huang et al., 2021; Morash et al., 2021), and is expected to play an even greater role in shaping fish biology and aquaculture production given accelerated climate change (Falconer et al., 2022; Islam et al., 2021; Reid et al., 2019). For example, sea surface temperatures over the past three decades have been higher than all other reliable observations since 1880 (Boyin et al., 2017), and are only expected to continue to increase (IPCC, 2022). Further, ocean heat waves are becoming more frequent and severe, and persisting for longer periods (Frölicher et al., 2018; Holbrook et al., 2019; Oliver et al., 2018).

Atlantic salmon farming is already experiencing challenges with increasing sea surface temperatures. In Tasmania, high water temperatures are negatively impacting growth, feed intake and quality traits like fillet pigmentation (Meng et al., 2022; Wade et al., 2019). More importantly, temperatures as high as 23°C at the surface can be concomitant with low oxygen conditions at the bottom of sea-cages, and this is leading to issues of crowding at intermediate depths (a potential welfare issue) (Stehfest et al., 2017). Further, there was a mass die-off of over 2.6 million farmed salmon in 2019 on the southern coast of Newfoundland after a prolonged period of sea surface temperatures > 18°C and concurrent sea lice (*Lepeophtheirus salmonis*) infestation and treatment (Burke et al., 2020). Collectively, this information points to climate change as a current (and future) threat to the sustainability of the salmon aquaculture industry, and suggests that mitigation strategies need to be developed, and implemented, in the short-term.

Upper thermal tolerance in fishes has commonly been estimated using the critical thermal maximum (CT_{Max}) test; an acute protocol in which temperature is increased rapidly at rates

between 2 and 18°C h^{-1} until the fish loses equilibrium (Lutterschmidt and Hutchison, 1997). The CT_{Max} of Atlantic salmon is well reported across strains, populations and life stages, and ranges from 24 to 33°C depending upon the rate of heating (Chapter 2; Anttila et al., 2015, 2013; Bowden et al., 2018; Corey et al., 2017; Elliott and Elliott, 1995; Gallant et al., 2017; Hines et al., 2019; Leeuwis et al., 2019; Penney et al., 2014; Shi et al., 2018). However, without standardized methodology, my ability to interpret and compare results between studies is difficult. Further, without ecological and/or practical reasons to use such rapid increases in temperature, the results often have little value beyond a mechanistic physiological perspective (Adams et al., 2022; Morash et al., 2018; Rodnick et al., 2004). For example, Atlantic salmon reared in sea-cages in the North Atlantic typically do not experience average temperature increases of more than ~1-2°C $week^{-1}$ during the seasonal transition to summer (Björnsson et al., 2007; Burt et al., 2012; Gamperl et al., 2021). Therefore, a more industrially-relevant approach has been recommended to measure a fish's upper thermal tolerance. The incremental thermal maximum (IT_{Max}) test is a relatively new method for assessing the survival of aquatic species when exposed to increases in temperature that more realistically mimics conditions in the natural environment and/or in sea-cages. The IT_{Max} test was first used to estimate the upper thermal tolerance of Atlantic cod (*Gadus morhua*) (Zanuzzo et al., 2019), but has since been employed in several studies on Atlantic salmon (Chapters 2 & 4; Bartlett et al., 2022; Gamperl et al., 2020). However, no studies to date have examined if family-based differences in salmon upper temperature tolerance can be identified using the IT_{Max} test.

Therefore, the objectives of the current study were to: 1) measure the incremental upper thermal tolerance (i.e., IT_{Max}) of 20 families of Atlantic salmon; 2) determine how these data compare with/are related to their acute upper thermal tolerance (i.e., CT_{Max}); and 3) perform

regression analyses to establish what relationships, if any, exist between phenotypic/morphometric characteristics (including those important for salmon aquaculture production) and these two measures of upper thermal tolerance. With genetic family-based selection now standard across the industry, artificial selection is helping to develop genotypes associated with farmed salmon robustness (e.g., resistance to environmental stressors, pathogens, etc.). The novel results presented in this paper are anticipated to be of great benefit to the salmon aquaculture industry, and provide validation as to why IT_{Max} is a better metric (at least as compared to CT_{Max}) to use in programs/research whose aim is to improve the tolerance of farmed finfish populations in the face of rising ocean temperatures.

5.3 Materials and methods

This study was approved by the Animal Care Committee of Memorial University of Newfoundland and Labrador (protocol #19-19-KG), and salmon husbandry and experimental procedures were performed in accordance with the Canadian Council on Animal Care Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (Canadian Council on Animal Care, 2005).

5.3.1 Experimental animals

Previously PIT (Passive Integrated Transponder)-tagged conventional mixed-sex, diploid, Atlantic salmon from 20 families of St. John River origin were transported from AquaBounty Canada (Prince Edward Island, Canada) to the Dr. Joe Brown Aquatic Research Building (JBARB; Ocean Sciences Centre, Memorial University of Newfoundland and Labrador). These families were produced from 20 dams and 13 sires (see Table 5-1 for cross structure) within the

same week, with no prior selection or knowledge of their thermal tolerance. The salmon were smolted and reared in a single 10 m^3 flow-through seawater tank (stocking density ~20 kg m^{-3}) at ambient temperatures (~5.5-8.0°C) for a period of ~5 months. The fish were then transferred to the Laboratory for Atlantic Salmon and Climate Change Research (LASCCR; Ocean Sciences Centre, Memorial University of Newfoundland and Labrador) and initially distributed amongst eight 2.2 m^3 tanks (~140 fish $tank^{-1}$) with flow-through seawater at 7°C prior to experimentation.

Table 5-1. Family cross structure of the Atlantic salmon used to assess thermal tolerance traits in this study.

Dam ID:	Sire ID:	Family ID:
D1	S1	F1
D2	S2	F2
D3	S3	F3
D4	S3	F4
D5	S4	F5
D6	S5	F6
D7	S6	F7
D8	S7	F8
D9	S7	F9
D10	S7	F10
D11	S7	F11
D12	S7	F12
D13	S8	F13
D14	S6	F14
D15	S9	F15
D16	S10	F16
D17	S11	F17
D18	S12	F18
D19	S13	F19
D20	S13	F20

5.3.2 Experimental design

The salmon were given 6 days to acclimate to the LASCCR facility before they were anesthetized (0.2 g L^{-1} AquaLife TMS; Syndel Laboratories Ltd, Nanaimo, BC, Canada), weighed, and re-distributed amongst ten 2.2 m^3 tanks; with an effort made to include ~6 fish per family within each tank (initial stocking density ~17 kg m^{-3}). Temperature was maintained at 7°C for another 3 days before increasing it at 0.5°C day^{-1} until all tanks reached 12°C, and this temperature was maintained for 2 weeks. At the end of this acclimation period, the fish were anesthetized (0.2 g L^{-1} TMS) and measured for weight and fork length. During this initial assessment, it was observed that 49 fish (4.4% of the total study population) had developed dermal ulcers similar to what was reported in Chapter 4. These fish were culled, and samples were taken to be analyzed by the Microbial Pathogenesis and Vaccinology Laboratory (Memorial University). However, the results were inconclusive (data not shown).

Figure 5-1 shows an overview of the protocol used to assess the growth performance and IT_{Max} of these salmon families. After the initial assessment was performed, the 'warm' treatment (5 tanks) was exposed to an incremental thermal maximum (IT_{Max}) challenge at a rate of +0.2°C day^{-1}. This level of increase was chosen to mimic conditions that these fish would be expected to encounter during a Newfoundland summer (Burt et al., 2012; Gamperl et al., 2021). In contrast, the 'control' treatment (5 tanks) had their temperature lowered to 10°C (-0.2°C day^{-1}) and they remained at this temperature for the duration of the study. This was done as past experience in our facility has shown that ulcers do not develop at this lower temperature, and to ensure the health/welfare of the fish. This small difference in acclimation temperature (i.e., lowering temperature from 12 to 10°C in the control treatment), however, is unlikely to have influenced the overall results of the study. A further 30 fish were removed from the warm treatment (during

the increase from 12 to 16°C) due to the presence of ulcers, and two Parasite S (Syndel Canada, Nanaimo, BC, Canada) treatments (250 ppm for 45 min) were given to all tanks 7 and 14 days after the initial assessment (i.e., after 2 weeks of acclimation to 12°C).

When the warm treatment reached 20°C (36 days after the temperature increase began), another assessment was performed wherein fish from both temperature groups were anesthetized (0.2 g L^{-1} TMS), had their weight and fork length recorded, and subsets of salmon from all families in both treatments were sampled for transcriptomic and genetic (single nucleotide polymorphism; SNP) analyses to be conducted through the Mitigating the Impact of Climate-Related Challenges on Salmon Aquaculture (MICCSA) project. These data are the focus of future papers. The temperature in these tanks was then increased at 0.2°C day^{-1} until all salmon became moribund (i.e., lost equilibrium/succumbed), with temperature recorded for each individual fish as their IT_{Max}. At this point, the salmon were euthanized, weight, fork length, liver weight, ventricle weight, sex and state of sexual maturity were recorded, and a fin clip taken for SNP analyses.

Water temperatures in this study were controlled by large plate heat-exchangers that supplied heated seawater to separate header tanks for each treatment. Each header tank supplied seawater to 5 individual tanks (Appendix D Figure D-1). Tank flow rates were set to 15 L min^{-1} and photoperiod was maintained at 14 h light: 10 h dark. Oxygen levels were >100% air saturation throughout the experiment, with diffusers in the tanks supplied with pure O_2 when necessary (e.g., when temperature in the tanks exceeded 20°C). Temperature and oxygen levels in all tanks were measured manually (using a YSI ProODO meter, Yellow Springs, OH, USA) at least once daily, and 2 tanks in each treatment were continually monitored (via a YSI 5500D Multi DO Optical Monitoring and Control Instrument) for these parameters. Fish were fed a

commercial salmon diet (Northeast Nutrition, Truro, NS, Canada), which transitioned from 3.0 mm (Signature Salmon FW; minimum 49% crude protein, 20% crude fat) to 4.0 mm (Signature Salmon PW; minimum 46% crude protein, 29% crude fat) feed as the study progressed / the fish grew. Daily rations were calculated based on feed intake data from Chapter 2 and predicted growth rate within both the control and warm treatments. Therefore, the control treatment was fed 0.8% body weight day^{-1} for the duration of the experiment while the warm treatment was fed 0.8-1.3% body weight day^{-1} as temperature rose. Fish were hand fed 30% of their ration in the morning, and then the remaining amount was provided through vibrational feeders set to shake for 0.5 s every 20 min over 8 h. However, salmon in the warm treatment were hand fed twice daily when feed intake decreased at high temperatures.

Once all the IT_{Max} data had been collected and analyzed (2 weeks after the IT_{Max} trial concluded), salmon from the 'bottom' 4 and 'top' 4 thermally tolerant families remaining in the control (10°C) treatment were re-sorted into a single 2.2 m^3 tank (8 fish family^{-1}; 64 fish total), and allowed to recover for two weeks before a CT_{Max} test was performed on these fish. To accomplish this, half of the fish (32 in total; 4 fish family^{-1}) were transferred into six 0.5 m^3 tanks (5 - 6 fish tank^{-1}) and held for 20 h before testing. The next day, temperature was continuously raised at 2°C h^{-1} (Rodnick et al., 2004), with temperature and oxygen values recorded in all 6 tanks every 15 min. Tank flows were set to 5 L min^{-1} and oxygen levels never fell below 100% air saturation. When a fish was unable to maintain equilibrium, the temperature was recorded as their CT_{Max}, and the salmon were netted out, euthanized (0.4 g L^{-1} TMS), and had their weight, fork length, liver weight, ventricle weight, sex and level of sexual maturity documented. This entire procedure was then repeated for the remaining 32 fish starting the following day.

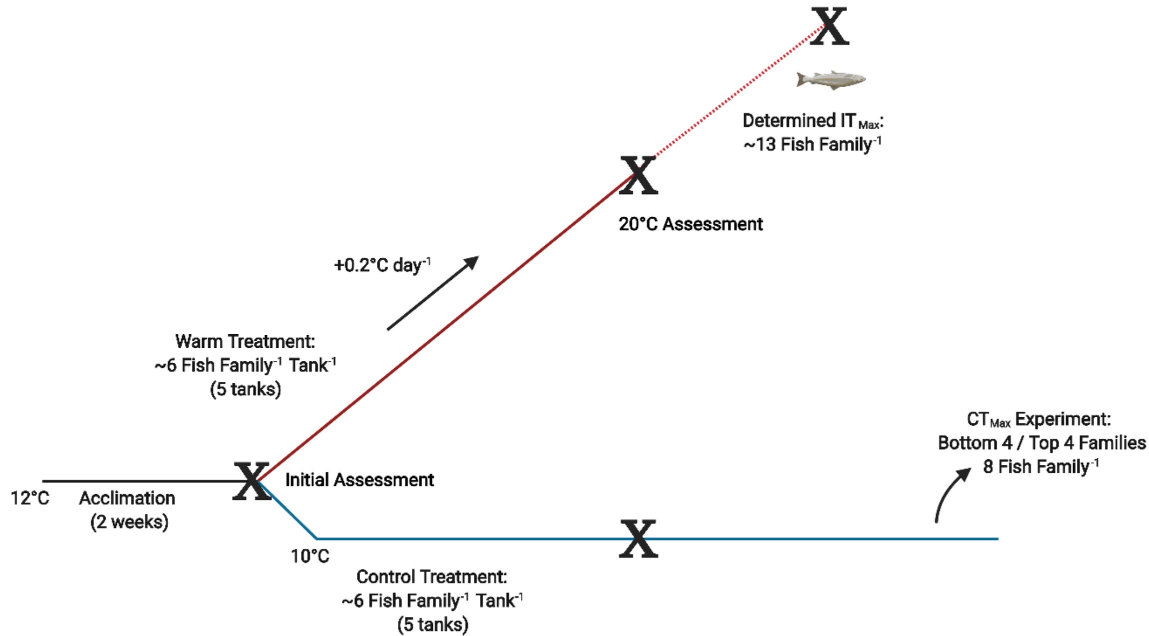

Figure 5-1. Overview of the protocol used to determine the incremental upper thermal tolerance (IT_{Max}) of 20 Atlantic salmon families and the acute upper thermal tolerance (CT_{Max}) of the bottom 4 and top 4 thermally tolerant families as identified in the IT_{Max} test. 'X' indicates time points when the morphometrics of all fish were determined. Created with BioRender.com.

5.3.3 Growth parameters

Weight gain was calculated for both treatments between the initial assessment, the assessment performed as the warm treatment reached 20°C and as fish reached their IT_{Max}, and each fish's thermal growth coefficient (TGC) was calculated to assess growth (Cho, 1992; Iwama and Tautz, 1981). TGC was calculated using the following formula:

$$TGC = \left(\frac{W_f^{1/3} - W_i^{1/3}}{\sum_{i=1}^{n} T_i}\right) \times 1000$$

where W_f and W_i are the final and initial fish body weights (in g), respectively, n is the number of days since W_i, and T_i is mean daily water temperature (in °C).

Specific growth rate (SGR) was also calculated using:

$$SGR = \left(\frac{\ln(W_f) - \ln(W_i)}{n}\right) \times 100$$

and the fish's Fulton's condition factor (K) was calculated as:

$$K = \frac{\text{Fish weight (g)}}{(\text{Fish fork length [cm]})^3} \times 100$$

Liver and ventricle weights were used to calculate each fish's hepatosomatic index (HSI) and relative ventricular mass (RVM), respectively, using the following equations:

$$HSI = \left(\frac{\text{Liver weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

$$RVM = \left(\frac{\text{Ventricle weight (g)}}{\text{Fish weight (g)}}\right) \times 100$$

5.3.4 Statistical analysis

The data were first assessed via Shapiro-Wilk's normality tests and $\log_{10}$-transformed if necessary to meet testing assumptions, and Levene's tests were performed to measure homoscedasticity. Then, linear mixed-effect models and ANOVAs were used to examine if the main factor of 'family', with 'tank' set as a random factor, influenced growth performance and thermal tolerance parameters. If a significant effect ($p < 0.05$) was found, a Tukey's HSD post-hoc test was performed. T-tests were used when comparisons were only made between two variables (e.g., comparisons between treatments or sexes). Linear mixed-effect models were also used to examine if weight, growth rate (i.e., TGC, SGR), HSI or RVM were related to IT_{Max} and/or CT_{Max}, using family and tank as fixed and random factors, respectively. In these models, a

single significant (Grubb's test; $p < 0.01$) outlier was removed in both the HSI and RVM datasets from the CT_{Max} experiment. All statistical procedures were carried out using R (v. 4.1.2) (R Studio Team, 2015) and a difference was considered significant if $p < 0.05$. Data are reported as mean ± standard error of the mean (SEM).

5.4 Results

5.4.1 Morphometric data and growth rate up to the 20°C assessment

Differences in weight, length and K between the families were evident ($p < 0.05$) at the start of the experiment after the fish were initially acclimated to 12°C (Table 5-2). Even though these salmon were all the same age and reared under the same conditions, there was almost a 400 g difference in weight between the largest (F6) and smallest (F16) families at this time point. However, initial morphometrics did not differ ($p > 0.05$) between fish split between the control and warm groups (Table 5-3). At the 20°C assessment, salmon from the warm group had higher ($p < 0.05$) weight, K, weight gain and SGR values, but lower ($p < 0.05$) TGC values, as compared to the controls maintained at 10°C (Table 5-3). No difference ($p > 0.05$) in length was observed between the groups. On average, regardless of rearing temperature, weight and length did increase ($p < 0.001$) in fish during this portion of the study. In contrast, K increased ($p < 0.001$) by ~4.1% within the warm group only. Family-based differences in morphometrics and growth rates within the control (Table 5-4) and warm (Table 5-5) groups were also identified ($p < 0.05$). The general patterns in size disparity between families continued with a difference of ~ 600 and ~ 540 g between F6 and F16 within the control and warm groups, respectively. From the subset of fish that were sampled (for purposes outside the scope of this paper) at the 20°C assessment point, morphometric and growth rate data were compared between sexes. Rearing

temperature influenced ($p < 0.05$) K, weight gain and SGR within both male and female salmon, with higher values observed in the warm group compared to the controls. However, no differences ($p > 0.05$) were detected for any parameter between the sexes in either the control or warm groups (Appendix D Table D-1).

Table 5-2. Initial morphometrics of family fish at 12°C before temperature was adjusted in the control and warm treatments.

Family	Control Treatment - n	Warm Treatment - n	Weight (g)	Length (cm)	K
F1	30	28	425.1 ± 18.2^{fg}	32.0 ± 0.4^{ef}	1.26 ± 0.01^{defg}
F2	25	25	484.3 ± 21.8^{g}	33.4 ± 0.5^{fg}	1.27 ± 0.02^{deh}
F3	30	31	484.7 ± 18.3^{g}	33.4 ± 0.4^{fg}	1.27 ± 0.01^{eh}
F4	26	28	426.1 ± 22.2^{fg}	31.9 ± 0.6^{ef}	1.24 ± 0.02^{bcef}
F5	29	32	459.8 ± 17.8^{fg}	33.3 ± 0.4^{fg}	1.21 ± 0.01^{ace}
F6	28	29	598.8 ± 19.6^{h}	35.3 ± 0.4^{g}	1.33 ± 0.01^{h}
F7	28	29	446.6 ± 17.9^{fg}	33.1 ± 0.4^{f}	1.20 ± 0.01^{ac}
F8	23	22	305.3 ± 14.4^{ce}	29.3 ± 0.5^{bd}	1.18 ± 0.01^{ac}
F9	23	22	314.2 ± 17.9^{ce}	29.4 ± 0.5^{bd}	1.18 ± 0.01^{ac}
F10	28	29	286.5 ± 11.5^{bcd}	27.9 ± 0.4^{abc}	1.28 ± 0.01^{fh}
F11	27	29	302.7 ± 13.0^{ce}	29.2 ± 0.4^{bd}	1.17 ± 0.01^{ab}
F12	26	25	336.6 ± 19.3^{de}	30.0 ± 0.6^{cde}	1.18 ± 0.01^{ab}
F13	24	25	459.4 ± 18.5^{fg}	33.5 ± 0.4^{fg}	1.20 ± 0.01^{acd}
F14	23	22	293.8 ± 17.4^{bcd}	27.6 ± 0.6^{ab}	1.32 ± 0.02^{gh}
F15	29	29	371.0 ± 17.0^{ef}	30.7 ± 0.4^{de}	1.25 ± 0.01^{cef}
F16	27	26	210.2 ± 9.1^{a}	25.9 ± 0.4^{a}	1.18 ± 0.01^{ab}
F17	25	26	253.3 ± 11.4^{ac}	27.4 ± 0.4^{ab}	1.19 ± 0.01^{ac}
F18	25	26	228.1 ± 9.3^{ab}	26.4 ± 0.3^{a}	1.20 ± 0.01^{ace}
F19	26	25	244.2 ± 10.1^{ac}	27.2 ± 0.4^{ab}	1.20 ± 0.04^{ac}
F20	26	27	241.4 ± 11.6^{ab}	27.1 ± 0.5^{ab}	1.16 ± 0.01^{a}

Average weight, length and K were calculated by pooling data collected from all tanks. Lower case letters denote significant differences ($p < 0.05$) between families. K, Fulton's condition factor

Table 5-3. Morphometrics and growth rates of the salmon organized by treatment at the initial assessment time point and when the warm treatment reached 20°C.

	Initial		**20°C Assessment**	
	Control (n = 528)	**Warm (n = 535)**	**Control (n = 516)**	**Warm (n = 437)**
Weight (g)	358.4 ± 7.0^{A}	367.0 ± 6.8^{A}	444.9 ± 9.5^{Ba}	474.1 ± 9.7^{Bb}
Length (cm)	30.2 ± 0.2^{A}	30.4 ± 0.2^{A}	32.3 ± 0.2^{B}	32.7 ± 0.2^{B}
K	1.22 ± 0.01	1.23 ± 0.01^{A}	1.23 ± 0.01^{a}	1.28 ± 0.01^{Bb}
Weight Gain (g)	-	-	92.4 ± 3.4^{a}	131.6 ± 3.9^{b}
TGC [$g^{1/3}$ (°C d)$^{-1}$]	-	-	1.55 ± 0.05^{b}	1.40 ± 0.03^{a}
SGR (% body weight day^{-1})	-	-	0.64 ± 0.02^{a}	0.94 ± 0.02^{b}

Lower case letters denote significant differences ($p < 0.05$) between treatments at the same assessment point. Whereas, upper case letters denote significant differences ($p < 0.05$) between assessment points within the same treatment. K, Fulton's conditions factor; TGC, thermal growth coefficient; SGR, specific growth rate. Note: the control group was held at a constant temperature of 10°C.

Table 5-4. Family morphometrics and growth rates of the salmon from the control treatment (10°C) at the 20°C assessment time point (i.e., 36 days after temperature began increasing in the warm treatment).

Family	n	Weight (g)	Length (cm)	K	Weight Gain (g)	TGC [g$^{1/3}$ (°C d)$^{-1}$]	SGR (% body weight day^{-1})
F1	28	570.4 ± 34.5^{h}	34.7 ± 0.6^{fgh}	1.32 ± 0.02^{def}	137.4 ± 14.2^{bd}	2.02 ± 0.15^{bd}	0.77 ± 0.05^{b}
F2	24	562.5 ± 44.6^{fgh}	35.0 ± 0.8^{fgi}	1.25 ± 0.03^{bce}	72.2 ± 17.7^{b}	0.97 ± 0.20^{bc}	0.36 ± 0.07^{b}
F3	30	547.2 ± 28.5^{gh}	35.0 ± 0.6^{gh}	1.24 ± 0.02^{bce}	74.5 ± 12.2^{b}	1.06 ± 0.17^{bc}	0.40 ± 0.06^{b}
F4	25	539.6 ± 49.9^{egh}	34.2 ± 0.9^{egh}	1.26 ± 0.03^{bce}	111.4 ± 21.5^{bc}	1.60 ± 0.23^{bd}	0.62 ± 0.08^{b}
F5	27	631.8 ± 39.7^{hi}	35.9 ± 0.7^{hi}	1.33 ± 0.02^{ef}	190.3 ± 17.3^{cd}	2.71 ± 0.19^{cd}	1.02 ± 0.07^{b}
F6	25	843.0 ± 46.0^{i}	38.8 ± 0.6^{i}	1.41 ± 0.02^{f}	236.1 ± 17.0^{d}	2.76 ± 0.15^{d}	0.94 ± 0.05^{b}
F7	28	457.4 ± 32.1^{dh}	33.7 ± 0.6^{egh}	1.16 ± 0.03^{ab}	29.0 ± 19.7^{a}	0.35 ± 0.29^{a}	0.12 ± 0.11^{a}
F8	23	391.5 ± 32.3^{bde}	31.3 ± 0.7^{bce}	1.23 ± 0.03^{ace}	88.2 ± 16.0^{bc}	1.59 ± 0.21^{bd}	0.68 ± 0.08^{b}
F9	23	410.5 ± 34.0^{bdg}	31.9 ± 0.8^{cg}	1.21 ± 0.02^{ace}	95.2 ± 12.1^{bc}	1.75 ± 0.13^{bd}	0.75 ± 0.05^{b}
F10	28	350.1 ± 18.6^{bd}	29.9 ± 0.5^{acd}	1.29 ± 0.03^{cf}	88.2 ± 7.8^{bc}	1.81 ± 0.13^{bd}	0.81 ± 0.06^{b}
F11	27	386.0 ± 26.0^{bde}	31.3 ± 0.6^{bce}	1.20 ± 0.02^{acd}	92.3 ± 11.5^{bc}	1.75 ± 0.18^{bd}	0.76 ± 0.07^{b}
F12	26	408.7 ± 33.5^{bdef}	31.9 ± 0.8^{cef}	1.20 ± 0.03^{ac}	82.9 ± 10.3^{bc}	1.55 ± 0.15^{bd}	0.68 ± 0.07^{b}
F13	22	572.7 ± 49.4^{gh}	36.0 ± 0.8^{hi}	1.17 ± 0.03^{ac}	104.6 ± 20.6^{bc}	1.39 ± 0.22^{bd}	0.50 ± 0.07^{b}
F14	23	374.8 ± 38.4^{bd}	29.7 ± 0.8^{acd}	1.33 ± 0.03^{def}	76.6 ± 17.2^{bc}	1.38 ± 0.25^{bd}	0.59 ± 0.10^{b}
F15	28	413.2 ± 28.7^{cdg}	31.9 ± 0.6^{deg}	1.23 ± 0.03^{ace}	64.6 ± 13.9^{b}	1.07 ± 0.07^{bd}	0.43 ± 0.07^{b}
F16	27	243.7 ± 17.3^{a}	27.5 ± 0.6^{a}	1.12 ± 0.03^{a}	38.8 ± 7.6^{ab}	0.89 ± 0.17^{b}	0.42 ± 0.08^{b}
F17	25	301.2 ± 15.1^{abc}	29.3 ± 0.5^{acd}	1.17 ± 0.02^{ac}	57.4 ± 7.3^{b}	1.33 ± 0.15^{bd}	0.63 ± 0.07^{b}
F18	25	285.2 ± 16.2^{ab}	28.4 ± 0.5^{ab}	1.21 ± 0.02^{ace}	59.6 ± 5.4^{b}	1.41 ± 0.10^{bd}	0.68 ± 0.05^{b}
F19	26	322.8 ± 16.3^{ad}	30.1 ± 0.5^{acd}	1.16 ± 0.02^{ab}	76.8 ± 5.6^{bc}	1.70 ± 0.10^{bd}	0.79 ± 0.05^{b}
F20	26	289.1 ± 17.6^{ab}	28.8 ± 0.6^{ac}	1.17 ± 0.02^{ac}	78.1 ± 5.5^{bc}	1.94 ± 0.12^{bd}	0.97 ± 0.07^{b}

Lower case letters denote significant differences ($p < 0.05$) between families. K, Fulton's condition factor; TGC, thermal growth coefficient; SGR, specific growth rate

Table 5-5. Family-based salmon morphometrics and growth rates from the warm treatment (20°C) at the 20°C assessment time point (i.e., 36 days after temperature began increasing in the warm treatment).

Family	n	Weight (g)	Length (cm)	K	Weight Gain (g)	TGC [g$^{1/3}$ (°C d)$^{-1}$]	SGR (% body weight day^{-1})
F1	24	570.6 ± 41.3^{eg}	34.4 ± 0.7^{fhi}	1.35 ± 0.01^{cd}	176.6 ± 14.9^{de}	1.69 ± 0.09^{cd}	1.05 ± 0.05^{bcd}
F2	16	556.6 ± 41.2^{deg}	34.5 ± 0.9^{fhj}	1.32 ± 0.03^{bcd}	130.7 ± 20.1^{acd}	1.22 ± 0.16^{bcd}	0.75 ± 0.09^{ad}
F3	19	624.2 ± 42.9^{eg}	35.9 ± 0.7^{hj}	1.31 ± 0.02^{bcd}	150.3 ± 17.8^{ce}	1.32 ± 0.14^{bcd}	0.78 ± 0.08^{bcd}
F4	17	546.8 ± 56.3^{cef}	33.5 ± 1.2^{cdghi}	1.37 ± 0.03^{cd}	175.4 ± 16.9^{ce}	1.77 ± 0.09^{cd}	1.16 ± 0.07^{cd}
F5	24	643.0 ± 46.2^{fg}	36.7 ± 0.8^{ij}	1.25 ± 0.03^{ac}	179.9 ± 23.7^{ce}	1.53 ± 0.18^{bcd}	0.90 ± 0.11^{bcd}
F6	16	815.8 ± 52.5^{g}	38.6 ± 0.8^{j}	1.39 ± 0.03^{d}	261.7 ± 25.3^{e}	1.99 ± 0.16^{d}	1.10 ± 0.08^{bcd}
F7	26	547.2 ± 43.7^{def}	34.9 ± 0.8^{ghj}	1.23 ± 0.03^{ab}	83.1 ± 25.1^{a}	0.71 ± 0.20^{a}	0.43 ± 0.12^{a}
F8	22	437.0 ± 30.9^{bce}	32.0 ± 0.7^{bcfgh}	1.28 ± 0.03^{ad}	129.6 ± 14.3^{bcd}	1.47 ± 0.12^{bcd}	1.00 ± 0.07^{bcd}
F9	20	408.2 ± 30.5^{bce}	31.4 ± 0.7^{afg}	1.27 ± 0.02^{ad}	121.5 ± 11.5^{acd}	1.45 ± 0.08^{bcd}	1.01 ± 0.06^{bcd}
F10	25	447.6 ± 30.0^{bcef}	31.6 ± 0.7^{bcfg}	1.37 ± 0.02^{de}	136.6 ± 11.7^{cd}	1.53 ± 0.08^{cd}	1.04 ± 0.05^{bcd}
F11	27	421.9 ± 27.2^{bce}	31.9 ± 0.6^{bcfgh}	1.26 ± 0.02^{ace}	122.1 ± 11.7^{bcd}	1.41 ± 0.08^{bcd}	0.97 ± 0.05^{bcd}
F12	24	477.5 ± 40.1^{bcef}	32.8 ± 1.0^{dfh}	1.27 ± 0.02^{ad}	135.1 ± 15.7^{cd}	1.43 ± 0.11^{bcd}	0.95 ± 0.07^{bcd}
F13	17	591.9 ± 32.1^{eg}	35.7 ± 0.6^{hj}	1.29 ± 0.02^{ad}	177.4 ± 20.5^{ce}	1.68 ± 0.18^{cd}	1.03 ± 0.11^{bcd}
F14	19	354.3 ± 28.3^{ac}	29.3 ± 0.8^{ac}	1.36 ± 0.03^{cd}	86.2 ± 10.7^{ac}	1.14 ± 0.13^{ad}	0.84 ± 0.10^{bcd}
F15	27	495.1 ± 36.3^{cef}	33.6 ± 0.71^{efhi}	1.25 ± 0.02^{ac}	111.8 ± 14.5^{acd}	1.11 ± 0.13^{ac}	0.70 ± 0.09^{ab}
F16	25	276.9 ± 21.6^{a}	28.1 ± 0.7^{a}	1.19 ± 0.03^{a}	65.7 ± 12.6^{ab}	0.95 ± 0.16^{ab}	0.74 ± 0.12^{ac}
F17	17	350.7 ± 20.7^{ac}	30.2 ± 0.7^{acd}	1.25 ± 0.03^{ad}	105.6 ± 7.3^{acd}	1.42 ± 0.08^{bcd}	1.04 ± 0.06^{bcd}
F18	23	328.1 ± 20.4^{ab}	29.1 ± 0.5^{ab}	1.29 ± 0.02^{ad}	106.3 ± 8.8^{acd}	1.49 ± 0.09^{bcd}	1.12 ± 0.06^{d}
F19	24	358.4 ± 20.6^{ac}	30.4 ± 0.6^{acde}	1.24 ± 0.01^{ac}	119.5 ± 6.3^{bcd}	1.63 ± 0.07^{cd}	1.22 ± 0.07^{d}
F20	25	387.3 ± 21.5^{bcd}	31.3 ± 0.6^{af}	1.23 ± 0.02^{ab}	120.7 ± 6.9^{bcd}	1.54 ± 0.07^{cd}	1.11 ± 0.06^{cd}

Lower case letters denote significant differences ($p < 0.05$) between families. K, Fulton's condition factor; TGC, thermal growth coefficient; SGR, specific growth rate.

5.4.2 Incremental upper thermal tolerance

Significant differences ($p < 0.05$) were observed in IT_{Max} between the families (Figure 5-2, Table 5-6). On average, IT_{Max} values ranged from 23.3°C at their lowest (F1) to 25.0°C at their highest (F19); with the IT_{Max} values for individual fish ranging from 20.7 to 26.7°C (Appendix D Figure D-2). Family-based differences ($p < 0.05$) were also detected in weight, length, K, weight loss, HSI and RVM at this terminal sampling point (Table 5-6). Irrespective of family, fish lost ~48 g on average since they were last assessed at 20°C. This was not surprising as feed intake declined sharply across all tanks in the warm group between 21 and 22°C; approximately 2-3°C before most fish reached their IT_{Max} (Appendix D Figure D-3). No differences ($p > 0.05$) were found when comparing IT_{Max}, weight, weight loss or RVM between male and female salmon (Appendix D Table D-2). However, females were longer ($p < 0.05$) than males by 3.4% on average, which was reflected in their lower values for K ($p < 0.05$). The HSI of female fish was also ~5% greater than that of male fish, but this difference was not significant ($p = 0.066$).

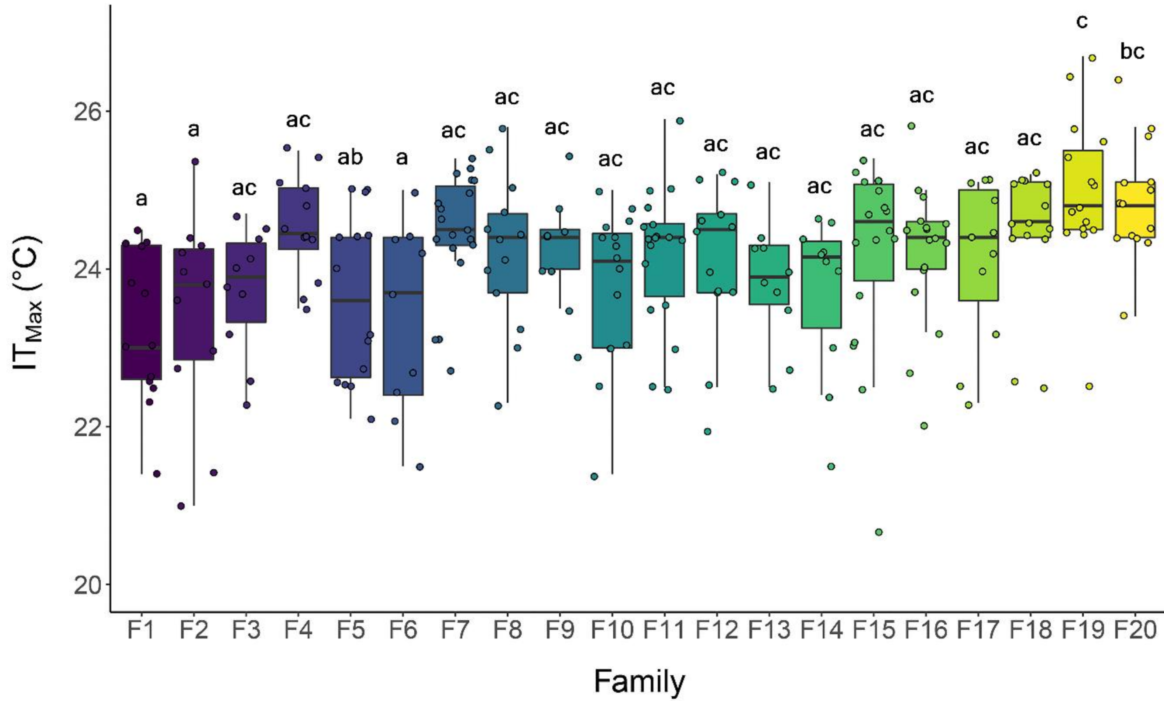

Figure 5-2. The incremental thermal maximum (IT_{Max}) of all families. Families without a letter in common are significantly different ($p < 0.05$). Boxplots illustrate the second and third quartiles, and the horizontal black lines indicate the median value for each family. The top and bottom whiskers show the upper and lower 25% of values, respectively. Circles represent individual fish.

Table 5-6. Family-based values for incremental upper thermal tolerance (IT_{Max}), morphometrics at IT_{Max}, and the weight loss of fish from the warm treatment from 20°C until they reached their IT_{Max}.

Family	n	IT_{Max} (°C)	Weight (g)	Length (cm)	K	Weight Loss (g)	HSI (%)	RVM (%)
F1	13	23.3 ± 0.3^{a}	530.3 ± 66.6^{beg}	34.7 ± 1.1^{bce}	1.19 ± 0.04^{bce}	-58.6 ± 8.0^{ac}	1.08 ± 0.04^{a}	0.084 ± 0.003^{bc}
F2	11	23.4 ± 0.4^{a}	505.3 ± 47.7^{beg}	35.0 ± 1.2^{bce}	1.15 ± 0.03^{ace}	-61.0 ± 11.0^{ac}	1.58 ± 0.23^{b}	0.080 ± 0.003^{ab}
F3	10	23.7 ± 0.3^{ac}	460.7 ± 45.2^{bceg}	34.3 ± 1.0^{ace}	1.12 ± 0.03^{ace}	-60.3 ± 7.2^{ac}	1.09 ± 0.07^{ab}	0.074 ± 0.004^{ab}
F4	12	24.5 ± 0.2^{ac}	453.7 ± 61.4^{abeg}	33.4 ± 1.5^{ace}	1.14 ± 0.03^{ace}	-65.7 ± 14.0^{a}	1.20 ± 0.05^{ab}	0.080 ± 0.003^{ab}
F5	14	23.5 ± 0.3^{ab}	601.8 ± 62.7^{fg}	37.3 ± 1.3^{e}	1.12 ± 0.02^{ace}	-77.9 ± 10.9^{ab}	1.47 ± 0.07^{bc}	0.095 ± 0.003^{c}
F6	9	23.4 ± 0.4^{a}	682.3 ± 62.2^{g}	37.8 ± 1.3^{e}	1.24 ± 0.03^{de}	-78.9 ± 6.8^{ac}	1.07 ± 0.04^{ab}	0.072 ± 0.003^{ab}
F7	19	24.5 ± 0.2^{ac}	536.0 ± 51.4^{eg}	36.1 ± 0.9^{de}	1.09 ± 0.03^{ab}	-54.2 ± 10.3^{ac}	1.02 ± 0.05^{a}	0.079 ± 0.003^{ab}
F8	13	24.2 ± 0.3^{ac}	372.8 ± 39.0^{abef}	31.9 ± 1.03^{acd}	1.10 ± 0.03^{acd}	-43.8 ± 7.5^{ac}	1.15 ± 0.06^{ab}	0.087 ± 0.003^{bc}
F9	9	24.2 ± 0.2^{ac}	380.8 ± 43.3^{abeg}	32.2 ± 1.2^{ace}	1.10 ± 0.03^{ace}	-49.0 ± 5.7^{ac}	1.20 ± 0.10^{ab}	0.080 ± 0.003^{ab}
F10	15	23.8 ± 0.3^{ac}	437.0 ± 39.3^{bceg}	32.6 ± 1.0^{ace}	1.21 ± 0.03^{ce}	-46.2 ± 6.5^{ac}	1.05 ± 0.04^{a}	0.081 ± 0.003^{ab}
F11	18	24.2 ± 0.2^{ac}	366.4 ± 30.6^{abef}	32.0 ± 0.9^{ace}	1.08 ± 0.02^{ab}	-50.1 ± 7.8^{ac}	1.07 ± 0.03^{a}	0.077 ± 0.002^{ab}
F12	13	24.1 ± 0.3^{ac}	521 ± 46.5^{bceg}	33.8 ± 1.7^{bce}	1.10 ± 0.02^{ace}	-62.1 ± 10.6^{ac}	1.26 ± 0.06^{ab}	0.081 ± 0.004^{ab}
F13	10	23.8 ± 0.3^{ac}	513.9 ± 40.7^{deg}	35.8 ± 0.8^{ce}	1.11 ± 0.03^{ace}	-64.0 ± 10.4^{ac}	1.32 ± 0.07^{ab}	0.080 ± 0.002^{ab}
F14	10	23.7 ± 0.3^{ac}	329.7 ± 38.0^{ae}	29.3 ± 1.2^{ab}	1.25 ± 0.03^{e}	-12.5 ± 8.0^{c}	1.12 ± 0.06^{ab}	0.079 ± 0.005^{ab}
F15	18	24.2 ± 0.3^{ac}	467.0 ± 44.3^{bceg}	34.3 ± 1.0^{bce}	1.10 ± 0.02^{acd}	-55.0 ± 6.9^{ac}	1.11 ± 0.07^{a}	0.068 ± 0.002^{a}
F16	17	24.2 ± 0.2^{ac}	264.8 ± 24.3^{a}	28.8 ± 0.9^{a}	1.07 ± 0.03^{a}	-20.3 ± 7.8^{c}	1.21 ± 0.08^{ab}	0.090 ± 0.005^{bc}
F17	11	24.1 ± 0.3^{ac}	296.2 ± 26.3^{ac}	30.3 ± 1.0^{ac}	1.03 ± 0.02^{a}	-32.2 ± 4.7^{bc}	1.30 ± 0.06^{ab}	0.092 ± 0.003^{bc}
F18	14	24.5 ± 0.2^{ac}	289.4 ± 20.8^{ab}	29.7 ± 0.6^{ab}	1.08 ± 0.02^{ac}	-24.7 ± 6.3^{bc}	1.23 ± 0.09^{ab}	0.091 ± 0.006^{bc}
F19	15	25.0 ± 0.3^{c}	317.4 ± 26.3^{abd}	30.5 ± 0.9^{ac}	1.08 ± 0.01^{ac}	-23.5 ± 6.5^{c}	1.11 ± 0.05^{ac}	0.081 ± 0.003^{ab}
F20	14	24.9 ± 0.2^{bc}	346.0 ± 31.1^{ae}	31.6 ± 1.0^{acd}	1.05 ± 0.02^{a}	-31.8 ± 5.2^{bc}	1.16 ± 0.07^{ab}	0.083 ± 0.003^{ac}

Lower case letters denote significant differences ($p < 0.05$) between families. K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass.

5.4.2 Acute upper thermal tolerance

CT_{Max} did not vary ($p > 0.05$) between families previously identified as the most and least thermally tolerant based on the IT_{Max} results (Figure 5-3, Table 5-7). Differences ($p < 0.05$) in weight and length were found between families held at 10°C, with there being almost a 600 g separation in average weight between F6 and F20 (Table 5-7). K, HSI and RVM were unaffected ($p > 0.05$) by family background. After pooling the data from the top and bottom thermally tolerant families, there were still no differences ($p > 0.05$) in CT_{Max}, HSI or RVM values (Table 5-8). However, on average, weight, length and K were 39.1%, 7.2% and 13.6% higher ($p < 0.05$), respectively, within the bottom families compared to the top families (Table 5-8). Further, after comparing the CT_{Max} and morphometric data between sexes, the only observable difference ($p < 0.05$) for fish acclimated at 10°C was for HSI, which was 12.4% higher on average in females as compared to males (Appendix D Table D-3).

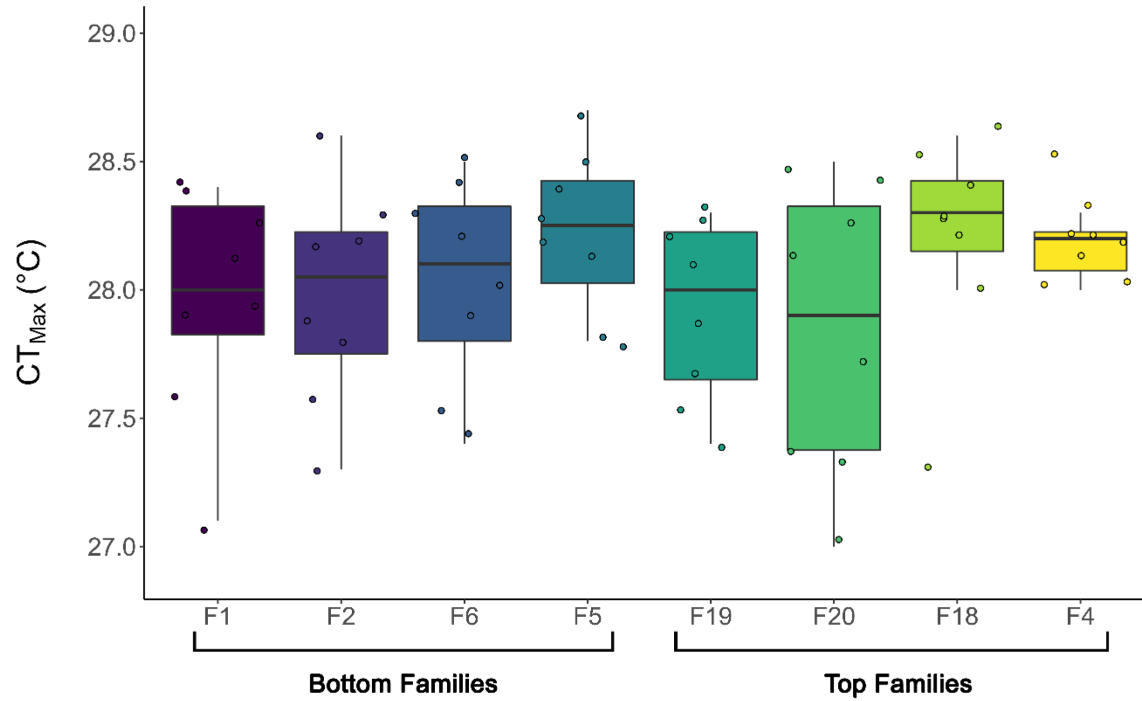

Figure 5-3. The critical thermal maximum (CT_{Max}) of the bottom (F1, F2, F6, F5) and top (F19, F20, F18, F4) thermally tolerant families (n = 8 family^{-1}) based on their IT_{Max}. No differences ($p > 0.05$) were detected between families. Boxplots illustrate the second and third quartiles, with the horizontal black lines indicating the median of each dataset. The top and bottom whiskers also show the upper and lower 25% of values, respectively. Circles represent individual fish.

Table 5-7. Comparison of the acute upper thermal tolerance (CT_{Max}) and morphometrics between the top (F19, F20, F18, F4) and bottom (F1, F2, F6, F5) thermally tolerant families (n = 8 family^{-1}) based on previous IT_{Max} results.

	Family	CTMax (°C)	Weight (g)	Length (cm)	K	HSI (%)	RVM (%)
Bottom	**F1**	28.0 ± 0.2	878.0 ± 110.8^{bc}	39.3 ± 1.5^{ab}	1.40 ± 0.05	1.31 ± 0.08	0.078 ± 0.018
	F2	28.0 ± 0.2	608.8 ± 58.8^{ab}	35.4 ± 1.9^{a}	1.47 ± 0.24	1.15 ± 0.07	0.064 ± 0.004
	F6	28.0 ± 0.1	1043.6 ± 88.0^{c}	42.6 ± 1.1^{b}	1.33 ± 0.03	1.28 ± 0.15	0.053 ± 0.002
	F5	28.2 ± 0.1	664.0 ± 86.6^{ab}	37.9 ± 1.6^{ab}	1.18 ± 0.05	1.25 ± 0.07	0.070 ± 0.003
Top	**F19**	27.9 ± 0.1	588.5 ± 46.7^{ab}	37.0 ± 0.8^{ab}	1.15 ± 0.03	1.13 ± 0.07	0.060 ± 0.004
	F20	27.8 ± 0.2	459.9 ± 36.9^{a}	33.9 ± 0.7^{a}	1.16 ± 0.03	1.30 ± 0.10	0.069 ± 0.007
	F18	28.2 ± 0.1	547.7 ± 40.9^{ab}	35.4 ± 1.0^{a}	1.22 ± 0.03	1.12 ± 0.06	0.062 ± 0.004
	F4	28.2 ± 0.1	699.8 ± 69.0^{abc}	38.5 ± 1.3^{ab}	1.20 ± 0.04	1.21 ± 0.08	0.058 ± 0.002

Lower case letters denote significant differences ($p < 0.05$) between families. K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass

Table 5-8. Comparison of the acute upper thermal tolerance (CT_{Max}) and morphometrics between the pooled bottom (F1, F2, F6, F5) and top (F19, F20, F18, F4) thermally tolerant Atlantic salmon families (n = 8 family^{-1}; n = 32 treatment^{-1}) based on previous IT_{Max} results.

	Bottom	Top
CT_{Max} (°C)	28.1 ± 0.1	28.0 ± 0.1
Weight (g)	798.6 ± 52.1[b]	574.0 ± 28.3[a]
Length (cm)	38.8 ± 0.9[b]	36.2 ± 0.6[a]
K	1.34 ± 0.06[b]	1.18 ± 0.02[a]
HSI (%)	1.25 ± 0.05	1.19 ± 0.04
RVM (%)	0.066 ± 0.005	0.062 ± 0.002

Lower case letters denote significant differences ($p < 0.05$) between groups. K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass. Note: these fish were held at 10°C prior to the CT_{Max} tests.

5.4.2 Relationships between upper thermal tolerance and phenotypic measurements

The relationships between IT_{Max} and weight, TGC, SGR, HSI and RVM are shown in Figure 5-4. The only significant ($p < 0.01$) finding was a negative linear relationship between IT_{Max} and HSI ($r^2 = 0.04$). Similarly, a negative relationship was found ($p < 0.01$) between CT_{Max} and HSI (Figure 5-5; $r^2 = 0.13$). No linear correlations were detected ($p > 0.05$) between CT_{Max} and either weight or RVM. Additionally, no relationship was observed ($p > 0.05$) when comparing average family values for IT_{Max} and CT_{Max}.

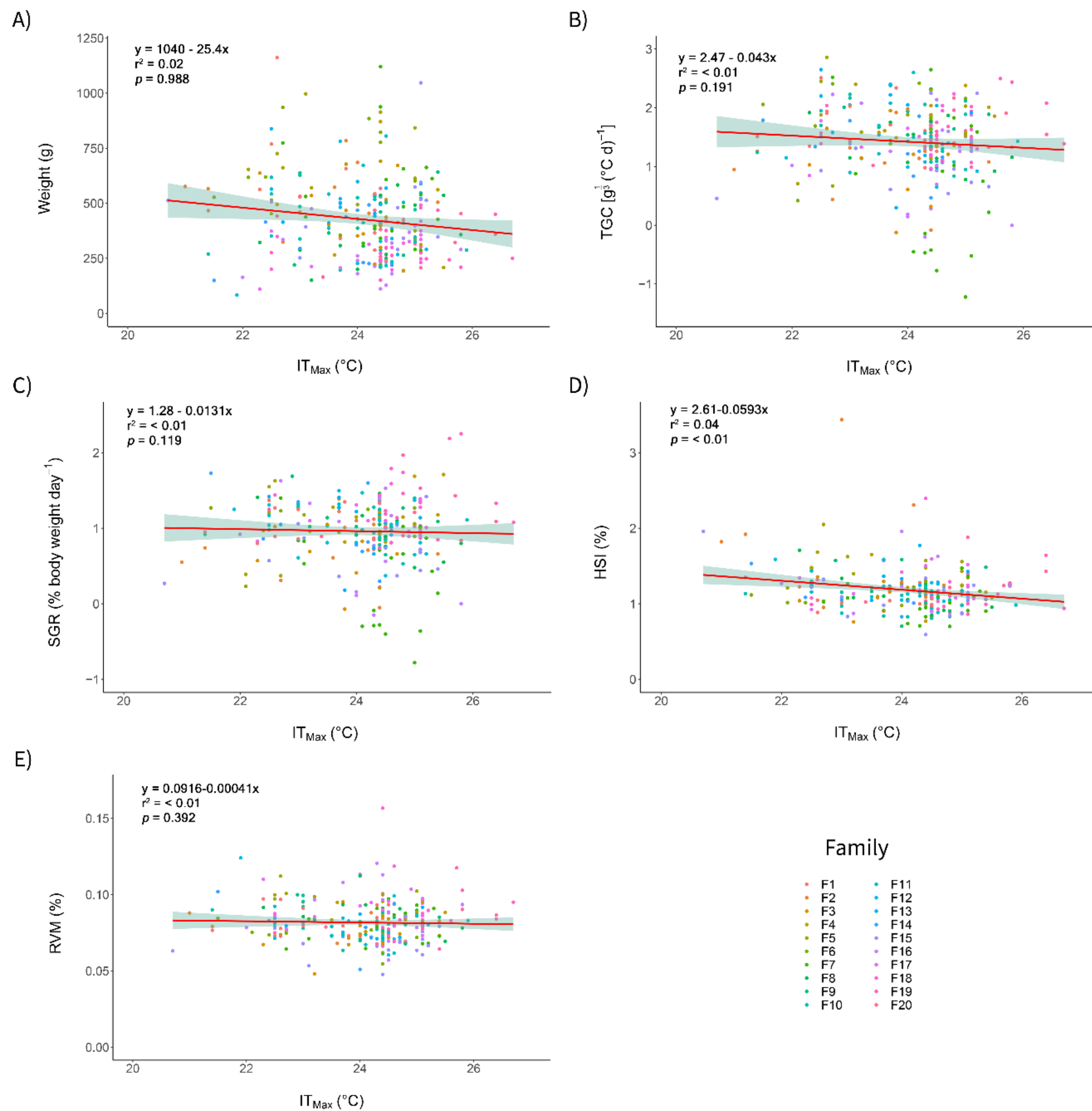

Figure 5-4. Relationships between incremental thermal maximum (IT_{Max}) and A) fish weight, B) thermal growth coefficient (TGC), C) specific growth rate (SGR), D) hepatosomatic index (HSI), and E) relative ventricular mass (RVM) (n = 265 in total between all 20 families). Each scatterplot was fitted with a linear relationship, with the shaded area surrounding it representing its standard error. The equation of the line, and the proportion of the variance attributable to the explanatory variable (r^2), are provided. The significance of the relationship was calculated using linear mixed-effect modeling, and is indicated by the *p*-value shown.

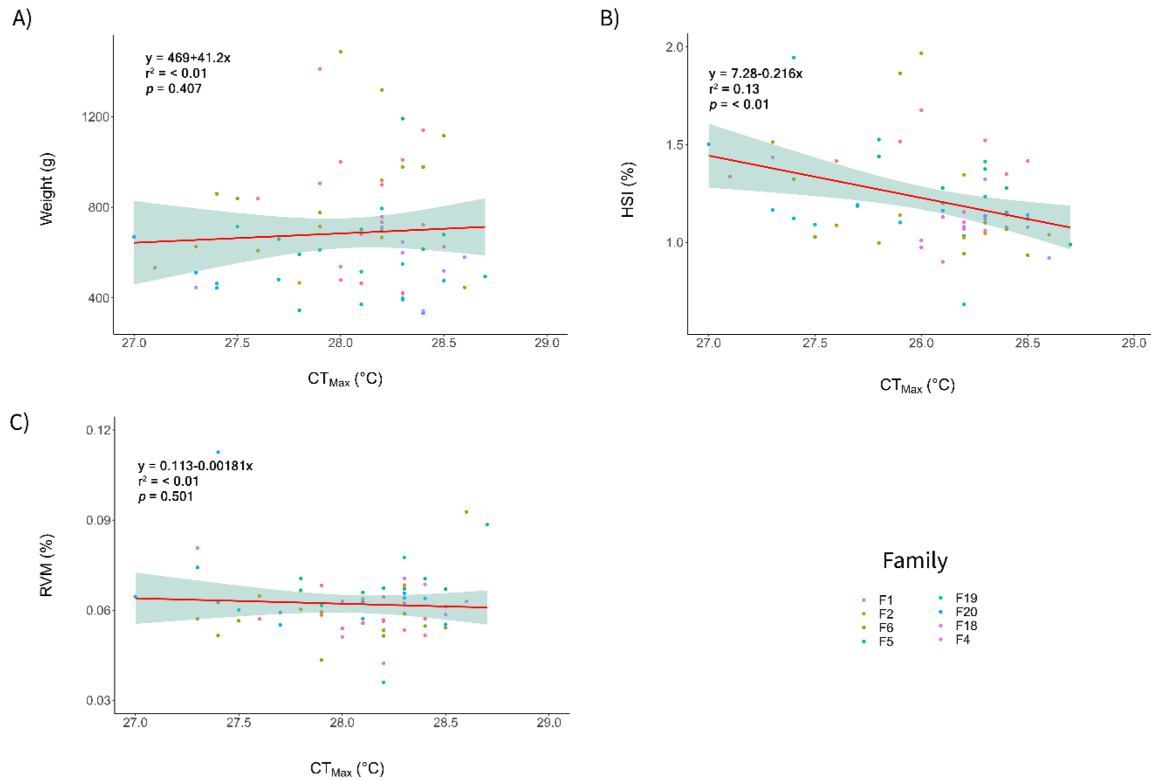

Figure 5-5. Relationships between critical thermal maximum (CT_{Max}) and A) fish weight, B) hepatosomatic index (HSI), and C) relative ventricular mass (RVM) within the bottom (F1, F2, F6, F5) and top (F19, F20, F18, F4) thermally tolerant families (n = 8 $family^{-1}$) as identified in the IT_{Max} experiment. Each scatterplot was fitted with a linear relationship, with the shaded area surrounding it representing its standard error. The equation of the line, and the proportion of the variance attributable to the explanatory variable (r^2), are provided. The significance of the relationship was calculated using linear mixed-effect modeling, and is indicated by the *p*-value shown.

5.5 Discussion

This study compared the upper thermal tolerance between families of Atlantic salmon using an incremental thermal maximum (IT_{Max}) test, and examined if there was a relationship between the CT_{Max} of the top and bottom thermally tolerant families from the IT_{Max} experiment. In addition, correlations between phenotypic traits and both measures of upper thermal tolerance were analyzed. Significant differences in IT_{Max} were detected between the families, but these results did not correspond to any variation in CT_{Max} values amongst the subset of families (i.e., most and least thermally tolerant) tested. A novel finding was that there were negative linear correlations between HSI and both IT_{Max} and CT_{Max}.

5.5.1 Production characteristics of salmon exposed to an incremental thermal challenge

Similar to Chapter 2, where I exposed post-smolt male Atlantic salmon sourced from AquaBounty Canada to the same incremental thermal challenge, fish in the warm group gained more weight by 20°C than salmon held at a constant lower temperature. Likewise, TGC was higher in the control group as compared to those exposed to an increase of +0.2°C day^{-1} (Chapter 2). This latter result indicates that salmon growth was suboptimal at elevated temperatures. The present study and Chapter 2 also show that salmon from AquaBounty Canada maintain high feed intake levels up to 21-22°C, a feature that surpasses the performance of other farmed populations of mixed sex diploid Atlantic salmon (Gamperl et al., 2020; Kullgren et al., 2013; Sambraus et al., 2017). However, in AquaBounty's female triploid population, growth was inconsistent and feed intake declined steeply at temperatures ≥ 19°C after 65 days at temperatures ≥ 16°C (Chapter 4). Therefore, further study is warranted to test the performance of AquaBounty's diploid populations to more prolonged exposure to elevated temperatures. The results of the

current study show that there was significant variation in weight gain and growth rates between families exposed to the incremental thermal challenge (Table 5-5). Therefore, it is likely that these traits could be genetically selected for, as has been shown for rainbow trout (*Oncorhynchus mykiss*) exposed to chronic heat stress (Gallardo-Hidalgo et al., 2021; Yoshida and Yáñez, 2021).

5.5.2 Incremental vs. acute upper thermal tolerance

A difference of 1.7°C was observed in the average IT_{Max} between the least (F1) and most (F19) thermally tolerant families (Figure 5-2, Table 5-6). Families from AquaBounty Canada were tested previously using the same IT_{Max} protocol as in the current study (Chapter 2), yet no differences were reported in upper thermal tolerance. However, Chapter 2 used a limited number of fish per family, and this likely explains why significant differences were not detected. Interestingly, the two families in the current study with the highest average IT_{Max} (i.e., F19, F20) were half-siblings. Thus, based on this latter finding and the reported difference in family IT_{Max} values, these data suggest that IT_{Max} in salmon is a heritable trait, a hypothesis that is actively being examined (Ignatz et al., in prep.).

Acute upper thermal tolerance has already been shown to be heritable or have a genetic basis in several salmonid species (Danzmann et al., 1999; Debes et al., 2021; Ihssen, 1986; Jackson et al., 1998; Penney et al., 2021; Perry et al., 2005a, 2005b, 2001; Somorjai et al., 2003). Based on Chapter 2, where the average IT_{Max} across the study was 25.2°C, AquaBounty could likely use genetics from other families that may have higher thermal tolerances as compared with families from the current study. For example, the average IT_{Max} of F11 from Chapter 2 was 25.8°C ($n = 5$). It also appears that variation is present across this population of salmon as female triploids from AquaBounty reached 50% mortality earlier (at ~24.2°C) after a similar IT_{Max}

challenge (Chapter 4). However, whether this difference was due to ploidy, genetics or variation in experimental design is uncertain. Regardless, salmon from AquaBounty appear to have a higher tolerance to a long-term (incremental) and industrially-relevant temperature challenge as compared to other farmed populations that have been tested in Atlantic Canada. For example, Gamperl et al. (2020) reported 30% mortality by 23°C in salmon post-smolts, and Bartlett et al. (2022) stated that the average IT_{Max} of naïve (not previously exposed to a CT_{Max} challenge) juvenile Atlantic salmon was ~22.8°C. In contrast, only 15.5% of fish in the current study died before temperature reached 23°C (Appendix D Figure D-2). This is an interesting finding considering that AquaBounty has never selected for thermal tolerance traits in their broodstock program, and has exclusively operated temperature-controlled land-based facilities for over 25 years.

Conversely, CT_{Max} did not vary between the top 4 and bottom 4 thermally tolerant families selected based on their measured IT_{Max} values (Figure 5-3). Both groupings of families had virtually the same acute upper thermal tolerance (i.e., ~ CT_{Max} 28°C; Table 5-7 & 5-8), and this value is very similar to the CT_{Max} of male AquaBounty Atlantic salmon also assessed when heated at 2°C h^{-1} in the same tank system (mean 28.5°C; Chapter 2). Nonetheless, this value is: much higher than a previous study on Atlantic salmon where CT_{Max} (acclimation temperature 4°C; +0.3°C min^{-1} to 22°C, then +0.1°C) was measured in juvenile Atlantic salmon from New Brunswick across 41 families, and average values ranged from 23.9 to 26.1°C (Anttila et al., 2013); ~ 2°C higher than reported in Leeuwis et al. (2019) for 10-12°C acclimated salmon from another commercial hatchery in Atlantic Canada (26.2°C; rate of heating also 2°C h^{-1}); and ~ 1°C higher than measured by Bartlett et al. (2021) who acclimated their salmon to 8.5°C and used a considerably faster rate of temperature increase (24°C h^{-1}) to determine their CT_{Max}.

Overall, it appears that CT_{Max} and IT_{Max} consistently differ by 3-5°C in Atlantic salmon (present study; Chapter 2; Bartlett et al., 2022). However, further study is required to determine whether this trend is consistent in larger, sexually maturing, Atlantic salmon.

While significant differences in CT_{Max} were detected between families in the Anttila et al. (2013) experiment, it is difficult to discern whether the discrepancy with the current study is due to methodology (e.g., faster heating regime, earlier life stage, lower acclimation temperature) or the limited number of families for which CT_{Max} was determined (n = 8); although Zanuzzo et al. (2019) was able to detect differences in CT_{Max} between 14 families of Atlantic cod (*Gadus morhua*) warmed at +2°C h^{-1}. Regardless, given that there was no link between the quicker and more convenient approach of testing thermal tolerance (CT_{Max}) and IT_{Max} (present study; Bartlett et al., 2021; Zanuzzo et al., 2019), it appears that the aquaculture industry will have to rely on measures of incremental thermal tolerance to provide more sensitive and realistic predictions of how well their salmon will survive in warming oceans, and possibly to select broodstock more resistant to the effects of warming sea-cage temperatures. Nonetheless, determining IT_{Max} across an entire farmed population may prove to be too time consuming and cost prohibitive. Thus, it may be more practical to identify SNP markers associated with increased IT_{Max} in salmon, to validate them, and to use these markers to select for this trait. This, however, would require that additional studies be performed.

5.5.3 Phenotypic traits and their correlation with measures of upper thermal tolerance

Anttila et al. (2013) reported a positive correlation ($r^2 = 0.21$) between CT_{Max} and RVM in cold-acclimated juvenile Atlantic salmon exposed to their more rapid rate of temperature increase. However, this in contrast to the present study and Bartlett et al. (2022) who failed to

report a relationship between these two parameters. Further, while I also report no relationship between RVM and IT_{Max}, such a relationship is reported by Bartlett et al. (2022); the r^2 for this relationship was 0.16. Clearly, there is no consistency between studies with regards to RVM being key to determining salmon upper thermal tolerance when measured acutely or chronically (incrementally). This may seem surprising given that it has been suggested that cardiac function is a primary determinant of fish high temperature tolerance (Eliason et al., 2011; Farrell, 2009; Gamperl et al., 2020; Wang and Overgaard, 2007). However, the strength of the reported above relationships are quite weak, and Anttila et al. (2014) suggest that physiological plasticity with respect to cardiac responses to warming temperatures in Atlantic salmon may obviate the need for adaptation at the population/genetic scale.

Interestingly, the present study is the first to my knowledge to examine the relationship between HSI and any measure of fish upper thermal tolerance. In this study, HSI was the only parameter measured that was significantly related to CT_{Max} and IT_{Max}, and it was negatively correlated with coefficient (r^2) values of 0.13 and 0.04, respectively (Figures 4D and 5B).

In contrast to my previous hypothesis (Chapter 2), I did not observe any differences in upper thermal tolerance (IT_{Max} or CT_{Max}) between female and male Atlantic salmon. Differences in survival during spawning migration have been reported between sexes of Pacific salmonids, with elevated river temperatures thought to play a role in higher female mortality (Crossin et al., 2008; Hinch et al., 2021). Unlike in coho salmon (*Oncorhynchus kisutch*), where higher RVM values were reported in males compared to females (Little et al., 2020), no such differences were detected in the present study. However, the salmon in my study were just starting to sexually mature (based on observational notes on their gonads) toward the end of the IT_{Max} experiment. This could also be why no sex differences in upper thermal tolerance were observed in the

present or past studies (Bartlett et al., 2022), as these fish were either sexually immature or just starting maturation. In contrast to RVM, HSI values for female salmon were greater as compared to males during both the IT_{Max} ($p = 0.066$) and CT_{Max} ($p < 0.05$) tests. In rainbow trout, HSI increases with sexual maturation in females, but not in males (Tveranger, 1985). This is likely due to increases in circulating 17β-estradiol stimulating vitellogenesis, as experimental injections of this hormone have increased the HSI of female salmonids previously (Benfey et al., 1989; Krisfalusi and Cloud, 1996). With this in mind, differences in these parameters might explain the lower survival of spawning female Pacific salmonids. Nonetheless, the fact that measures of thermal tolerance were not different between male and female fish, despite the greater HSI in the latter, suggests that it was not simply the size of the liver that was responsible for the significant relationships between this parameter and thermal tolerance when the relationships between all fish were examined (Figures 4D and 5B). In fact, the lower HSI of salmon that survived to higher temperatures could be reflective of their energy stores being depleted over time due to reduced feeding in the IT_{Max} trial. However, this would not explain the relationship observed between HSI and CT_{Max}, as feed was only restricted for 1 day before testing. Further, higher fat content in the liver of moribund post-smolt Atlantic salmon was reported following an acute mortality event in sea-cages during the winter at low ambient temperatures (i.e., ~4°C) (Dessen et al., 2020). Similarly, HSI was approximately two-fold higher in moribund post-smolt Atlantic salmon when reared at colder temperatures (between 8 and 1°C), and plasma markers of liver damage (e.g., aspartate aminotransferase) were also greatly elevated in these fish (Vadboncoeur et al., submitted). Therefore, liver composition and function could potentially be linked to the survival of salmonids at both their upper and lower thermal limits. Further exploration into this hypothesis, however, is required.

5.6 Conclusion

In summary, the lack of a difference in the CT_{Max} of salmon families with high vs. low IT_{Max} values, and of a significant relationship between these two measures of thermal tolerance (present study, Bartlett et al., 2022; Zanuzzo et al., 2019), strongly suggest that IT_{Max} provides a more accurate and meaningful metric of the Atlantic salmons' upper thermal tolerance, and that IT_{Max} experiments will be a more useful tool/metric of upper thermal tolerance for the aquaculture industry. This interpretation would be consistent with the fact that mortalities during IT_{Max} tests on Atlantic salmon populations begin around 22°C, whereas those in CT_{Max} trials are generally not seen below 26°C (present study; Chapter 2; Bartlett et al., 2022; Gamperl et al., 2020); and the former temperature is much closer to the maximum cage-site temperatures that salmon are likely to encounter, even with climate change (Gamperl et al., 2021; Stehfest et al., 2017; Wade et al., 2019).

More research is required, however, to determine what physiological characteristics determine a salmon's IT_{Max}. For example, additional research is needed into the relationships between upper thermal tolerance and heart and liver size/function, and into whether sexual maturity influences measures of Atlantic salmon upper thermal tolerance. Finally, genomic analyses [e.g., genome-wide association studies (GWAS); RNA-sequencing] could help elucidate why certain fish/families survive longer at elevated temperatures. An improved understanding of factors determining the upper thermal tolerance of salmon could enable the selection of broodstock for producing farmed Atlantic salmon that are better able to withstand future ocean temperatures that will pose a major threat to the sustainability of the aquaculture industry.

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CHAPTER 6: Characterization and transcript expression analyses of four Atlantic salmon (*Salmo salar*) *serpinh1* paralogues provide evidence of evolutionary divergence

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6.1 Abstract

Atlantic salmon (*Salmo salar*) are not only the world's most economically important farmed fish in terms of total value, but as a salmonid they are invaluable for studies of the evolutionary fate of genes following multiple whole-genome duplication (WGD) events. In this study, four paralogues of the molecular chaperone *serpinh1* were characterized in Atlantic salmon, as while this gene is considered to be a sensitive biomarker of heat stress in salmonids, mammalian studies have also identified it as being essential for collagen structural assembly and integrity. The four salmon paralogues were cloned and sequenced to conduct *in silico* analyses at the nucleotide and deduced amino acid levels. In addition, qPCR was used to measure: paralogue- and sex-specific constitutive *serpinh1* expression across 17 adult tissues, and their expression in the liver and head kidney of male Atlantic salmon as affected by stress phenotype (high vs. low responder), increased temperature and injection with a multi-valent vaccine. Compared to the other three paralogues, *serpinh1a-2* had a unique constitutive expression profile across the 17 tissues assessed. Although stress phenotype had minimal impact on the transcript expression of the four paralogues, injection with a commercial vaccine containing several formalin inactivated bacterins typically increased the expression of all paralogues (by 1.1 to 4.5-fold) across both tissues. At 20°C, the expression levels of *serpinh1a-1* and *serpinh1a-2* were generally lower (by -1.1- to -1.6-fold), and *serpinh1b-1* and *serpinh1b-2* were 10.2- to 19.0-fold greater, in comparison to salmon held at 12°C. With recent studies suggesting a putative link between *serpinh1* and upper thermal tolerance in salmonids, the current research is a valuable first step in elucidating the potential mechanisms involved. This research: supports the use of *serpinh1b-1* and *serpinh1b-2* as a biomarkers of heat stress in salmon; and provides evidence of

neo- and/or subfunctionalization between the paralogues and important insight into how multiple genome duplication events can potentially lead to evolutionary divergence.

6.2 Introduction

The serine protease inhibitor (serpin) superfamily consists of a large array of genes/proteins that typically use conformational change to inhibit target enzymes (Law et al., 2006). In eukaryotes, they are divided phylogenetically into 16 clades (A-P) (Irving et al., 2000), of which *serpinh1* [alias *heat shock protein 47* (*hsp47*)] is the only member of clade H (Heit et al., 2013) and of group V6 in the indel-based classification system (Kumar, 2015). SERPINH1 is one of the few members of the serpin superfamily that possesses a non-inhibitory reactive center loop, and is the only serpin found in the endoplasmic reticulum (Ito and Nagata, 2017; Law et al., 2006). Another unique feature of SERPINH1, is that unlike other molecular chaperones, it can recognize the folded conformation of its target (Koide et al., 2006, 2000; Ono et al., 2012; Tasab et al., 2000; Widmer et al., 2012). In mammals, SERPINH1 serves as a collagen-specific molecular chaperone with two main functions: i) it promotes collagen synthesis by inhibiting procollagen unfolding in the endoplasmic reticulum; and ii) it inhibits procollagen aggregation following triple-helix formation (Ito and Nagata, 2017; Nagata, 1996; Nagata et al., 1988). In mice (*Mus musculus*), *serpinh1* knockout is lethal as it is required for the formation of rigid triple-helical structures of type I collagen (Nagai et al., 2000). Missense mutations and variants of *serpinh1* also cause osteogenesis imperfecta in mammals (Christiansen et al., 2010; Drögemüller et al., 2009; Marshall et al., 2016).

Given its relevance to human disease, and that mammals are endotherms, there has been less focus on *serpinh1*'s role during heat shock in these higher vertebrates. In contrast, *serpinh1* has been identified as one of the best biomarkers of heat stress in salmonids (Akbarzadeh et al., 2018; Beemelmanns et al., 2021c; Houde et al., 2019; Pandey et al., 2021; Verleih et al., 2015). This gene, however, has not yet been fully characterized in any member of the Salmonidae

family, and only two out of the four paralogues of *serpinh1* have been previously identified in a salmonid species (Akbarzadeh et al., 2018; Rebl et al., 2013). As a pseudotetraploid (Lien et al., 2016) and a socioeconomically important species, Atlantic salmon (*Salmo salar*) are ideal for the study of this gene. Further, with anthropogenic climate change increasing global aquatic ecosystem temperatures (IPCC, 2022), it will be important to better understand how species like Atlantic salmon will respond to thermal stress. Although it is assumed that heat shock proteins like *serpinh1* will be acutely upregulated following exposure to environmental stressors (e.g., temperature, hypoxia, toxins) to help refold or remove misfolded proteins that accumulate during disrupted proteostasis (Roberts et al., 2010; Storey and Storey, 2022), prior to the current study it was unknown if this response was universal across the four Atlantic salmon gene duplicates.

In addition, little is known about whether the regulation of these paralogues varies between salmon with different stress-related phenotypic characteristics, or how they respond to bacterial immune stimulation. Atlantic salmon and other teleosts can be characterized as either low responders (LR) or high responders (HR) based on exhibiting a proactive or reactive stress coping phenotype, respectively, and this is associated with having different post-stress cortisol levels (Chapter 2; Hori et al., 2012; Koolhaas et al., 1999). Since *serpinh1* is a stress-activated chaperone, it was hypothesized that its expression may also differentiate LR and HR Atlantic salmon. A small number of studies have also identified that *serpinh1* plays a role in Atlantic salmon immune physiology (Chapter 3; Eslamloo et al., 2022; Umasuthan et al., 2020). In humans, SERPINH1 has important roles in regulating the immune system [e.g., correlating negatively with $CD8^{+}$ T cells and positively with M2 macrophages (i.e., anti-inflammatory, pro-healing)] (Zhong et al., 2022). Studying how *serpinh1* expression is affected by stress phenotype

and immune stimulation will provide novel insights into whether this gene is involved in additional biologically important pathways.

Therefore, the goals of the present study were to: i) identify and sequence all *serpinh1* paralogues in Atlantic salmon; ii) compare the nucleotide and putative protein sequences/structures of *serpinh1* within and between species; iii) quantify the constitutive transcript expression of each paralogue across a panel of 17 tissues in adult salmon; and iv) measure paralogue-specific expression in the liver and head kidney in salmon of different stress phenotypes, and when exposed to thermal stress (12 vs. 20°C) and formalin inactivated pathogenic bacterins. Collectively, this information is valuable as it will not only to help characterize a key gene in an industrially relevant species, but also to study how multiple genome duplication events can impact host regulation and lead to evolutionary divergence. This is particularly important as Atlantic salmon populations are threatened by rising sea surface temperatures (IPCC, 2022), and more frequent and severe marine heatwaves (Oliver et al., 2018), that can increase the risk of disease and mortality in these fish (Burke et al., 2020; Dalvin et al., 2020).

6.3 Materials and methods

6.3.1 *serpinh1* cDNA cloning

The full-length cDNA sequences for four Atlantic salmon *serpinh1* paralogues were obtained using a commercial kit for 5' and 3' rapid amplification of cDNA ends (RACE) [SMARTer® RACE 5'/3' Kit (Takara Bio, Mountain View, CA, USA)] following the manufacturer's instructions. Briefly, gene-specific primers (GSPs) were designed (Table 6-1) using Primer3web v.0.4.0 (bioinfo.ut.ee/primer3-0.4.0/) and the predicted cDNA sequences from

NCBI GenBank. A pool of total RNA from eight brain, eight head kidney and eight spleen samples collected from four female and four male salmon from the multi-tissue expression qPCR analysis (see below for details) was used as template for the 5' and 3' RACE cDNA synthesis reactions of *serpinh1a-1* and *serpinh1a-2*. A second set of 5' primers for both paralogues was required to ultimately capture the full sequences (i.e., GSP-2 in Table 6-1). In contrast, a pool of total RNA of 5 liver samples from Chapter 3 was used as template for the 5' and 3' RACE cDNA synthesis reactions of *serpinh1b-1* and *serpinh1b-2*. All cDNAs were then diluted by adding 10 μL (*serpinh1a-1*, *serpinh1a-2*) or 40 μL (*serpinh1b-1*, *serpinh1b-2*) of Tricine-EDTA buffer prior to their use in the 5' and 3' RACE touchdown PCR reactions. The touchdown PCR cycling conditions were: 94°C for 1 min; 5 cycles of (94°C for 30 s, 72°C for 3.5 min); 5 cycles of (94°C for 30 s, 70°C for 30 s, 72°C for 3.5 min); 25 cycles of (94°C for 30 s, 68°C for 30 s, 72°C for 3.5 min) and 1 final extension cycle of 72°C for 10 min. Five microliters of the primary 5' and 3' RACE PCR products were then diluted by adding 245 μL of Tricine-EDTA buffer to generate templates for nested PCR. The nested PCR cycling conditions were: 94°C for 1 min; 20 (or 35 for *serpinh1a-1* and *serpinh1a-2* GSP-2 5' RACE, and *serpinh1a-2* GSP-1 3' RACE) cycles of (94°C for 30 s, 68°C for 30 s, 72°C for 3.5 min) and 1 final extension cycle of 72°C for 10 min.

The nested 5' and 3' RACE PCR products were electrophoretically separated on a 1.0% agarose gel, excised, and purified using the NucleoSpin® Gel and PCR Clean-up Kit (Takara Bio). They were then cloned into the linearized pRACE vector using the In-Fusion HD Cloning Kit (Takara Bio), and transformations were performed using One Shot Top10 Chemically Competent *E. coli* cells (Invitrogen/Thermo Fisher Scientific, Burlington, ON, Canada) and standard molecular biology techniques. Plasmid DNA was extracted from individual clones

using the QIAprep Spin Miniprep Kit (QIAGEN, Mississauga, ON, Canada). These protocols were all performed following the manufacturer's instructions. Insert sizes were verified by restriction enzyme analysis using *Eco*RI and *Hind*III (Invitrogen/Thermo Fisher Scientific), followed by 1.0% agarose gel electrophoresis and visual comparison to a DNA size marker (1 kb Plus DNA Ladder; Invitrogen/Thermo Fisher Scientific).

Eight clones from each of the 5' and 3' RACE PCR products were sequenced at Génome Québec CES (Montréal, QC, Canada). Sanger sequencing was performed in both directions (with M13 primers) using a 3730xl DNA Analyzer (Applied Biosystems/Thermo Fisher Scientific). Vector NTI and AlignX (Vector NTI Advance 11.5; Invitrogen/Thermo Fisher Scientific) were then used to analyze and assemble the complete cDNA sequence for each *serpinh1*paralogue.

Table 6-1. RACE gene-specific primers (GSPs) used for characterizing the paralogues of Atlantic salmon *serpinh1*.

Paralogue	Nucleotide sequence (5'-3')	Application
serpinh1a-1 GSP-1	F: ACCTGGAAGATCAGCAACCGCATCT	3'RACE PCR
	R: TTTTATGGGCCAGGGGTATGCTCAG	5'RACE PCR
serpinh1a-1 Nested GSP-1	F: TGCACCGCACAGGTCTCTATGGTTT	3'RACE PCR
	R: AGATGCGGTTGCTGATCTTCCAGGT	5'RACE PCR
serpinh1a-1 GSP-2	R: CTGCCGAGGAAGAGGATGGAGTTGG	5'RACE PCR
serpinh1a-1 Nested GSP-2	R: ACACAGCCACAGCCGTCTCCTTCAG	5'RACE PCR
serpinh1a-2 GSP-1	F: ACAGACAAGGTCCTGAGCAACCACG	3'RACE PCR
	R: ATCCTTGGTTACCTCGGGCAGCTTG	5'RACE PCR
serpinh1a-2 Nested GSP-1	F: GCTCCACCACCTCCCAGATCAAGAC	3'RACE PCR
	R: GCCCAATCGTTGATGGACTTCAGAG	5'RACE PCR
serpinh1a-2 GSP-2	R: TCCTAAGTGGTGCAGTGGTCGAAGGC	5'RACE PCR
serpinh1a-2 Nested GSP-2	R: GTGTTGTCCGGGTTAGGTTTGGCCG	5'RACE PCR
serpinh1b-1 GSP	F: CCTGGTGACCCGTTCATTCACAGTC	3'RACE PCR
	R: CAACTTGGGGTTCCTCAGCTTCTCG	5'RACE PCR
serpinh1b-1 Nested GSP	F: CCCTATCACCTGGAACCCCTTGACA	3'RACE PCR
	R: GATGTTGGACAGATCGGCCTTGGTT	5'RACE PCR
serpinh1b-2 GSP	F: ACATGGAAGATCAGCAACCGCCTCT	3'RACE PCR
	R: CAGAGGCGTGGAACACATTGGAGAG	5'RACE PCR
serpinh1b-2 Nested GSP	F: CTCGGTCACCTTTGCCGATGACTTT	3'RACE PCR
	R: GTCAGCAGTTTCTCCAGCCTGTCCA	5'RACE PCR

6.3.2 *In silico* analyses

The Ensembl Genome Browser (http://www.ensembl.org/) was used to determine the gene structure of the *serpinh1* paralogues (i.e., exonic and intronic regions) and genome location, through alignment to the Atlantic salmon genome (Ensembl Assembly: Ssal_v3.1, Accession number: GCA_905237065). Neighbouring genes of each salmon paralogue were also identified using Ensembl, and the gene structure of each paralogue was visualized using WormWeb (http://wormweb.org/exonintron). The amino acid (AA) sequence of each SERPINH1 paralogue was predicted using Vector NTI to identify and annotate the open reading frame (ORF). Putative orthologous AA sequences for SERPINH1 in other vertebrates were collected from the NCBI

GenBank non-redundant (nr) protein database (Appendix E Table E-1). Sea lamprey (*Petromyzon marinus*) was included as a representative outgroup as the only agnathan in the analysis. An invertebrate outgroup was not possible as *serpinh1* is absent in all known invertebrate genomes (Kumar et al., 2017). Other vertebrate species were selected to include representatives from ray-finned fishes (including several teleosts), cartilaginous fishes, lobe-finned fishes and tetrapods. The paralogous Atlantic salmon SERPINH1 sequences, and the selected putative orthologous SERPINH1 sequences from other species, were included in a multiple sequence alignment (MSA) using MUSCLE, and this MSA was used to construct a phylogenetic tree in MEGA 11 (v.11.0.13) (Tamura et al., 2021) using the Neighbour-joining method (bootstrapped 10,000 times) and the default parameters.

6.3.3 Multi-tissue sampling

A full description of the sampling procedure can be found in Crossman et al. (2023), as the same tissue set was used. In summary, 17 different tissues were collected from four male (1135.8 ± 155.6 g; mean ± SE) and four female (1128.3 ± 182.5 g) adult Atlantic salmon to examine the constitutive transcript expression profiles of the *serpinh1* paralogues. Blood samples were collected from the caudal vein in 300 μL aliquots without heparin within 2 min of death. Eye, brain, gill, heart, head kidney, posterior kidney, spleen, liver, gonad (i.e., testes or ovaries), stomach, pyloric caecum, midgut, hindgut, skin, muscle and fin were then quickly dissected out, immediately flash-frozen in liquid nitrogen, and then stored at -80°C until RNA extractions could be performed.

6.3.4 Multi-tissue RNA preparation

Total RNA was extracted from tissues as described in Crossman et al. (2023). Briefly, tissue samples were homogenized (TissueLyser II, QIAGEN) in TRIzol®, then frozen on dry ice and stored at -80°C. The homogenates were later thawed on wet ice and passed through QIAshredder (QIAGEN) spin columns following the manufacturer's instructions. Additional TRIzol® was added and total RNA extractions were then completed following the manufacturer's instructions. For eight of the tissues (brain, midgut, hindgut, pyloric caecum, eye, gonad, liver and blood), RNA subsamples were re-extracted using the phenol-chloroform phase separation method (Xu et al., 2013) due to either low 260/230 absorbance ratios or high levels of genomic DNA (gDNA) contamination. All RNA samples were then DNaseI-treated (6.8 Kunitz units added to 30 µg total RNA; RNase-Free DNase Set, QIAGEN) and column-purified using the RNeasy MinElute Kit (QIAGEN) following the manufacturer's instructions. Using NanoDrop UV spectrophotometry (Thermo Fisher) all column-purified RNA samples were determined to be of high purity (i.e., A260/280 ratios > 2.0 and A260/230 ratios > 1.9). Further, RNA quality was visually assessed using 1.0% agarose gel electrophoresis, which showed tight 28S and 18S ribosomal RNA bands (with those for 28S being approximately twice as intense as for 18S).

6.3.5 Stress phenotype tissue sampling

These samples originated from Chapter 2 and were collected as described in Chapter 3. Briefly, male Atlantic salmon were initially subjected to a series of four monthly net stresses, and after each stressor post-stress plasma cortisol was measured and used to characterize individual fish. Fish were identified as LR or HR based on whether their total Z-score fell into the lower or upper quartile ranges of the population, respectively (Chapter 2; Hori et al., 2012;

Weil et al., 2001). Salmon were then exposed to either an incremental temperature increase from 12°C to 20°C (+0.2°C day^{-1}) or held at 12°C. Once the 'warm' treatment reached 20°C, head kidney and liver tissues from LR and HR salmon at both temperatures were sampled and used to measure the constitutive expression of the target transcripts (i.e., at time zero). Then, fish of each stress phenotype at each temperature were intraperitoneally (IP) injected with either an equal volume of phosphate-buffered saline (PBS; Thermo Fisher) or a commercial multivalent vaccine (Forte Micro; Elanco Limited, Charlottetown, PE, Canada). Forte Micro contains formalin inactivated cultures of *Aeromonas salmonicida*, *Vibrio anguillarum* serotypes I and II, *V. ordalii*, and *V. salmonicida* serotypes I and II. Once fish were injected, they were moved into holding tanks supplied with 12°C or 20°C seawater. The head kidney and liver of fish at 20°C were sampled at 12 h post-injection (hpi) and fish at 12°C were sampled at 24 hpi in an attempt to capture peak innate antibacterial immune responses in salmon based on past results from our research group (Zanuzzo et al., 2020). All tissues were initially flash-frozen in liquid nitrogen and then stored at -80°C until RNA extractions could be performed.

6.3.6 Stress phenotype tissue RNA preparation

One LR fish sampled at time zero and one LR salmon injected with PBS, both reared at 20°C, were excluded from the study as they were sexed as females. Head kidney and liver samples (n = 8-9 fish per phenotype/sampling/tissue) were homogenized in TRIzol® before they were centrifuged through QIAshredder columns and extracted following the manufacturer's instructions. Liver samples underwent a second extraction using the phenol-chloroform phase separation method (Xu et al., 2013). For both tissues, a subset of RNA per sample was DNase I-treated (6.8 Kunitz units added to 25 µg total RNA; QIAGEN RNase-Free DNase Set) and

column-purified (QIAGEN RNeasy Mini Kit) following the manufacturer's protocols. A single liver sample (HR salmon at 20°C injected with Forte Micro) was excluded from the study as its RNA repeatedly failed to meet quality standards.

6.3.7 cDNA synthesis

First-strand cDNA templates for qPCR were synthesized in 20 µL reactions from 1 µg of DNaseI-treated, column-purified, total RNA (from either the multi-tissue or stress phenotype studies) using random primers (250 ng; Invitrogen/Thermo Fisher Scientific), dNTPs (0.5 mM final concentration; Invitrogen), M-MLV reverse transcriptase (200 U; Invitrogen) with the manufacturer's first strand buffer and DTT (10 mM final concentration) at 37°C for 50 min.

6.3.8 Multi-tissue qPCR

PCR amplifications were performed in 13 µL reactions using 1X Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific), 50 nM of both the forward and reverse primers, and the indicated cDNA quantity using the QuantStudio 6 Flex Real Time PCR system (Applied Biosystems). The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of (95°C for 15 s and 60°C for 1 min). Fluorescence was detected at the end of each 60°C step and was followed by dissociation curve analysis.

Paralogue-specific *serpinh1* qPCR primers were designed using Primer3web v.0.4.0 with the sequences obtained from cDNA cloning (Table 6-2). The most stable normalizers were previously determined as *eif3d* and *rpl32* in the multi-tissue study (Crossman et al., 2023), with these primers coming from previous research (Caballero-Solares et al., 2017; Xue et al., 2015).

Amplification efficiencies for all primers pairs were calculated (Pfaffl, 2001) by generating separate standard curves using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of input total RNA from various tissues (i.e., eye, fin and gill for paralogue-specific *serpinh1* primer pairs, and head kidney, brain, gill and muscle for normalizer primer pairs; the inputted efficiencies are the average for their corresponding tissues), and included no-template controls (NTCs). Primer pair efficiencies for the *serpinh1* paralogues can be found in Table 6-2, while those of the normalizers are in Appendix E Table E-2. Further, melt curves indicated sharp, single, peaks with no evidence of primer dimers.

The experimental qPCR analyses were conducted according to MIQE guidelines (Bustin et al., 2009). cDNA corresponding to 5 ng of input total RNA was used as template in the PCR reactions and in all cases, technical triplicates were analyzed. As expression levels of each transcript in the sample set were assessed across four plates, a plate linker sample (i.e., a sample that was assessed for each transcript on all plates in the study) was also included to ensure there was no plate-to-plate variability. In addition, no reverse transcriptase (no-RT) controls created by pooling RNA from each individual tissue (Crossman et al., 2023) and an NTC were also included; there was no amplification in the no-RT controls and the NTCs for all of the transcripts.

To determine calibrated normalized relative quantities (CNRQ), raw cycle threshold (C_T) data were generated using the QuantStudio Real Time PCR Software (version 1.3). Briefly, for each individual plate (i.e., ".eds" file), technical replicate outliers were removed. A gene expression study (i.e., ".edm" file) was then created containing the four plates for each transcript. For each transcript, this sets the level at which the C_T is determined at the same value across all of the plates. The C_T values for each transcript (i.e., the four *serpinh1* paralogues and the two

normalizers) were then determined for each sample and exported as an MS-Excel file. These data were then imported into qbase+ (Biogazelle, Ghent, Belgium; (Hellemans et al., 2007). Similar to Crossman et al. (2023), geNorm confirmed that *eif3d* and *rpl32* were stable normalizers (mean geNorm M value and coefficient of variation of 0.380 and 0.133, respectively). CNRQs (Vandesompele et al., 2002) were calculated incorporating the C_T values of linker samples across plates and amplification efficiencies for each primer pair (Table 6-2, Appendix E Table E-2), which were then $\log_2$-transformed in Microsoft Excel.

Table 6-2. Paralogue-specific qPCR primers used for assessing the expression of Atlantic salmon *serpinh1*.

Paralogue (GenBank Accession Number)	Nucleotide sequence (5'-3')	Amplification Efficiency (%)					Amplicon Size (bp)
		Eye[a]	Fin[a]	Gill[a]	Liver[b]	Head Kidney[b]	
serpinh1a-1 (OQ814177)	F: GGGCGAGAAGATGAGAGATG	97.2	92.4	98.1	95.0	96.0	199
	R: GCACGAATGTTGGCACATAG						
serpinh1a-2 (OQ814179)	F: CCAATGTCTTCCATGCCTCT	89.6	93.2	93.5	93.8	93.0	94
	R: TTGGGGTTCTTCAGCTTGTC						
serpinh1b-1 (OQ814180)	F: CCAAAGTCAGCATGGAGGTT	93.7	89.9	84.6	91.2	89.4	151
	R: ATTGAAGAGGCGTGGAACAC						
serpinh1b-2 (OQ814182)	F: CTCATCATGCCCTACCACCT	86.3	95.2	94.2	100.0	105.0	188
	R: TCCACAGCTTCAGTCACACC						

[a] Efficiencies were assessed for the multi-tissue study; the average efficiency for these 3 tissues was used to calculate the calibrated normalized relative quantities (CNRQ).
[b] Efficiencies were assessed for the stress phenotype study; the respective tissue-specific efficiency was used to calculate the normalized relative quantities (NRQs).

6.3.9 Stress phenotype qPCR

The same paralogue-specific qPCR primers (Table 6-2) were used to assess expression in the liver and head kidney of LR and HR salmon. Normalizer genes from Chapter 3 were tested, which were also previously published (Caballero-Solares et al., 2017; Jones et al., 2007; Xu et al., 2013). Primer amplification efficiencies were calculated (Pfaffl, 2001) by generating separate standard curves using a 5-point 1:2 dilution series starting with cDNA representing 10 ng of input total RNA from either tissue, and included NTCs. The *serpinh1* paralogue and normalizer primer efficiencies can be found in Table 6-2 and Appendix E Table E-2, respectively. No-RT controls, created from pooling the RNA of randomized samples (n = 23-24 per pool), were previously tested and showed no signs of gDNA contamination (Chapter 3). Aside from *ef1a*, which had a slight shoulder to the left of the primary peak, all other primer sets had sharp, single peaks in their melt curves.

PCR amplifications were carried out in the same manner as described above, with a few exceptions: 1) cDNA representing 10 ng of input total RNA was used in each reaction; 2) amplifications were performed using the ViiA7 Real-Time PCR system (Applied Biosystems); 3) each transcript was run on a single, independent, 384-well plate. Therefore, normalized relative quantities (NRQs) were calculated from raw C_T data (with technical outliers removed) and amplification efficiencies for each primer pair (Table 6-2, Appendix E Table E-2). Similar to above, these were then log_2-transformed in Excel. Testing was performed concurrently with the analysis described in Chapter 3. Therefore, the normalizers chosen for liver (*ef1a*, *pabpc1*) and head kidney (*eif3d*, *pabpc1*) are the same as in that study. A single head kidney sample (LR salmon at 20°C injected with PBS) was removed from the analysis as it consistently failed to amplify ($C_T > 35$) during qPCR.

6.3.10 Statistical analyses

For the multi-tissue qPCR study, CNRQ values were $\log_{10}$-transformed if necessary to meet normality assumptions before comparisons were made using one-way ANOVAs, followed by Tukey's HSD *post-hoc* tests to examine differences between tissues. T-tests were performed to assess differences between sexes within each tissue with *p*-values adjusted for false discovery rate (FDR) to account for multiple comparisons.

The same statistical approach described in Chapter 3 was used to analyze the stress phenotype qPCR data in the current study. In summary, I split statistical analysis into six independent parts: 1) the effects of stress phenotype and temperature on constitutive expression; 2) the effects of stress phenotype and IP injection of Forte Micro at 12°C; 3) the effects of stress phenotype and IP injection of Forte Micro at 20°C; 4) the effect of IP injection of PBS at 12°C; 5) the effect of IP injection of PBS at 20°C; and 6) the effect of stress phenotype on fold-changes calculated between fish IP injected with PBS and Forte Micro. Data were first assessed for normality and were $\log_{10}$-transformed if necessary to meeting testing assumptions. To address parts 1-5 listed above, two-way ANOVAs followed by Tukey's HSD *post-hoc* tests were used to assess the described variables. T-tests were used to compare fold-changes between LR and HR salmon at a given sampling point/temperature.

All data were statistically analyzed and graphed using RStudio (R Studio Team, 2015; R v.4.1.2). Statistical differences were considered significant at $p < 0.05$, with trends noted at $0.05 \leq p < 0.10$.

6.4 Results

6.4.1 Characterization of four Atlantic salmon *serpinh1* paralogue sequences

From the RACE sequencing reads, two cDNAs for *serpinh1a-1* were assembled with 2470 and 2299 bp (both excluding the poly-A tail but based on alternate poly-A sites; GenBank accessions OQ814177 – isoform 1 & OQ814178 – isoform 2, respectively; Appendix E Figure E-1). The predicted cDNAs consisted of a 196 bp 5'-untranslated region (UTR), a 1266 bp (422 AA) ORF and a 1008 or 837 bp 3'-UTR based on the alternate poly-A sites. One polyadenylation signal (AUUAAA) was found in the 3'-UTR starting 21 bp upstream of the first poly-A site (Appendix E Figure E-1). Relying on only high-quality sequence results, the poly-A tail started at the first poly-A site in 2 clones, while the poly-A tail started at the second poly-A site in 4 clones. Using the Ensembl database it was found that *serpinh1a-1* contains 4 exons (i.e., 1: 830 bp, 2: 99 bp, 3: 233 bp, 4: 1308 or 1137 bp up to each of the poly-A tails of isoform 1 and isoform 2, respectively) and 3 introns (i.e., 1: 124 bp, 2: 148 bp, 3: 316 bp) (Figure 6-1). This paralogue is located on *Salmo salar* chromosome 4 (accession GCA_905237065, Primary_assembly 4: 53,713,310-53,716,445).

Assembly of *serpinh1a-2* generated a 3007 bp cDNA (excluding poly-A tail; GenBank accession OQ814179; Appendix E Figure E-2). The predicted *serpinh1a-2* cDNA consisted of a 165 bp 5'-UTR, a 1230 bp (410 AA) ORF and a 1612 bp 3'-UTR. One polyadenylation signal (AAUAAA) was found in the 3'-UTR (Appendix E Figure E-2). According to the Ensembl database, *serpinh1a-2* contains 5 exons (i.e., 1: 142 bp, 2: 621 bp, 3: 99 bp, 4: 233 bp, 5: 1912 bp) and 4 introns (i.e., 1: 1835 bp, 2: 119 bp, 3: 133 bp, 4: 276 bp) (Figure 6-1). *Salmo salar serpinh1a-2* is found on chromosome 13 in the genome (accession GCA_905237065, Primary_assembly 13: 80,986,392-80,997,828).

Two cDNA sequences for *serpinh1b-1* were assembled, one 2128 bp and the other 2084 bp in length (both excluding the poly-A tail; GenBank accessions OQ814180 – isoform 1 & OQ814181 – isoform 2, respectively; Appendix E Figure E-3). The *serpinh1b-1* cDNAs were predicted to consist of a 142 or 98 bp 5'-UTR, a 1218 bp (406 AA) ORF and a 768 bp 3'-UTR. Two polyadenylation signal (AAUAAA) sequences were found in the 3'-UTR (Appendix E Figure E-3). Using the Ensembl database it was determined that the first isoform of *serpinh1b-1* (OQ814180) contained 6 exons (i.e., 1: 61 bp, 2: 44 bp, 3: 623 bp, 4: 99 bp, 5: 233 bp, 6: 1068 bp up to the poly-A tail) and 5 introns (i.e., 1: 887 bp, 2: 1124 bp, 3: 142 bp, 4: 124 bp, 5: 167 bp) (Figure 6-1). In contrast, the second isoform (OQ814181) contained 5 exons (i.e., 1: 61 bp, 2: 623 bp, 3: 99 bp, 4: 233 bp, 5: 1068 bp up to the poly-A tail) and 4 introns (i.e., 1: 2011 bp, 2: 142 bp, 3: 124 bp, 4: 167 bp) (Figure 6-1). Therefore, the difference between the two isoforms of *serpinh1b-1* is that isoform 2 is missing the second exon found in isoform 1. This paralogue is located on *Salmo salar* chromosome 20 (accession GCA_905237065, Primary_assembly 20: 72,318,578-72,329,248).

For *serpinh1b-2*, a 2116 bp cDNA (excluding poly-A tail) was assembled using the RACE sequencing reads (GenBank accession OQ814182; Appendix E Figure E-4). The predicted *serpinh1b-2* cDNA sequence consisted of a 156 bp 5'-UTR, a 1215 bp (405 AA) ORF and a 745 bp 3'-UTR. Two polyadenylation signal (AAUAAA/AUUAAA) sequences were found in the 3′-UTR (Appendix E Figure E-4). It was determined using the Ensembl database that *serpinh1b-2* contains 5 exons (i.e., 1: 119 bp, 2: 620 bp, 3: 99 bp, 4: 233 bp, 5: 1045 bp up to the poly-A tail) and 4 introns (i.e., 1: 1504 bp, 2: 146 bp, 3: 112 bp, 4: 164 bp) (Figure 6-1). *serpinh1b-2* is located on chromosome 9 in the *Salmo salar* genome (accession GCA_905237065, Primary_assembly 9: 138,804,399-138,809,245). Both the original GenBank

(XM_014214962) and Ensembl (ENSSSAT00000046979) entries include an extra 44 bp exon that would fit in the middle of the first 1504 bp intron (i.e., 833 bp intron, 44 bp exon, 671 bp intron). However, this exon region was absent from all 8 clones that were sequenced for *serpinh1b-2*. This identical nucleotide fragment was also not found in 7 out of 8 sequenced clones of *serpinh1b-1*, and is the difference between GenBank entries OQ814180 and OQ814181 as noted above.

The intron/exon structure and synteny of the four Atlantic salmon *serpinh1* paralogues, as well as the two zebrafish paralogues and the one human copy of *serpinh1* were compared (Figure 6-1). Although all seven *serpinh1* genes have between 4 and 6 exons, and correspondingly 3-5 introns, each *serpinh1* regardless of species has protein-coding exons of 99 and 233 bp in length. All *serpinh1* genes also have a copy of *glycerophosphodiester phosphodiesterase domain-containing 5* (*gdpd5*) located directly upstream. Other similarities include *alkaline ceramidase 3* (*acer3*) directly flanking *serpinh1* downstream (*S. salar serpinh1a-1*, *serpinh1a-2*, *D. rerio serpinh1a*), as well as genes like *nuclear receptor interacting protein 1* (*nrip1*), *ubiquitin specific peptidase 25* (*usp25*), *SAM domain, SH3 domain and nuclear localization signals 1* (*samsn1*), *retinal guanylyl cyclase 2* (*gucy2f*), *matrix metallopeptidase 13* (*mmp13*) and *30* (*mmp30*) being found within close proximity to several paralogues of *serpinh1* in Atlantic salmon and zebrafish.

Generally, Atlantic salmon *serpinh1a-1* and *serpinh1a-2* sequences were more similar (i.e., 85.9% identity at the nucleotide level between *serpinh1a-1* isoform 1 and *serpinh1a-2*), and the same was true for *serpinh1b-1* and *serpinh1b-2* (i.e., 82.4% identity at the nucleotide level between *serpinh1b-1* isoform 1 and *serpinh1b-2*) (Appendix E Table E-3). qPCR primers were designed within the coding region of each paralogue, with at least one nucleotide different between all other paralogues in the forward and reverse primers (Appendix E Figure E-5). As the

coding sequence is well-conserved between paralogues, it was challenging to design primers in regions with higher levels of variation. To increase stringency/specificity, the annealing temperature was set at the melting temperature (T_m) of the primers (i.e., 60°C). Further, no evidence of multiple products/binding sites was ever found during primer quality control testing (see methods), and therefore, we are confident that the differences we report in paralogue expression are accurate.

Based on the four deduced Atlantic salmon SERPINH1 AA sequences and 40 putative orthologous AA sequences from 27 selected species, an MSA (Appendix E Figure E-6) and phylogenetic tree (Figure 6-2) were constructed. In the phylogenetic tree, putative orthologues were grouped and sub-grouped based on taxonomic classification. For example, all teleosts grouped together by superorder, with all SERPINH1A or SERPINH1B sequences from species within superorder *Protacanthopterygii* [Atlantic salmon, Arctic charr (*Salvelinus alpinus*) and northern pike (*Esox lucius*)] sharing a single high-trust (bootstrap value 100) branch point in the phylogenetic tree. As the other representative of the Salmonidae family, the Arctic charr possesses four putative copies of SERPINH1 similar to the Atlantic salmon. All other teleosts included in the analysis have two putative copies of SERPINH1. In contrast, within the Sarcopterygii taxon, the West Indian Ocean coelacanth (*Latimeria chalumnae*) grouped together with the tetrapods, and only one copy of SERPINH1 was found in each of these taxa. As expected, the sea lamprey was separated from all gnathostomes in the tree. In the case of Arctic charr, the deduced SERPINH1 sequences clustered individually with the Atlantic salmon's four paralogues. In other teleosts, if only two putative orthologues were identified, SERPINH1A clustered with Atlantic salmon SERPINH1A-1 and -2 while SERPINH1B clustered with Atlantic salmon SERPINH1B-1 and -2. The sterlet sturgeon (*Acipenser ruthenus*) contain four putative

SERPINH1 orthologues, while spotted gar (*Lepisosteus oculatus*) have two putative copies, and Polypteriformes (i.e., reedfish, *Erpetoichthys calabaricus* and Senegal bichir, *Polypterus senegalus*) possess only one putative copy of SERPINH1. Two copies of SERPINH1 in sterlet sturgeon (i.e., SERPINH1_1 and _2) and one in spotted gar (i.e., SERPINH1_1), as well as SERPINH1 in the Polypteriformes, cluster as the basal actinopterygians between the Sarcopterygii taxon and the teleosts. In contrast, the remaining SERPINH1 orthologues in sterlet sturgeon (i.e., SERPINH1_3 and _4) and spotted gar (i.e., SERPINH1_2) cluster with the one cartilaginous fish included in this analysis, the whale shark (*Rhincodon typus*).

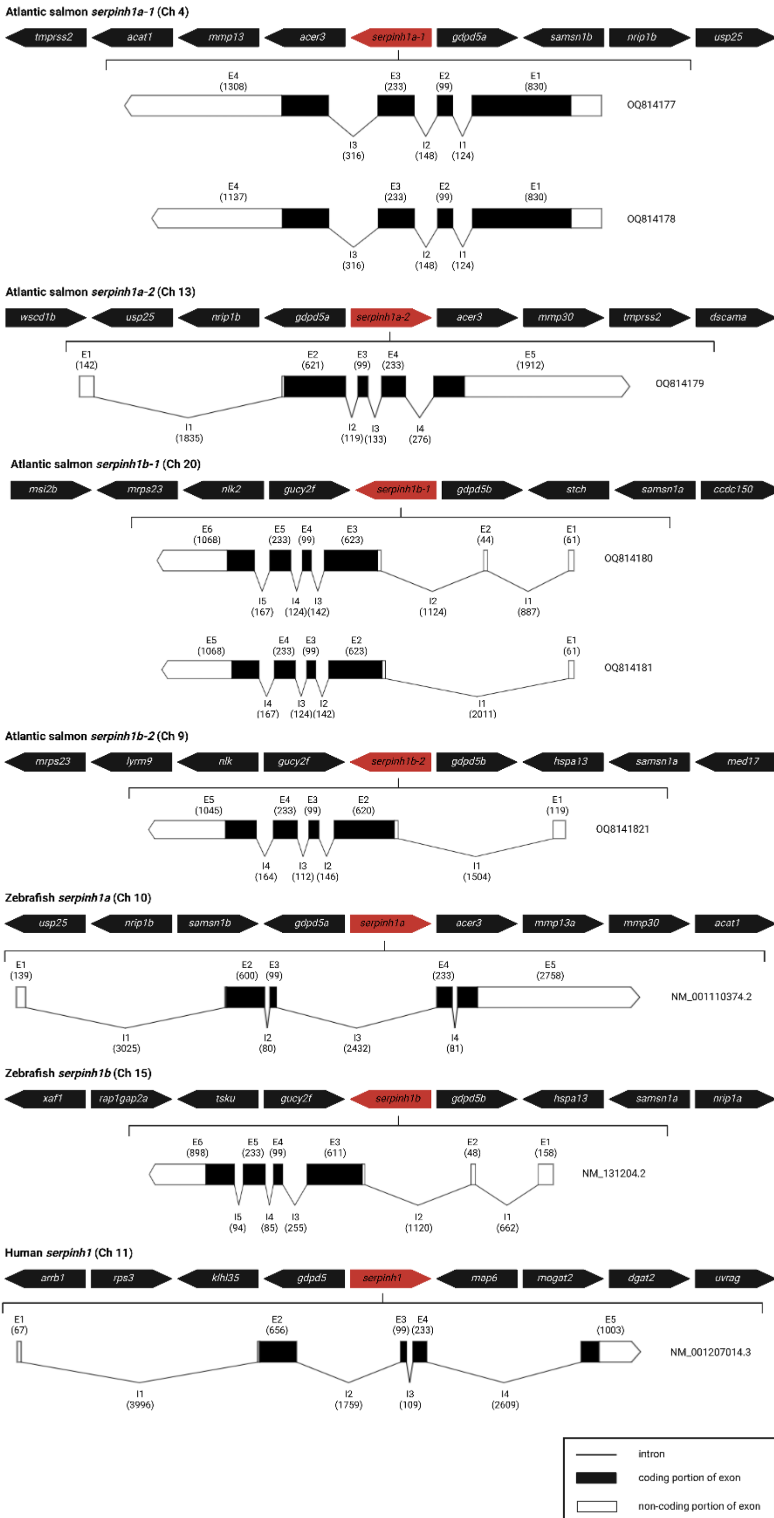

Figure 6-1. Genome organization and synteny analysis of *serpinh1* in Atlantic salmon, zebrafish and human. The Ensembl genome browser (http://www.ensembl.org) was used to determine neighbouring transcripts, and arrow tips are used to indicate the transcription direction for each transcript. The Ensembl database was also used to identify exonic and intronic regions within

each copy of *serpinh1*. The GenBank Accession numbers of each transcript are provided to the right of each schematic structure. Coding sections of exons (E) are depicted as black boxes, while 5′ and 3′ untranslated regions (UTRs) are depicted as white boxes. Introns (I) are depicted as lines. The scale is similar between structures, but not uniform. Numbers in parentheses indicate the bp length of each region.

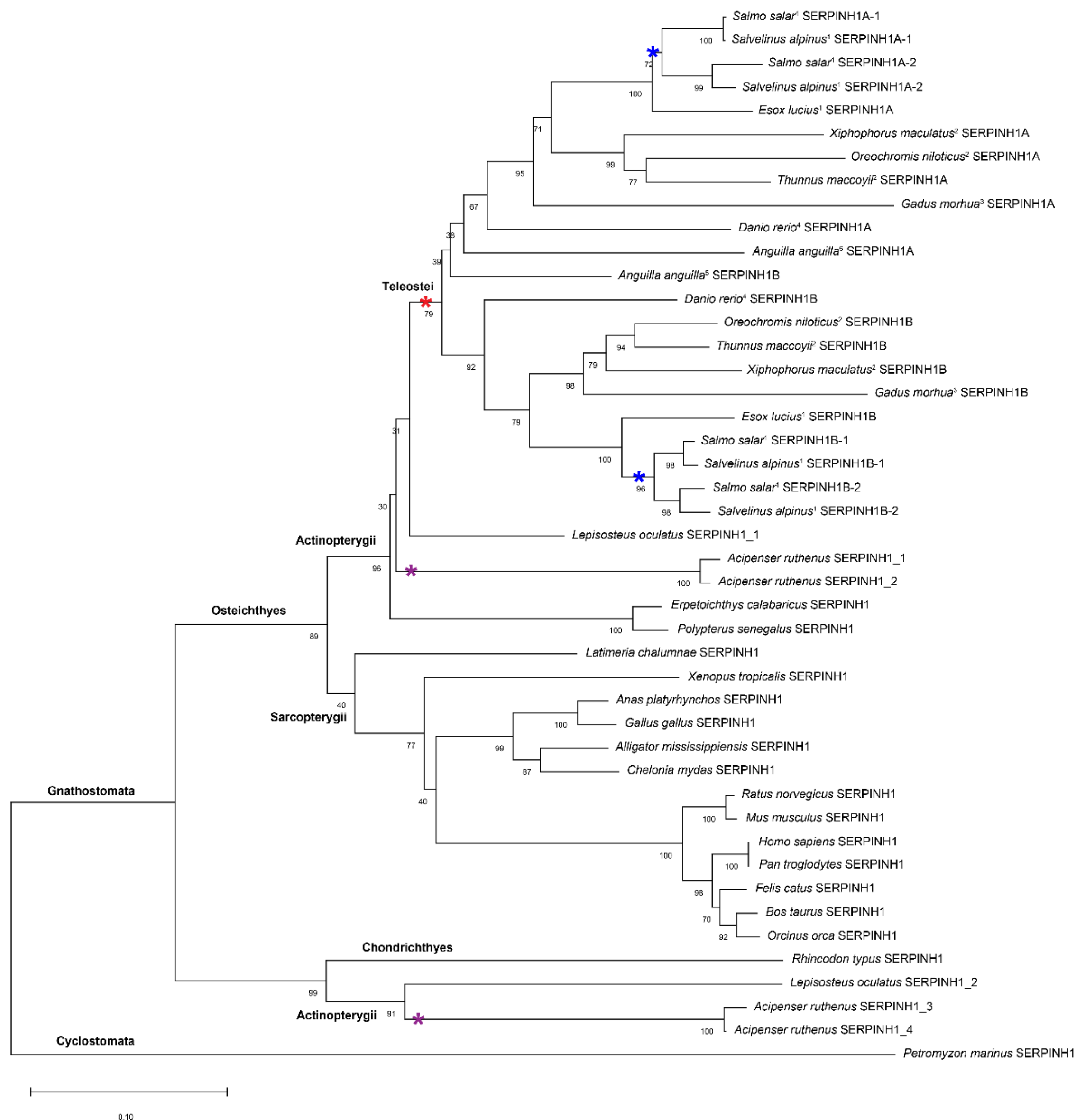

Figure 6-2. Phylogenetic tree analysis of putative SERPINH1 orthologues across various species. Putative SERPINH1 amino acid sequences obtained from the NCBI non-redundant protein database (see Appendix E Table E-1) were used to infer the evolutionary relationship among SERPINH1 orthologues. The phylogenetic tree was generated via the Neighbour-joining method and bootstrapped 10,000 times using MEGA 11 (v.11.0.13) software. The numbers at the branch points represent the bootstrap value and branch length, and these are proportional to the calculated evolutionary distances. The scale represents the number of substitutions per site. Coloured asterisks denote the ancient acipencerid WGD (purple), teleost-specific WGD (red) and the salmonid-specific WGD (blue). Superscript numbers represent the superorder of the teleost fish: Protacanthopterygii[1], Acanthopterygii[2], Paracanthopterygii[3], Ostariophysi[4], Elopomorpha[5]. *Salmo salar* (Atlantic salmon), *Salvelinus alpinus* (Arctic charr), *Esox lucius* (northern pike), *Xiphophorus maculatus* (southern platyfish), *Oreochromis niloticus* (Nile tilapia), *Thunnus maccoyii* (southern bluefin tuna), *Gadus morhua* (Atlantic cod), *Danio rerio* (zebrafish), *Anguilla anguilla* (European eel), *Lepisosteus oculatus* (spotted gar), *Acipenser ruthenus* (sterlet

sturgeon), *Erpetoichthys calabaricus* (reedfish), *Polypterus senegalus* (Senegal bichir), *Latimeria chalumnae* (West Indian Ocean coelacanth), *Xenopus tropicalis* (tropical clawed frog), *Anas platyrhynchos* (mallard duck), *Gallus gallus* (chicken), *Alligator mississippiensis* (American alligator), *Chelonia mydas* (green sea turtle), *Rattus norvegicus* (Norway rat), *Mus musculus* (mouse), *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Felis catus* (domestic cat), *Bos taurus* (cow), *Orcinus orca* (orca), *Rhincodon typus* (whale shark), *Petromyzon marinus* (sea lamprey).

6.4.2 Constitutive expression of Atlantic salmon *serpinh1* paralogues across tissues

The constitutive transcript expression levels of the Atlantic salmon's four *serpinh1* paralogues varied across the 17 different tissues (Figure 6-3). Visually, *serpinh1a-1*, *serpinh1b-1* and *serpinh1b-2* share comparable expression patterns. For all three of these paralogues, the lowest detectable expression levels (considering both sexes together) were detected in the midgut. However, the highest expression levels (considering both sexes together) were found in the eye for *serpinh1a-1* and in the fin for both *serpinh1b-1* and *serpinh1b-2*. In contrast, the overall expression of *serpinh1a-2* was lowest in liver (with the exception of blood where transcript expression was not detected for all four paralogues) and highest in gill. A few instances of sex-specific differences within individual paralogues were also noted. For example, the expression of *serpinh1a-2* was 3.1-fold higher in testis than in ovary (Figure 6-3B), while the expression of *serpinh1b-2* was 3.2-fold higher in ovary compared with testis. The expression of *serpinh1a-2* was also 5.0-fold higher in the pyloric caecum of male vs. female fish. Appendix E Figures E-7 and E-8 show the tissue-specific expression of the four paralogues in only female or male salmon, respectively.

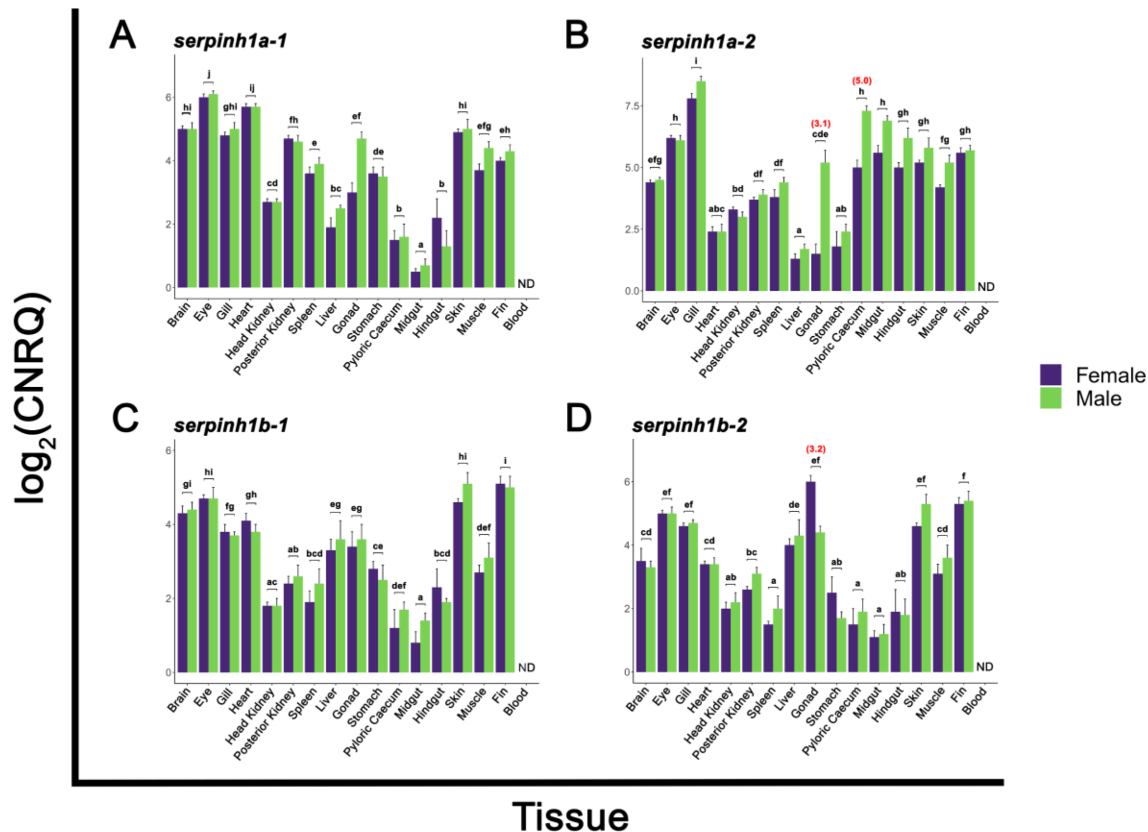

Figure 6-3. Constitutive expression of A) *serpinh1a-1*, B) *serpinh1a-2*, C) *serpinh1b-1* and D) *serpinh1b-2* in different tissues of adult Atlantic salmon. Calibrated normalized relative quantities (CNRQ) are presented as means ± 1 standard error (n = 4 per sex). Values without a letter in common denote significant ($p < 0.05$) differences between tissues (1-way ANOVA; data pooled between sexes, n = 8). Red numbers in parentheses denote significant fold-change values between female and male Atlantic salmon (t-test; FDR-adjusted $p < 0.05$). In all cases, the letter 'a' signifies the lowest value within a comparison. ND, not detected.

6.4.3 Paralogue-specific transcript expression in response to heat stress and bacterial immune stimulation between stress phenotypes

Expression levels of the four *serpinh1* paralogues in the liver were affected by stress phenotype, rearing temperature and Forte Micro vaccine injection (Figure 6-4). No significant ($p > 0.05$) differences in the expression of the paralogues were found between stress phenotypes, and the only trend ($p = 0.076$) observed was at 12°C for *serpinh1b-2*, where LR salmon exhibited a greater response to Forte Micro relative to PBS injection at 24 hpi (4.5-fold LR vs.

1.6-fold HR). However, temperature influenced the expression of all four paralogues at time zero. The constitutive expression of *serpinh1a-1* and *serpinh1a-2* were both lower at 20°C compared to 12°C ($p < 0.01$ for temperature's overall interaction term in both cases); although this effect was more evident in LR fish for both paralogues. In contrast, *serpinh1b-1* and *serpinh1b-2* were both strongly upregulated ($p < 0.0001$) at the higher temperature regardless of stress phenotype (average 11.2- and 10.7-fold, respectively). Interestingly, the expression of *serpinh1a-1* and *serpinh1a-2* was downregulated at 12°C after PBS-injection, and *serpinh1a-1* expression was also lower in PBS-injected HR salmon at 20°C relative to time zero. In many cases, injection of Forte Micro had a significant, but weak, overall effect of increasing expression across the four paralogues compared to PBS controls. However, the only significant difference between time-matched PBS- and Forte Micro-injected fish within a particular phenotype was for *serpinh1b-2* at 12°C in LR salmon at 24 hpi.

In head kidney, the Atlantic salmon's *serpinh1* paralogues were also responsive to the same treatments/stimulations described above (Figure 6-5). In this organ, the only significant ($p < 0.05$) difference between stress phenotypes was a higher fold-change value for *serpinh1a-1* in response to Forte Micro compared to PBS controls at 20°C in LR salmon at 12 hpi relative to HR salmon (1.4- vs. 1.0-fold, respectively). In head kidney, expression of *serpinh1a-1* was not affected by temperature at time zero. Similar to liver tissue, though, *serpinh1a-2* was downregulated at 20°C (-1.3-fold on average; $p < 0.05$ for temperature's interaction term; more evident in HR salmon). Likewise, *serpinh1b-1* and *serpinh1b-2* were both upregulated at 20°C compared to 12°C at time zero (average 16.7- and 10.4-fold, respectively; $p < 0.0001$). Relative to time zero, the expression of *serpinh1a-1* and *serpinh1a-2* was lower following PBS injection at 12°C in HR salmon. Injection of Forte Micro only increased the expression of the four

paralogues moderately (by 1.2- to 2.7-fold) as compared to PBS controls in many cases (i.e., there was a significant overall interaction for injection at 12 or 24 hpi). Similar to the results for the liver, only for *serpinh1b-2* in LR fish at 24 hpi was there a significant difference between time-matched PBS- and Forte Micro-injected fish. The complete set of statistical results generated by comparing transcript expression in the liver or head kidney is available in Appendix E Table E-4.

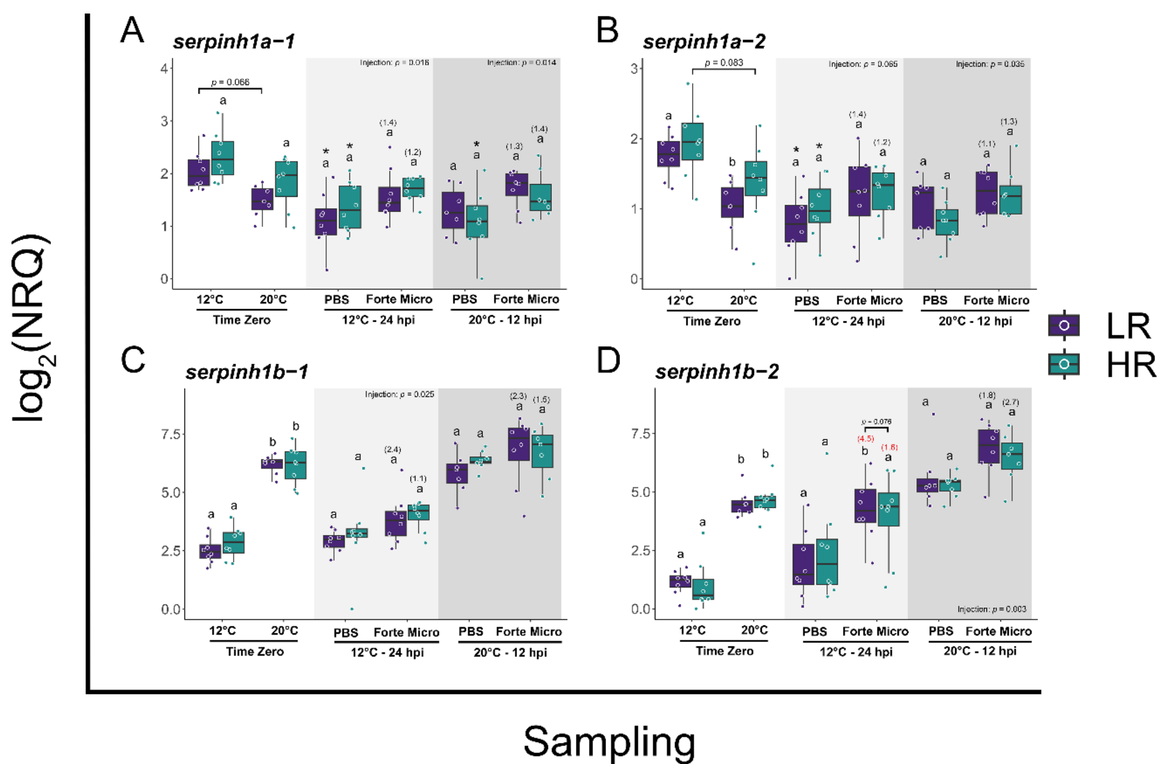

Figure 6-4. Expression of A) *serpinh1a-1*, B) *serpinh1a-2*, C) *serpinh1b-1* and D) *serpinh1b-2* in the liver of male Atlantic salmon characterized as low (LR) or high (HR) stress responders when sampled at 12 or 20°C. Samples were taken prior to injection (time zero; i.e., these represent constitutive expression), and 12 h (20°C) or 24 h (12°C) post-injection (hpi) with PBS or Forte Micro. Lower and upper box boundaries indicate the 2nd and 3rd quartiles, respectively, the line inside the box is the median value, and the top and bottom whiskers show the upper and lower 25% of values, respectively. The individual symbols (solid circles) represent individual fish. Normalized relative quantities (NRQs) were compared by two-way ANOVAs ($p < 0.05$; n = 7-8 per sampling/phenotype). Lower case letters denote significant differences between temperatures within a phenotype at time zero, or between injection treatments within a phenotype at 12 or 24 hpi. Numbers in parentheses denote mean fold-change values between

time-matched PBS and Forte Micro treatments, with red lettering signifying differences between phenotypes (t-test; $p < 0.05$, unless indicated by brackets above). Asterisks indicate significant differences between temperature and phenotype-matched time zero and PBS-injection measurements (two-way ANOVA; $p < 0.05$). Significant interactions and those which did not quite reach significance ($p < 0.05$, $0.05 \leq p < 0.10$, respectively) are also indicated at the margin of each panel if significant effects have not already been noted.

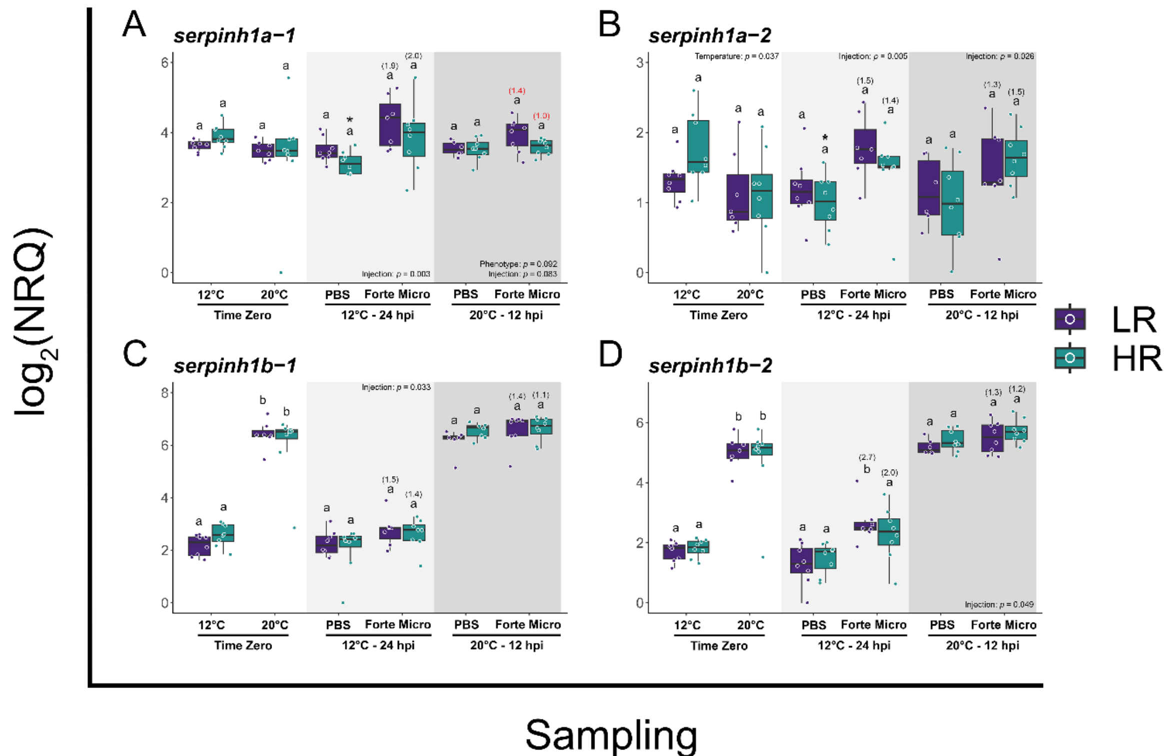

Figure 6-5. Expression of A) *serpinh1a-1*, B) *serpinh1a-2*, C) *serpinh1b-1* and D) *serpinh1b-2* in the head kidney of male Atlantic salmon characterized as low (LR) or high (HR) stress responders sampled at 12 or 20°C. Samples were taken prior to injection (time zero; i.e., these represent constitutive expression), and 12 h (20°C) or 24 h (12°C) post-injection (hpi) with PBS or Forte Micro. Lower and upper box boundaries indicate the 2nd and 3rd quartiles, respectively, the line inside the box is the median value, and the top and bottom whiskers show the upper and lower 25% of values, respectively. The individual symbols (solid circles) represent individual fish. Normalized relative quantities (NRQs) were compared by two-way ANOVA ($p < 0.05$; n = 6-8 per sampling/phenotype). Lower case letters denote significant differences between temperatures within a phenotype at time zero, or between injection treatments within a phenotype at 12 or 24 hpi. Numbers in parentheses denote mean fold-change values between time-matched PBS and Forte Micro treatments, with red lettering signifying differences between phenotypes (t-test; $p < 0.05$). Asterisks indicate significant differences between temperature and phenotype-matched time zero and PBS-injection measurements (two-way ANOVA; $p < 0.05$). Significant interactions and those which did not quite reach significance ($p < 0.05$, $0.05 \leq p < 0.10$, respectively) are also indicated at the margin of each panel if significant effects have not already been noted.

6.5 Discussion

6.5.1 *serpinh1* transcript characterization

A single ancestral *serpinh1* was duplicated during the teleost-specific WGD, resulting in paralogues *a* and *b*. Paralogues *a-1* and *a-2* are derived from the salmonid-specific WGD, as *S. salar* chromosomes 4 and 13 contain homeologous blocks (Lien et al., 2016). Similarly, paralogues *b-1* and *b-2* resulted from the salmonid-specific WGD, as *S. salar* chromosomes 20 and 9 also contain homeologous blocks (Lien et al., 2016). As expected, there are genes in synteny comparing these *serpinh1* genome regions in Atlantic salmon and zebrafish. Since Atlantic salmon *serpinh1a-1* has seven out of eight flanking genes in common with zebrafish *serpinh1a* (i.e., *acat1*, *mmp13*, *acer3*, *gdpd5a*, *samsn1b*, *nrip1b* and *usp25*), whereas Atlantic salmon *serpinh1a-2* has only five of eight in common (i.e., *acer3*, *gdpd5a*, *nrip1b*, *usp25* and *mmp30*), I hypothesize that Atlantic salmon *serpinh1a-1* was the original *serpinh1a* in this species' lineage with *serpinh1a-2* arising from the salmonid-specific WGD event. Likewise, since Atlantic salmon *serpinh1b-1* has three of eight flanking genes in common with zebrafish *serpinh1b* (i.e., *gucy2f*, *gdpd5b* and *samsn1a*), whereas the Atlantic salmon *serpinh1b-2* has four of eight flanking genes in synteny with zebrafish *serpinh1b* (i.e., *gucy2f* immediately downstream, and *gdpd5b*, *hspa13*, and *samsn1a* immediately upstream, with the same gene orientations relative to *serpinh1b* between the species), I hypothesize that *serpinh1b-2* was the original *serpinh1b* in the Atlantic salmon lineage and that *serpinh1b-1* arose from the salmonid-specific WGD event.

We found two isoforms of *serpinh1b-1* based on the presence/absence of a 44 bp exon section in the 5’-UTR, and this region was completely missing in *serpinh1b-2* in comparison to the GenBank and Ensembl database sequences. A similar feature was reported in mice, where

heat shock induced alternative splicing removed a section in the 5' noncoding exon region of *serpinh1* (Takechi et al., 1994). The liver samples used for RACE PCR of these two paralogues were taken from salmon reared at 20°C, which may explain why this section was spliced out of 7 out of 8 *serpinh1b-1* and all *serpinh1b-2* cDNA sequences. These samples were a subset of those analyzed in the stress phenotype qPCR assays, and show high upregulation of *serpinh1b-1* and *serpinh1b-2* at 20°C compared to 12°C, indicating heat stress induction. Therefore, alternative splicing of these two paralogues is potentially evolutionarily conserved as it appears present in salmon and mice (Takechi et al., 1994). This would also explain the difference in the number of introns/exons between Atlantic salmon *serpinh1a-1* and zebrafish *serpinh1a*, as this splicing event in salmon removes an entire exon. The brain, head kidney and spleen RNA samples contributing to the pooled RNA used in RACE PCR for *serpinh1a-1* and *serpinh1a-2* were taken from salmon reared at 6.6°C (Crossman et al., 2023). Thus, I do not know whether splicing would also occur in these paralogues under high temperature stress. Interestingly, previous work identified eight CpG sites within the first intron and second exon of Atlantic salmon *serpinh1b-2,* and found that four of these sites became highly methylated in the liver following an incremental temperature increase of +1°C week^{-1} from 12°C to 20°C (Beemelmanns et al., 2021a). Further, the degree of methylation of these four sites was significantly correlated with transcript expression. However, after four weeks spent at 20°C, the CpG sites were no longer significantly differentially methylated (Beemelmanns et al., 2021a). Thus, DNA methylation of *serpinh1b-2* in salmon is reversible following acclimation to high temperatures, and this suggests that methylation helps elicit a strong rapid response to thermal stress but can be modified if the stress becomes chronic (Beemelmanns et al., 2021a). It is likely that methylation and/or splicing of the

sequence before or within the first exon of the coding sequence plays key regulatory roles during thermal stress in Atlantic salmon.

Kumar et al. (2017) previously hypothesized that multiple copies of *serpinh1* in fish may be required to properly fold the collagen produced from duplicated collagen synthesis genes (Duran et al., 2015). This hypothesis is supported by the strong positive correlations between transcript expression of *collagen type I alpha 1 chain* (*col1a1*) and *serpinh1b*, and between *col1a2* and *serpinh1a*, in grass carp (*Ctenopharyngodon idella*) muscle following aerobic exercise (Liang et al., 2021). However, I acknowledge that this hypothesis requires validation in Atlantic salmon and should involve quantification at both the transcript and protein levels. Since each paralogue encodes for a unique SERPINH1 protein in Atlantic salmon, there may be functional differences amongst them.

6.5.2 Multiple sequence alignment and phylogenetic analyses

Kumar et al. (2017) previously reported that there were 3-4 copies of *serpinh1* in all ray-finned fishes. In contrast, my analysis shows that the actinopterygian species examined herein have 1, 2 or 4 copies of this gene. The genomes of many of these species have been re-annotated and former loci thought to be paralogues of *serpinh1* have now been reclassified as *serpinh2*. For example, a third putative *serpinh1* paralogue in zebrafish located on chromosome 7, described in Kumar et al. (2017), is now designated as *serpinh2*. In addition, this past study did not include any Polypteriformes or any other actinopterygians that did not experience the teleost-specific WGD in their analyses (Kumar et al., 2017), which would explain the lack of identification of any ray-finned fishes with a single copy of *serpinh1*. Therefore, the phylogenetic tree in the current study represents a more accurate and comprehensive depiction of the evolutionary

relationships between putative copies of *serpinh1* across species compared to past work (i.e., Kumar et al., 2017; Liang et al., 2021).

The protein sequences assessed in the MSA shared 69.4% identity on average, ranging between 41.2% (i.e., between sea lamprey and whale shark) and 100% (i.e., between human and chimpanzee, *Pan troglodytes*). The four Atlantic salmon paralogues share between 75.6 and 95.1% identity at the amino acid level. The serpin superfamily domain is particularly well conserved among species, with most variation present at the N-terminus, and all SERPINH1 orthologues contain an ER retention sequence. The high level of conservation in SERPINH1 sequences among species agrees with past comparisons (Kumar et al., 2017; Liang et al., 2021; Wang et al., 2007).

6.5.3 Constitutive expression of *serpinh1* paralogues across tissues

A few studies have characterized tissue-specific *serpinh1* expression in other fish species, but typically only targeted a single paralogue (Pearson et al., 1996; Wang et al., 2007). In goldfish (*Carassius auratus*), RT-PCR detected relatively similar and abundant expression of *serpinh1* in the brain, heart, skeletal muscle, kidney and gill, with lower levels found in the liver (Wang et al., 2007). In contrast, qPCR revealed that grass carp had the highest expression of *serpinh1a* and *serpinh1b* in the heart, lowest in the intestine and muscle, with intermediate levels detected in swim bladder, liver and skin (Liang et al., 2021). Neither the goldfish or grass carp expression profiles match that of the Atlantic salmon paralogues, and this suggests species-specific effects on constitutive expression of *serpinh1* transcripts. It was not surprising that none of the Atlantic salmon *serpinh1* paralogues amplified in the blood as mature red blood cells (a main component of blood) lack an endoplasmic reticulum (Sekhon and Beams, 1969). Zebrafish

serpinh1b is constitutively expressed at relatively low levels throughout embryonic development at this species' typical rearing temperature (i.e., 28.5°C), but is strongly induced by heat stress at 34 and 37°C during the gastrula to the 3-day larval stages (Pearson et al., 1996). SERPINH1 is also essential for neural plate development during gestation in rats (Walsh et al., 1997). The importance of SERPINH1 production in mammals is further evidenced by lethal knockout and mutation/variants causing bone disorders (Christiansen et al., 2010; Drögemüller et al., 2009; Marshall et al., 2016; Nagai et al., 2000). Thus, *serpinh1* is likely a necessary modulator of collagen structural integrity and responds to heat stress throughout the entire life cycle of teleost fishes and mammals.

In addition, levels of *serpinh1* varied by sex in a few instances in this study. Expression of *serpinh1a-2* was more than 3-fold higher in salmon testis vs. ovary, while the opposite was true for *serpinh1b-2*. This is the first sex-specific comparison of *serpinh1* expression in fish, but in humans there does not appear to be a major difference in abundance of *serpinh1* between the testis and ovary (Fagerberg et al., 2014). SERPINH1 levels did not change in the Leydig cells of hamsters (*Mesocricetus auratus*) between 6 and 24 months of age (Beltrán-Frutos et al., 2016), and protein abundance in hamsters and mRNA levels in rats (*Rattus norvegicus*) were only higher in regressed testes compared to sexually developed organs (Beltrán-Frutos et al., 2016; Syntin et al., 2001). What role these paralogues have in Atlantic salmon remains to be determined. Based on the observed sex-specific expression of two *serpinh1* paralogues in gonad, I hypothesize that *serpinh1a-2* evolved to have specific functions in Atlantic salmon testes, with *serpin1b-2* evolving to have specific functions in the Atlantic salmon ovary. Alternatively, it could be that the overexpression of *serpinh1a-2* in testis is simply balanced by reduced expression of *serpinh1b-2* and the inverse could be true of these paralogues in ovary. Clearly,

these hypotheses require further testing, and should include salmon at other stages of sexual development.

6.5.4 Response to elevated temperature between paralogues of *serpinh1*

serpinh1 is one of the most consistently upregulated heat stress biomarkers in salmonids at the mRNA (Akbarzadeh et al., 2018; Beemelmanns et al., 2021c, 2021b; Jeffries et al., 2014, 2012; Manzon et al., 2022; Rebl et al., 2013; Tomalty et al., 2015) and protein (Nuez-Ortín et al., 2018; Quan et al., 2021) levels. This is true for several other fish species as well (e.g., Dettleff et al., 2020; Hori et al., 2010; Swirplies et al., 2019). Interestingly, we found this pattern of high expression at elevated temperatures evident in only two of the Atlantic salmon paralogues (i.e., *serpinh1b-1* and *serpinh1b-2*). In contrast, levels of *serpinh1a-1* and *serpinh1a-2* were lower at 20°C than 12°C in the liver, and the same was true for expression of *serpinh1a-2* in the head kidney. These results agree with Ignatz et al. (2022), where *serpinh1b-1* was upregulated and *serpinh1a-1* was downregulated in the liver of ~800 g AquAdvantage Salmon (growth hormone transgenic female triploid Atlantic salmon) reared at 16.5°C as compared to at 10.5°C. Although the impact of cold temperatures on *serpinh1* expression has not been well studied, Vadboncoeur et al. (2023) reported that levels of *serpinh1b-2* in post-smolt Atlantic salmon liver were unaffected by a gradual (1°C per week) decrease in temperature from 8 to 1°C. As noted in the aforementioned study, it would be interesting to analyze the expression of all four paralogues to determine if any of the other paralogues are cold-responsive.

Candidate single nucleotide polymorphisms (SNPs) associated with survival under thermal stress have been identified in redband trout (*Oncorhynchus mykiss gairdneri*), and the most significant SNP in this study was found in the 3’-UTR of *serpinh1* (Narum et al., 2013).

Ongoing research has also found that the four Atlantic salmon *serpinh1* paralogues were differentially expressed between the most and least thermally tolerant Atlantic salmon families (Ignatz et al., in final prep.). It is hypothesized, based on rainbow trout transcript expression data, that SERPINH1 helps neutralize reactive oxygen species (ROS) during heat shock (Wang et al., 2016), and that this protein aids in synthesizing and stabilizing collagen molecules to keep hepatocyte structures intact in Atlantic salmon at high temperatures (Beemelmanns et al., 2021b, 2021c). Further, phenotypic plasticity allows rainbow trout to modulate collagen deposition in the ventricle during thermal acclimation (Johnston and Gillis, 2022), and cardiac performance is a key determinant of upper temperature limits in fish (Farrell, 2002; Farrell et al., 2009). Thus, *serpinh1* is a strong candidate for further validation studies to establish what role(s) it plays in mediating the upper thermal tolerance of salmonids.

A few studies have examined what impact other external factors, in conjunction with high temperature stress, have on *serpinh1* expression in teleosts. For example, while hypoxia (60 – 70% air saturation) at high temperatures (i.e., ≥ 18°C) did not have an additive effect on *serpinh1b-2* expression in the liver of post-smolt Atlantic salmon, the combined impact of hypoxia and long-term exposure to 16°C, but not this temperature alone, increased the expression of this paralogue relative to individuals held at 12°C (Beemelmanns et al., 2021b). Both *serpinh1b-1* and *serpinh1b-2* were found to be suitable biomarkers of heat stress in juvenile Chinook salmon (*Oncorhynchus tshawaytscha*) gill, but not correlated to salinity or hypoxic stress (Houde et al., 2019). Exposure to venlafaxine (an antidepressant) diminished the response of *serpinh1* (by ~60%) in zebrafish gill following a critical thermal maximum (CT_{Max}) test, whereas this effect was not seen in liver (Weber et al., 2023). This latter result highlights a localized/tissue-specific effect at the site of direct contact with the drug (Weber et al., 2023).

Rainbow trout hepatocytes at 18 and 24°C had higher expression of *serpinh1* when incubated with selenium nanoparticles (up to 5.0 μg mL^{-1}), and at the higher temperature exposure to these particles increased hepatocyte viability as well as glutathione peroxidase and superoxide dismutase activity (Sun et al., 2022). However, supplemental dietary cholesterol did not impact the expression of *serpinh1b-2* in the liver of post-smolt female triploid Atlantic salmon reared at 18°C (Ignatz et al., 2022b). Finally, these concomitant effects do not only involve elevated temperatures. In the current study, *serpinh1a-1* and *serpinh1a-2* were downregulated at 12°C in the liver following PBS-injection in comparison to time zero (i.e., pre-injection) in both LR and HR individuals. In addition, the expression of *serpinh1a-1* was lower in the liver of HR salmon at 20°C, and *serpinh1a-1* and *serpinh1a-2* were both downregulated in the head kidney of HR salmon at 12°C. These effects: showcase that these paralogues are responsive to handling stress, but that this is dependent on the tissue and rearing temperature examined; and further highlight differences in comparison to the other two paralogues.

6.5.5 Response to bacterial immune stimulation between paralogues of *serpinh1*

The pleiotropic nature of *serpinh1* is shown by the fact that all four paralogues of this gene in Atlantic salmon responded to elevated temperature and bacterial stimulation (i.e., Forte Micro injection). Other transcriptomic studies have reported that *serpinh1* is differentially expressed in disease-challenged Atlantic salmon. For example, in *Moritella viscosa*-infected post-smolt Atlantic salmon, *serpinh1b-2* was downregulated at the site of the skin lesion at 29 days post-challenge compared to uninfected skin samples (Eslamloo et al., 2022). Whereas, *serpinh1b-2* expression was unaffected in the skin sampled ~1 cm away from the edge of the lesion of infected fish (Eslamloo et al., 2022), indicating a highly localized immune response for

this transcript. Since SERPINH1 is a collagen-specific chaperone (Ishida and Nagata, 2011), and *M. viscosa* causes large lesions in the skin of infected fish, SERPINH1 could play a role in wound healing and structural repair of skin damage in Atlantic salmon. In zebrafish, knockout of *serpinh1b* was shown to reduce fin length, segment length and cell proliferation in regenerating tissue, highlighting its important role in bone growth and skeletal patterning in this species (Bhadra and Iovine, 2015). However, *serpinh1a-1*, *serpinh1b-1* and *serpinh1b-2* were all downregulated in the fin of post-smolt Atlantic salmon infected with chalimus I stage sea lice (*Lepeophtheirus salmonis*) relative to the uninfected controls (Umasuthan et al., 2020). It is well established that sea lice secrete/excrete numerous enzymes and other proteins at the site of attachment that suppress the immune system and the wound healing ability of salmonids (Fast et al., 2007, 2003; Hamilton et al., 2018). Thus, it is likely that sea lice inhibit the expression of these 3 *serpinh1* paralogues in Atlantic salmon. Interestingly, the expression of *serpinh1a-2* was not significantly affected by sea lice infection (Umasuthan et al., 2020), further hinting at the functional divergence of this paralogue.

6.6 Conclusion

This is the first study to characterize the four paralogues of the collagen-mediated molecular chaperone *serpinh1* in a salmonid species at the nucleotide and deduced protein sequence levels. Based on our results, we hypothesize that *serpinh1* was duplicated during the teleost-specific WGD (i.e., producing *serpinh1a-1* and *serpinh1b-2*), and that these two copies were duplicated during the salmonid-specific WGD (i.e., producing *serpinh1a-2* and *serpinh1b-1*, respectively). Since the most recent WGD event ~80 million years ago (Lien et al., 2016), it appears that *serpinh1a-2* has diverged evolutionarily based in part on constitutive expression

profiles across tissues. Stress phenotype had minimal impact on paralogue-specific expression, but bacterin exposure did increase the expression of all paralogues moderately compared to saline-injected controls in most cases. Contrary to expectations, only *serpinh1b-1* and *serpinh1b-2* were upregulated at high temperatures (i.e., 20°C), while the remaining two paralogues were downregulated relative to salmon reared at 12°C. As heat shock proteins like *serpinh1* are generally characterized by their increase in expression during thermal stress, this suggests subfunctionalization of *serpinh1a-1* and *serpinh1a-2*. Further evidence of divergence is shown by the levels of these first two paralogues decreasing following handling/injection, while no such effect was observed for *serpinh1b-1* and *serpinh1b-2*. Although more research, and importantly methods to quantify tissue SERPINH1 protein levels, are required to further understand this gene's/protein's association with upper thermal tolerance in fishes, this study provides unique insights into the effects of genome duplication of an essential mediator of survival.

6.7 References

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CHAPTER 7: Summary

7.1 Summary of research findings

As climate change continues to threaten the sustainability of the Atlantic salmon aquaculture industry, proactive mitigation strategies must be implemented to offset the negative consequences of rising sea surface temperatures. Prior to this thesis, there was considerable knowledge on the general effects of high temperatures on Atlantic salmon growth, feed intake, fillet pigmentation, behaviour and gene expression (e.g., Beemelmanns et al., 2021b, 2021a; Gamperl et al., 2021, 2020; Stehfest et al., 2017; Wade et al., 2019). Building upon this information, this thesis applied selective approaches with potential to improve the upper thermal tolerance and other commercially relevant traits of farmed Atlantic salmon grown in sea-cages in the North Atlantic. To improve the reliability and relevance of the data, all trials were conducted using the same incremental thermal challenge (+0.2°C day^{-1} from their acclimation temperature), that mimicked conditions salmon would naturally experience in sea-cages during the summer (Burt et al., 2012; Gamperl et al., 2021; Johansson et al., 2007, 2006). Three main factors were tested to determine their influence on the incremental thermal maximum (IT_{Max}) of these salmon: i) stress phenotype; ii) supplemental dietary cholesterol; and iii) family/genetic background. In addition, four paralogues of a key thermal stress biomarker gene, *serpinh1*, were characterized. Altogether, this novel research provides the aquaculture industry with vital information that can help inform and improve production strategies.

The first hypothesis tested whether stress phenotype would impact the IT_{Max} of male Atlantic salmon (Chapter 2; Ignatz et al., 2021). I was able to consistently characterize low responders (LR) and high responders (HR) based on repeatedly measuring post-stress cortisol levels in fish reared at 9°C. It was originally thought that LR salmon with proactive stress phenotypes might be more prone to mortality in sea-cages as active avoidance behaviour at high

temperatures in a confined environment may be detrimental compared to the reactive HR phenotype (Gamperl et al., 2020; Hvas et al., 2017). However, stress phenotype was not predictive of upper thermal tolerance as no differences in IT_{Max} were detected between LR and HR fish. Additionally, stress phenotypes were no longer distinguishable from one another based on post-stress cortisol levels measured at high temperatures (i.e., 23.6°C). Importantly, when compared to the more conventional approach of measuring acute thermal tolerance, a large separation was found between the IT_{Max} and critical thermal maximum (CT_{Max}; measured at +2°C h^{-1}) of these salmon (i.e., IT_{Max} = 25.3°C vs. CT_{Max} = 28.5°C). This result highlighted that IT_{Max} likely provides a more realistic estimate of survival at high temperatures compared to the convenient, and routinely used, CT_{Max} method. While selection of fish based on stress phenotype at lower temperatures (i.e., 9°C) may not be beneficial to incorporate into Atlantic salmon breeding programs if the goal is to improve survival at high temperatures, there were several unanswered questions regarding how LR and HR may differ (e.g., in immune responses at optimal vs. elevated temperatures).

Therefore, samples that were collected during Experiment #2 in Chapter 2 were used to assess the impact of stress phenotype, elevated temperature, and bacterin exposure on male Atlantic salmon growth, stress and immune biomarker gene expression (Chapter 3; Ignatz et al., submitted #1). It was anticipated that HR fish may mount a less robust immune response based on past studies that showed lower survival during disease challenges among HR salmonids as compared to LR fish (Fevolden et al., 1993, 1992). However, based on the expression of 10 head kidney immune-related transcripts, HR salmon mounted a greater innate antibacterial immune response to Forte Micro (i.e., vaccine) injection than LR salmon at 20°C. In contrast, a greater ($p < 0.06$) response in LR vs. HR fish was evident in seven liver stress-related transcripts following

injection with Forte Micro at 12°C. Nonetheless, these data do not provide conclusive evidence that the selection of a particular phenotype would have major implications for the health (e.g., resistance to pathogens and/or environmental stressors) of salmon at the two assessed temperatures (i.e., 12 and 20°C).

In Chapter 4 it was hypothesized that the inclusion of supplemental dietary cholesterol would improve the IT_{Max} of female triploid Atlantic salmon (Ignatz et al., 2022). Supplemental cholesterol was chosen as I thought it could aid in maintaining cell rigidity, reducing stress and the need to mobilize astaxanthin from muscle stores, and improve salmon growth and survival at high rearing temperatures (Chimsung et al., 2014, 2013; Farkas et al., 2001; Hazel, 1979; Irvine et al., 1957). Adding cholesterol to the diet did not affect the salmon's IT_{Max} or growth, but inclusion of +1.30 and 1.76% cholesterol did reduce fillet bleaching above 18°C (i.e., SalmoFan™ scores only significantly decreased at these temperatures in the control treatment). A key finding of this study was that ≤ 5% of the female triploid Atlantic salmon died, irrespective of diet, before temperature reached 22°C. As previous work has suggested that triploid salmonids are more susceptible to mortality at high temperatures than diploids (Hyndman et al., 2003; Ojolick et al., 1995), the fact that the IT_{Max} of triploid salmon was comparable to diploids in other studies (Bartlett et al., 2022; Gamperl et al., 2020) is promising. This is particularly relevant as the commercial production of female triploid Atlantic salmon is ongoing on the South coast of Newfoundland.

Chapter 5 tested whether family-based differences in IT_{Max} could be detected in Atlantic salmon (Ignatz et al., 2023). Previous work in Atlantic cod (*Gadus morhua*) did not find differences in IT_{Max} between families and suggested that genetic selection for this trait could prove difficult (Zanuzzo et al., 2019). However, I found a 1.7°C separation in average IT_{Max}

between the most and least thermally tolerant salmon families, although the CT_{Max} of the top four and bottom four temperature tolerant families (based on IT_{Max}) did not differ. These results further suggest that IT_{Max} provides a more accurate and meaningful metric of the Atlantic salmons' upper thermal tolerance, and that IT_{Max} experiments will be a more useful tool/metric of upper thermal tolerance for the aquaculture industry. Interestingly, hepatosomatic index (HSI) was negatively correlated with both IT_{Max} and CT_{Max}, and was the only phenotypic measurement assessed that significantly correlated with either measure of upper thermal tolerance. Overall, this study shows that IT_{Max} varies between families, and thus, the industry can likely select for this trait.

The final part of my thesis characterized the four paralogues of *serpinh1* [alias *heat shock protein 47* (*hsp47*)] in Atlantic salmon (Chapter 6; Ignatz et al., submitted #2). This gene is a sensitive biomarker of heat stress in salmonids (Akbarzadeh et al., 2018; Houde et al., 2019) and mammalian studies have also identified it as being essential for collagen structural assembly and integrity (Ito and Nagata, 2017; Nagata, 1996; Nagata et al., 1988). It is likely that *serpinh1* was duplicated during the teleost-specific whole genome duplication (WGD) event (i.e., producing *serpinh1a-1* and *serpinh1b-2*), and that these two copies were duplicated during the salmonid-specific WGD (i.e., producing *serpinh1a-2* and *serpinh1b-1*, respectively). This research provided evidence to support the hypothesis that *serpinh1a-2* has neofunctionalized. For example, there are unique sex-specific differences in the expression of *serpinh1a-2* in the gill, pyloric caecum, midgut and hindgut that are not evident in any other paralogue. There are also signs of subfunctionalization of *serpinh1a-1* and *serpinh1a-2* as they were both downregulated at elevated temperature (i.e., 20 vs. 10°C), in contrast to *serpinh1b-1* and *serpinh1b-2* which were upregulated at 20°C as expected for a heat shock protein. This gene is pleiotropic, in that all four

paralogues responded to elevated temperature and bacterial immune stimulation (i.e., Forte Micro injection). Overall, this study provides important insights into the regulation of a key heat stress biomarker, and demonstrates how multiple genome duplication events can lead to the evolutionary divergence of paralogues.

In summary, this thesis reports numerous novel findings pertaining to how farmed Atlantic salmon will respond to rising average ocean temperatures and current seasonal changes in temperature. While selecting for stress phenotype or providing supplemental dietary cholesterol may not help improve the survival of salmon in sea-cages during the warm summer months, family-based selection of upper thermal tolerance is a promising avenue for future study. The characterization and expression analyses of all four *serpinh1* paralogues in Atlantic salmon are also valuable as it improves our mechanistic understanding of how this species regulates thermal stress, and how multiple whole genome duplications can lead to differences in the regulation and potential function of paralogues in a salmonid. Collectively, this research will be of significant use to the Atlantic salmon aquaculture industry as it navigates the pervasive threat of accelerated climate change.

7.2 Future research

While this thesis greatly expands our understanding of the thermal biology of Atlantic salmon, much remains unknown regarding which factors are the most influential in regulating/determining this species' upper thermal tolerance. Family was shown to impact the IT_{Max} of salmon, thus genomic analyses [e.g., genome-wide association studies (GWAS); RNA-sequencing] could help elucidate why certain fish/families survive longer at elevated temperatures and whether IT_{Max} is heritable. In fact, expansion of Chapter 5 to address these

exact questions is ongoing (Ignatz et al., in prep. #1). A GWAS was conducted using fin clips from salmon that reached their IT_{Max} across all 20 families (n = 265) and the North American 50K single nucleotide polymorphism (SNP) chip. SNP- and pedigree-based heritability of IT_{Max} and thermal growth coefficient (TGC) were also assessed. RNA-seq analyses of liver samples (n = 5-6 $family^{-1}$ $temperature^{-1}$), collected from the 4 most and 4 least tolerant families at 10 and 20°C, were also used to provide insights into potential mechanisms modulating the Atlantic salmon's thermal tolerance. Real-time quantitative polymerase chain reaction (qPCR) validation of the RNA-seq results included further analysis of the four *serpinh1* paralogues. Co-localization analysis also found overlap between the GWAS and RNA-seq datasets, identifying several transcripts that could be included in a follow-up functional validation study. These results not only build directly on my existing work, but also provide additional value to the aquaculture sector. Importantly, identifying and validating genetic markers predictive of upper thermal tolerance would reduce the need to conduct the stressful, and ultimately lethal, IT_{Max} tests. This would be advantageous for the salmon aquaculture industry as these protocols are labour and time intensive, and limit the impacts of selection for this trait on fish welfare.

Another way to expand on the results of this thesis would be to study the impact of supplemental dietary cholesterol on the tissue composition and innate immune responses of female triploid Atlantic salmon (Ignatz et al., in prep. #2 & #3). Using the liver and white muscle samples collected from Chapter 4, total lipid class and fatty acid composition have been assessed between dietary treatments and at different temperatures. Notably, supplemental cholesterol impacted the amount of omega-3 fatty acids in both the liver and muscle, and correlation analysis between the tissue composition data and the expression of target genes related to lipid/fatty acid metabolism is underway. In addition, innate antibacterial and antiviral immune responses were

assessed between the dietary treatments at 18°C following injection with Forte Micro or polyriboinosinic polyribocytidylic acid (pIC; a double-stranded RNA analog that elicits a potent antiviral-like response). It was found that adding 1.30% cholesterol to the diet modulated immune-related gene expression both pre- and post-injection compared to salmon fed the control diet. Collectively, these data show that supplemental dietary cholesterol had more effects on salmon than elucidated in Chapter 4. In future work, it would be helpful to include full-sibling diploids to test alongside triploids to parse out dietary, temperature and ploidy effects. In addition, future studies should measure dietary cholesterol and phytosterols levels separately to help determine how that could impact the bioavailability of cholesterol.

No additional work (aside from Chapters 2 and 3) with the stress phenotypes was conducted or is actively planned. However, much is still unknown about LR and HR salmon, and consequently, there are several avenues for future research. In particular, Chapter 3 was limited to a single sampling time point at each temperature [i.e., 12 or 24 h post-injection (hpi) at 20 and 12°C, respectively]. If this experiment were to be repeated, it would be beneficial to include several more time points (e.g., 0, 1, 3, 6, 12, 18, 24 hpi), as well as to sample plasma for the measurement of cortisol and other stress hormones (e.g., catecholamines). Head kidney and liver tissue samples could also be analyzed by RNA-seq to give a more holistic view of how LR and HR salmon may differ in their immune responses and other biological processes. It would also be helpful to use pIC to study LR vs. HR antiviral responses at different temperatures. Live pathogen testing (e.g., bacterial, viral, parasitic) and/or co-infection trials (e.g., sea lice and *Renibacterium salmoninarum*) in a biocontainment facility would also provide more industrially relevant results, and survival data that could be collected/compared. Behavioural thermoregulation could also be assessed with these trials if LR and HR salmon were provided

access to a gradient of temperatures (e.g., 12-20°C) and their movements between temperature zones were monitored (Boltana et al., 2018; Haddad et al., 2023; Sanhueza et al., 2023). I hypothesize that proactive (i.e., LR) fish would be more likely to initiate a behavioural fever response by spending more time at high temperatures to combat an infection than reactive (i.e., HR) fish. It would also be interesting to determine at what temperature stress phenotypes can no longer be distinguished. In Chapter 2, post-stress cortisol levels between LR and HR salmon consistently differed by ~ 90 ng mL^{-1} at 9°C. However, this separation was not found when reassessed at 23.6°C after the first few mortalities were recorded during the IT_{Max} challenge. A study could be conducted using the same rate of temperature increase (+0.2°C day^{-1}) where net stress samplings of LR and HR salmon are performed at every +1°C. I hypothesize that post-stress cortisol levels would remain distinct between stress phenotypes until the fish stop feeding (i.e., ~22°C). I believe this point would mark the general takeover of chronic thermal stress, and that this would alter responses to additional acute stressors. Chronic stress reduces feed intake in Atlantic salmon and alters the regulation of the hypothalamic-pituitary-interrenal (HPI) axis resulting in changes to cortisol production (Madaro et al., 2015). While no differences in upper thermal tolerance were detected between stress phenotypes, it will still be important to further study LR and HR salmon as a particular phenotype may potentially influence other production traits (e.g., long-term growth, disease resistance, fillet quality).

If time and resources were available, another interesting follow up to my thesis would be to run a large-scale experiment that tested whether sex and sexual maturity influence the upper thermal tolerance of Atlantic salmon. This work would use related (full- or half-sibling) diploid salmon, and measure IT_{Max} across several life stages (e.g., fry, parr, smolt, pre-adult and adult). This would involve using subsets from the base population at each of these assessment points (as

the protocol is lethal). Blood samples could be taken to sex the fish via PCR (King and Stevens, 2020) and passive integrated transponder (PIT) tags could be inserted for individual identification of fish at the parr stage. If a fish were large enough when it succumbs to high temperature, gonads would be scored on a standardized basis for sexual maturity and samples would be taken for histology. Other morphometric data would also be collected (e.g., fish weight, fish length, ventricle weight, liver weight). The design of the IT_{Max} challenge itself could also be improved for this study. While a gradual average increase in temperature (i.e., +0.2°C day^{-1}) could still be achieved, it would be more realistic to use a diurnal cycle where temperature fluctuates slightly (e.g., ± 2°C) throughout the day (Gamperl et al., 2021; Gollock et al., 2006). This fluctuation could increase at temperatures >20°C (e.g., to ± 4°C) to simulate a heatwave as well. These experimental changes would better reflect the natural conditions that these fish experience. Periodic blood sampling of random fish throughout the experiment could also be used to quantify circulating sex hormones (e.g., testosterone, estrogen). Altogether, such a study would determine whether sexual maturity influences sex-based survival at high temperatures in Atlantic salmon, and could also further examine HSI's relationship to IT_{Max}.

A myriad of other studies could be designed to improve or expand upon my thesis. While that list is too long to include here, a final example would be to use CRISPR-Cas9 gene editing to further research the function of each *serpinh1* paralogue. Individual gene knockouts could be used to test whether each paralogue is necessary for survival, like *serpinh1* is in mice (Nagai et al., 2000). In contrast, alterations could be made in the promoter region of each paralogue to induce changes in expression (Cleveland et al., 2018). Salmon with altered genotypes could then be challenged in IT_{Max} or CT_{Max} experiments to determine if increases or decreases in expression alter the Atlantic salmon's upper thermal tolerance. Certainly, there is ample opportunity for

future research to build upon the data collected in this thesis. In fact, additional studies, and information, will be required before the Atlantic salmon aquaculture industry can sustainably maintain and/or expand production volumes in the climate-vulnerable North Atlantic.

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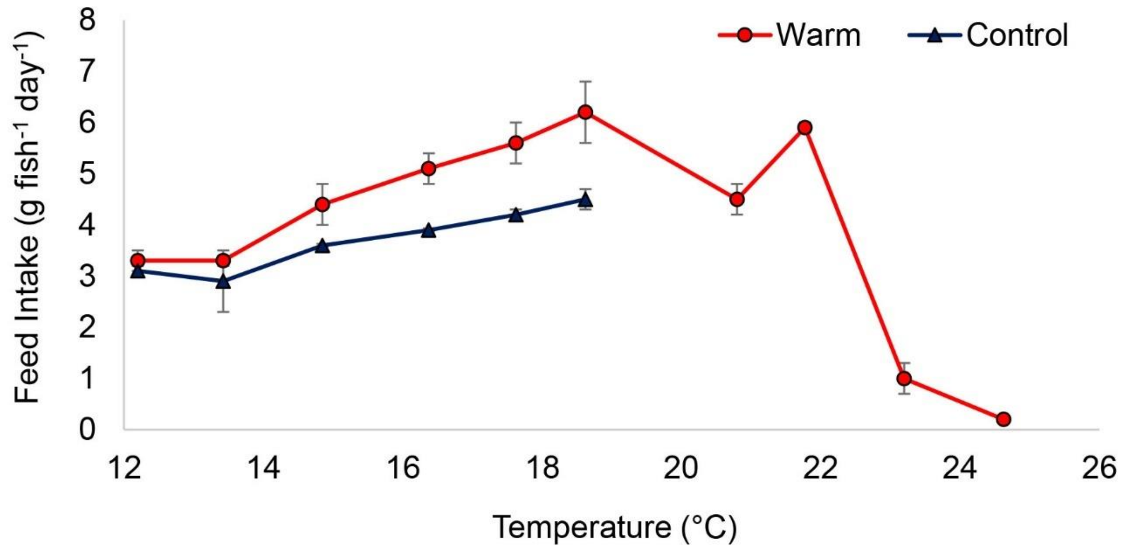
APPENDICES

Appendix A Table A-1. Family cross structure of fish used in the phenotypic stress response study.

Dam ID:	Sire ID:	Family ID:
D1	S1	F1
D2	S2	F2
D3	S2	F3
D4	S3	F4
D5	S3	F5
D6	S4	F6
D7	S5	F7
D8	S6	F8
D9	S7	F9
D10	S8	F10
D11	S10	F11
D12	S10	F12
D13	S11	F13
D14	S11	F14
D15	S12	F15
D16	S12	F16
D17	S1	F17



Appendix A Figure A-1. Feed intake of fish exposed to an incremental temperature increase (warm treatment) and of fish held at a constant 12°C (control). Feed intake data were collected in the control treatment only until the 20°C assessment was completed. Error bars represent standard errors. No statistics could be performed due to lack of replication in the control group (n=2).

Appendix B Table B-1. Fold-change comparisons between head kidney cDNA pools assessed during the preliminary qPCR assays on sample pools.

	alox12	*c3a*	*campb*	*ccl19b*	*ch25ha*	*cox2*	*ctsla*	*cxcr1*	*cyp11a*	*cyp11b*	*cyp1a1a*	*cyp1a1b*	*ftm*	*hampa*	*hsd3b*	*il1b*	*irf1a*	*junba*	*lect2a*	*mmp9a*	*pgds*	*saa5*	*stara*	*starb*	*stlr5a*	*wap65a*
12°C - HR PBS / LR PBS	1.1	0.7	1.5	0.3	0.9	1.0	1.0	0.6	0.9	1.0	1.8	1.5	1.0	2.0	1.1	1.0	0.9	1.1	0.9	0.9	1.4	0.8	2.7	1.0	0.6	1.2
20°C - HR PBS / LR PBS	0.9	2.1	1.1	1.3	0.7	0.7	0.9	0.5	1.0	1.1	0.3	0.9	0.9	0.9	0.8	0.7	1.0	0.8	0.9	1.1	0.8	1.1	0.8	0.8	0.8	7.4
12°C - HR FM / LR FM	1.1	1.0	1.2	0.5	1.2	1.0	1.0	2.2	0.8	0.9	1.1	0.9	1.2	1.3	1.2	1.1	1.2	0.9	0.8	1.3	1.2	1.4	1.1	1.2	1.1	0.9
20°C - HR FM / LR FM	1.2	3.1	1.7	1.3	1.8	1.7	1.2	1.3	1.1	1.4	3.4	1.2	1.3	2.3	1.2	1.4	1.5	1.2	1.5	1.6	1.2	1.1	0.4	1.3	1.8	58.2
12°C - HR FM / HR PBS	0.8	7.7	11.3	2.6	6.4	10.0	1.3	0.2	0.3	0.3	1.0	0.6	1.3	24.7	0.4	3.4	1.1	1.5	0.8	1.4	0.6	14.1	0.2	0.4	6.1	1.2
12°C - LR FM / LR PBS	0.8	5.6	13.4	1.6	4.7	9.8	1.3	0.1	0.3	0.3	1.7	1.0	1.0	37.6	0.3	3.1	0.8	1.9	0.9	1.0	0.6	7.5	0.6	0.3	3.5	1.4
20°C - HR FM / HR PBS	1.1	6.1	37.3	1.6	4.4	8.3	1.8	0.2	0.4	0.4	3.0	0.6	1.2	11.9	0.5	3.6	1.4	1.8	1.7	1.6	0.8	30.3	0.5	0.5	4.8	3.8
20°C - LR FM / LR PBS	0.8	4.0	24.4	1.6	1.7	3.4	1.3	0.1	0.4	0.3	0.2	0.4	0.8	4.4	0.4	1.7	0.9	1.4	1.1	1.2	0.6	32.8	1.0	0.3	2.2	0.5

Appendix B Table B-2. Fold-change comparisons between liver cDNA pools assessed during the preliminary qPCR assays on sample pools.

	campb	*ch25ha*	*cirbp*	*f2*	*ftm*	*ghra*	*ghrb*	*gra*	*grb*	*hpx*	*hsp70*	*hsp90aa1*	*hsp90ab1*	*igf1*	*ndufa1*	*pgds*	*serpinh1*	*ucp2*
12°C - HR PBS / LR PBS	0.9	0.4	1.0	1.0	1.0	0.8	1.0	0.8	0.8	1.0	0.9	0.6	0.8	1.3	1.2	1.3	2.7	1.2
20°C - HR PBS / LR PBS	2.1	1.5	2.3	1.2	1.2	2.4	1.7	1.2	1.6	1.5	1.2	0.8	1.0	1.5	1.2	1.5	0.6	1.1
12°C - HR FM / LR FM	0.5	0.7	0.5	0.6	0.6	0.4	0.6	0.7	0.7	1.0	0.5	0.3	0.6	0.6	0.4	0.6	0.5	0.4
20°C - HR FM / LR FM	2.0	2.1	2.1	1.5	1.5	0.8	1.1	1.0	1.3	1.0	1.1	1.3	1.4	1.6	1.6	1.2	1.0	1.3
12°C - HR FM / HR PBS	95.7	14.2	0.4	0.6	0.6	0.5	0.5	0.4	0.3	0.6	1.3	23.6	0.7	0.2	0.5	0.4	1.1	0.8
12°C - LR FM / LR PBS	172.2	8.5	0.7	0.9	1.0	0.9	0.8	0.4	0.3	0.6	2.2	50.3	1.0	0.5	1.3	0.8	5.8	2.4
20°C - HR FM / HR PBS	283.9	20.9	0.5	1.2	1.8	2.8	1.2	0.6	0.5	0.8	4.9	35.8	1.5	1.0	1.5	1.3	4.7	6.1
20°C - LR FM / LR PBS	299.4	14.5	0.6	1.0	1.4	8.1	1.9	0.7	0.6	1.3	5.4	22.5	1.0	0.9	1.2	1.5	2.8	5.2

Appendix B Table B-3. Primers used for preliminary qPCR testing using sample pools, but ultimately not chosen for the main study.

Gene name (GenBank accession number)	Nucleotide sequence (5'-3')	Amplicon size (bp)	Source
Head Kidney Gene List			
arachidonate 12-lipoxygenase, 12S-type (*alox12*) (BT072280)	F: CTCGCTCACTTGCTTCTCCT	155	Xue et al. (2021)
	R: CCCAATCAAAGGACCAGAGA		
CC chemokine 19 b (*ccl19b*) (CO471983)	F: GTGGGTGATCCCTGAACA	140	This study[a]
	R: TGCATCCACCCTACAATGAG		
cathepsin L1a (*ctsla*) (BT057383)	F: GTTCAGGAAGACTGGCAAGC	130	This study[a]
	R: GGCGTTGTCCTGGATGTACT		
cytochrome P450 1A1a (*cyp1a1a*) (BT045666)	F: AGGTGGGAATGACTCGTACTC	136	Beemelmanns et al. (2021)
	R: GATGTATCCTTGACTGTGCAGT		
cytochrome P450 1A1b (*cyp1a1b*) (AF361643)	F: AGGTGGGATTGAATCGCACTC	136	This study[a]
	R: GATGTATCCTTGATCGTGCAGT		
ferritin, middle subunit (*ftm*) (BT045310)	F: TGCACAAGATTGCCTCTGAC	107	Xue et al. (2021)
	R: GATGTGGTCACCCAGCTTCT		
interferon regulatory factor 1a (*irf1a*) (BT048538)	F: GCAATGAAGTAGGCACAGCA	100	Caballero-Solares et al. (2017)
	R: CGCAGCTCTATTTCCGTTTC		
transcription factor jun-B a (*junba*) (BT044843)	F: CCGAGAACTATCGGAACCAA	129	This study[a]
	R: GGATGATCAATCGCTCCAGT		
leukocyte cell derived chemotaxin 2a (*lect2a*) (BT059281)	F: CAGATGGGGACAAGGACACT	150	Smith et al. (2018)
	R: GCCTTCTTCGGGTCTGTGTA		
matrix metalloproteinase-9a (*mmp9a*) (BT045896)	F: CCATGTACCAACGGTAGAATCAG	127	Umasuthan et al. (2020)
	R: GGTTATATCTCTCTGGACCCTAGTC		
warm temperature acclimation protein a (*wap65a*) (XM_014174610)	F: CTGTGTCTGTGCTTGGTGCT	82	This study
	R: CCTCATGTCCCTCATGTCCT		
60S ribosomal protein 32 (*rpl32*) (BT043656)[b]	F: AGGCGGTTTAAGGGTCAGAT	119	Xue et al. (2015)
	R: TCGAGCTCCTTGATGTTGTG		

Liver Gene List			
prothrombin (*f2*) (EG773276)	F: GGCTTCAAACCAGAGGAACA R: TCCCTGTCACATCCTTCTCC	137	Emam et al. (2022)
ferritin, middle subunit (*ftm*) (BT045310)	F: TGCACAAGATTGCCTCTGAC R: GATGTGGTCACCCAGCTTCT	107	Xue et al. (2021)
hemopexin-like (*hpx*) (CK896897)	F: GTGGATGCCGTCTTCTCCTA R: AGCACCTCCTTCAAGGGTTT	125	Emam et al. (2022)
insulin-like growth factor (*igf1*) (EF432852)	F: CCTGTTCGCTAAATCTCACTTC R: TACAGCACATCGCACTCTTGA	226	Bower & Johnston (2010)
NADH dehydrogenase 1 alpha subcomplex subunit 1 (*ndufa1*) (BT046880)	F: TGATGGAGAGAGACAGACGAGT R: AGGTGAGATCTGGGATTAGTGGA	89	Beemelmanns et al. (2021)
60S ribosomal protein 32 (*rpl32*) (BT043656)[b]	F: AGGCGGTTTAAGGGTCAGAT R: TCGAGCTCCTTGATGTTGTG	119	Xue et al. (2015)

[a] Primers were designed as part of two Genomic Applications Partnership Program projects [GAPP #6604, Biomarker Platform for Commercial Aquaculture Feed Development project; and GAPP #6607, Integrated Pathogen Management of Co-infection in Atlantic salmon (IPMC) project] awarded to MLR. These projects were funded by the Government of Canada through Genome Canada and Genome Atlantic, and EWOS Innovation (now part of Cargill, Incorporated). The IPMC project was also funded by the Government of Newfoundland and Labrador's Department of Tourism, Culture, Industry and Innovation (Leverage R&D award #5401-1019-108). These primers were designed by Drs. Jennifer R. Hall and Albert Caballero-Solares.
[b] Normalizer gene

Appendix B Table B-4. Head kidney and liver gene expression statistical results compared between stress phenotypes. The table continues through pages 327-331.

			Time Zero									
			Two-Way ANOVA						*Tukey's HSD Post-Hoc*			
			Phenotype		Temperature		Phenotype:Temperature		LR vs. HR - 12°C	LR vs. HR - 20°C	LR - 12°C vs. LR - 20°C	HR - 12°C vs. HR - 20°C
			F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
Head Kidney	Fig. 3-2A	*stara*	0.001	0.984	0.109	0.744	0.018	0.894	-	-	-	-
	Fig. 3-2B	*starb*	2.036	0.165	0.004	0.953	0.981	0.331	-	-	-	-
	Fig. 3-2C	*cyp11a*	0.408	0.528	0.166	0.687	1.735	0.199	-	-	-	-
	Fig. 3-2D	*cyp11b*	1.098	0.304	0.009	0.924	0.686	0.415	-	-	-	-
	Fig. 3-2E	*hsd3b*	1.150	0.293	0.001	0.987	0.418	0.524	-	-	-	-
	Fig. 3-3A	*stlr5a*	0.017	0.898	36.178	**2.03E-06**	1.960	0.173	0.626	0.864	**0.019**	**7.69E-05**
	Fig. 3-3B	*cxcr1*	0.553	0.463	1.316	0.261	2.217	0.148	-	-	-	-
	Fig. 3-3C	*campb*	0.781	0.385	9.602	**0.005**	0.026	0.873	0.969	0.911	0.200	0.114
	Fig. 3-3D	*hampa*	0.497	0.487	0.600	0.445	0.235	0.632	-	-	-	-
	Fig. 3-3E	*ch25ha*	1.014	0.323	1.520	0.228	0.159	0.693	-	-	-	-
	Fig. 3-4A	*il1b*	0.634	0.433	0.045	0.833	1.260	0.272	-	-	-	-
	Fig. 3-4B	*saa5*	2.724	0.110	0.504	0.484	0.949	0.339	-	-	-	-
	Fig. 3-4C	*c3a*	0.231	0.635	13.165	**0.001**	0.620	0.438	0.851	0.989	**0.023**	0.191
	Fig. 3-4D	*cox2*	0.239	0.629	1.509	0.230	0.333	0.569	-	-	-	-
	Fig. 3-4E	*pgds*	1.314	0.262	2.026	0.166	0.942	0.340	-	-	-	-
Liver	Fig. 3-6A	*gra*	1.828	0.188	0.674	0.419	0.006	0.940	-	-	-	-
	Fig. 3-6B	*grb*	0.476	0.496	15.485	**0.001**	0.403	0.531	0.834	0.999	**0.018**	0.103
	Fig. 3-6C	*cirbp*	0.020	0.888	3.576	0.069	1.125	0.298	-	-	-	-
	Fig. 3-6D	*hsp70*	0.099	0.755	131.005	**7.26E-12**	0.114	0.738	0.998	0.991	**1.00E-08**	**1.00E-07**
	Fig. 3-6E	*hsp90aa1*	3.048	0.092	216.136	**2.09E-14**	0.402	0.531	0.545	0.975	**1.00E-08**	**1.00E-08**
	Fig. 3-6F	*serpinh1*	1.458	0.238	187.888	**1.12E-13**	0.162	0.690	0.993	0.849	**1.00E-08**	**1.00E-08**
	Fig. 3-6G	*ucp2*	0.245	0.625	251.107	**3.38E-15**	2.671	0.114	0.686	0.633	**1.00E-08**	**1.00E-08**
	Fig. 3-7A	*ghra*	5.075	0.033	0.292	0.593	2.419	0.132	0.945	0.055	0.455	0.899
	Fig. 3-7B	*ghrb*	0.002	0.968	30.088	**8.30E-06**	0.665	0.422	0.886	0.973	**0.016**	**0.001**
	Fig. 3-7C	*campb*	0.001	0.990	3.502	0.072	0.850	0.365	-	-	-	-
	Fig. 3-7D	*ch25ha*	0.133	0.719	0.115	0.737	0.375	0.546	-	-	-	-
	Fig. 3-7E	*pgds*	2.361	0.136	9.369	**0.005**	0.886	0.355	0.981	0.357	0.481	**0.039**

12°C (24 hpi) - PBS vs. Forte Micro Injection										
Two-Way ANOVA						*Tukey's HSD Post-Hoc*				
Phenotype		Injection		Phenotype:Injection		PBS LR vs. HR	Forte Micro LR vs. HR	LR - PBS vs. LR - Forte Micro	HR - PBS vs. HR - Forte Micro
F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
0.331	0.569	0.440	0.512	0.459	0.503	-	-	-	-
0.006	0.937	0.190	0.666	0.001	0.972	-	-	-	-
0.486	0.492	2.280	0.142	0.341	0.564	-	-	-	-
0.002	0.966	0.036	0.850	0.012	0.912	-	-	-	-
0.144	0.707	0.979	0.331	0.014	0.907	-	-	-	-
0.004	0.949	22.913	**4.97E-05**	2.564	0.121	0.646	0.700	0.134	**0.001**
3.016	0.094	32.146	**5.07E-06**	2.268	0.144	0.986	0.097	**1.60E-04**	**0.026**
0.006	0.938	37.356	**1.35E-06**	0.014	0.906	0.999	0.999	**0.001**	**0.001**
0.080	0.780	54.816	**4.61E-08**	0.055	0.817	0.999	0.983	**5.25E-05**	**1.29E-04**
0.002	0.967	10.683	**0.003**	0.442	0.512	0.971	0.959	0.280	**0.045**
0.518	0.478	13.680	**0.001**	0.023	0.880	0.978	0.926	0.081	0.051
0.007	0.933	38.653	**1.02E-06**	0.062	0.805	0.995	0.999	**0.001**	**0.001**
0.005	0.943	8.281	**0.008**	0.269	0.608	0.988	0.975	0.359	0.099
0.089	0.768	53.159	**6.15E-08**	0.252	0.620	0.999	0.941	**2.66E-04**	**3.91E-05**
1.366	0.252	2.778	0.107	0.255	0.617	-	-	-	-
0.559	0.461	17.769	**2.35E-04**	0.015	0.902	0.926	0.971	**0.035**	**0.023**
2.777	0.107	53.873	**5.42E-08**	1.609	0.215	0.992	0.186	**8.30E-06**	**0.001**
0.576	0.454	5.638	**0.025**	0.150	0.701	0.994	0.849	0.230	0.507
1.006	0.325	27.837	**1.30E-05**	0.017	0.898	0.926	0.854	**0.004**	**0.006**
1.030	0.319	71.008	**3.65E-09**	0.452	0.507	0.995	0.636	**3.30E-06**	**4.20E-05**
0.037	0.848	8.243	**0.008**	0.138	0.713	0.978	0.999	0.124	0.310
0.001	0.996	23.925	**3.73E-05**	4.075	0.053	0.491	0.496	**2.11E-04**	0.201
3.547	0.070	0.484	0.493	0.003	0.957	-	-	-	-
0.020	0.887	0.677	0.418	0.017	0.898	-	-	-	-
0.147	0.705	99.481	**1.02E-10**	0.698	0.411	0.988	0.824	**1.00E-07**	**3.10E-06**
0.062	0.805	54.717	**4.69E-08**	0.023	0.881	0.999	0.992	**6.23E-05**	**1.11E-04**
0.153	0.699	0.171	0.682	0.001	0.998	-	-	-	-

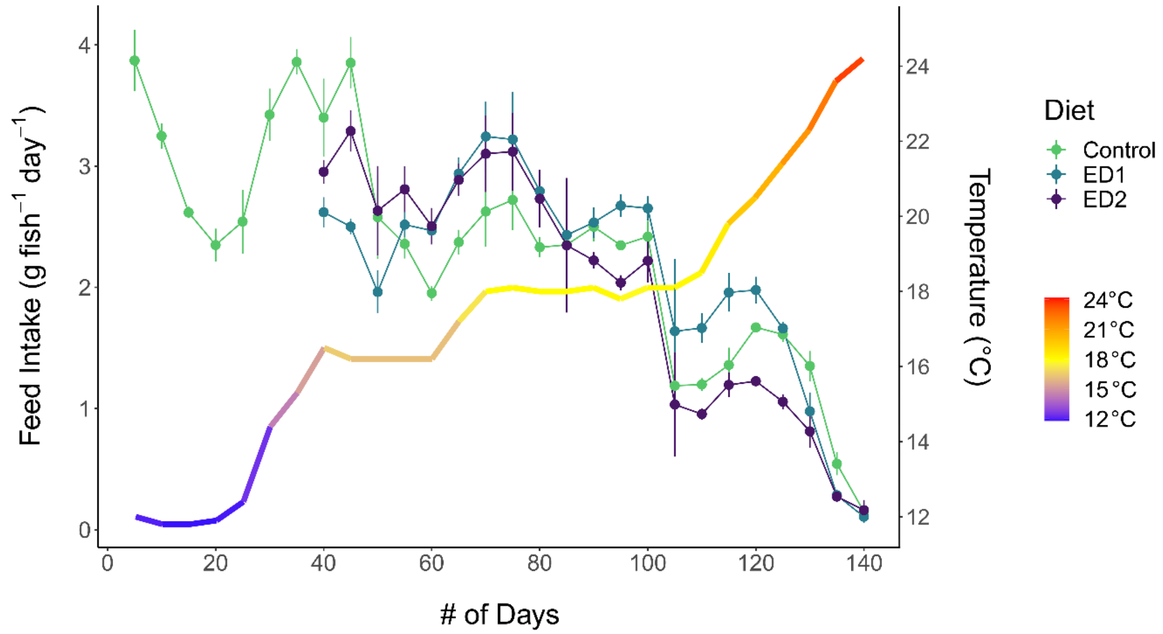
20°C (12 hpi) - PBS vs. Forte Micro Injection									
Two-Way ANOVA						*Tukey's HSD Post-Hoc*			
Phenotype		Injection		Phenotype:Injection		PBS LR vs. HR	Forte Micro LR vs. HR	LR - PBS vs. LR - Forte Micro	HR - PBS vs. HR - Forte Micro
F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
0.147	0.704	0.385	0.541	0.799	0.379	-	-	-	-
3.704	0.065	2.340	0.138	0.242	0.629	-	-	-	-
0.001	0.996	3.033	0.093	0.645	0.429	-	-	-	-
2.454	0.129	3.450	0.075	0.125	0.727	-	-	-	-
1.481	0.235	3.876	0.060	0.052	0.821	-	-	-	-
0.056	0.815	55.976	**6.07E-08**	3.481	0.073	0.832	0.281	**0.005**	**2.10E-06**
0.002	0.962	57.237	**4.96E-08**	1.260	0.272	0.658	0.978	**1.54E-05**	**3.24E-04**
0.471	0.499	153.182	**2.11E-12**	0.236	0.631	0.996	0.964	**1.00E-07**	**1.00E-07**
0.001	0.975	70.940	**6.65E-09**	1.317	0.262	0.976	0.605	**2.56E-04**	**1.30E-06**
0.142	0.709	26.229	**2.44E-05**	3.142	0.088	0.566	0.634	0.159	**2.06E-04**
0.590	0.449	24.118	**4.25E-05**	2.588	0.120	0.474	0.859	0.163	**4.12E-04**
0.959	0.336	125.259	**1.93E-11**	0.609	0.442	0.900	0.978	**1.10E-06**	**1.00E-08**
0.129	0.722	5.249	**0.030**	0.032	0.859	0.961	0.994	0.350	0.419
0.484	0.493	46.033	**3.36E-07**	0.320	0.576	0.980	0.602	**0.002**	**7.26E-05**
0.101	0.754	4.942	**0.035**	0.004	0.950	0.982	0.990	0.420	0.403
0.130	0.721	106.491	1.10E-10	0.759	0.392	0.831	0.980	**1.00E-07**	**2.50E-06**
0.119	0.733	83.389	**1.36E-09**	0.738	0.398	0.856	0.975	**1.00E-06**	**2.06E-05**
0.001	0.978	4.039	0.055	1.276	0.269	-	-	-	-
3.747	0.064	72.257	**5.57E-09**	0.202	0.657	0.581	0.916	**3.09E-05**	**6.10E-06**
2.054	0.164	126.462	**1.74E-11**	0.099	0.755	0.894	0.994	**2.00E-07**	**1.00E-07**
1.535	0.226	9.891	**0.004**	0.008	0.930	0.859	0.910	0.161	0.127
3.230	0.084	89.288	**6.81E-10**	0.196	0.661	0.671	0.956	**5.50E-06**	**1.10E-06**
0.023	0.881	24.544	**3.79E-05**	0.334	0.568	0.950	0.992	**0.003**	**0.023**
0.367	0.550	5.419	**0.028**	0.286	0.597	0.999	0.898	0.205	0.591
0.408	0.529	312.441	**5.22E-16**	0.577	0.454	0.999	0.794	**1.00E-08**	**1.00E-08**
0.740	0.397	221.200	**3.15E-14**	2.410	0.133	0.997	0.101	**1.00E-08**	**1.00E-08**
0.226	0.638	0.067	0.798	0.154	0.698	-	-	-	-

12°C - Time Zero vs. PBS Injection							
Two-Way ANOVA						*Tukey's HSD Post-Hoc*	
Phenotype		Injection		Phenotype:Injection		LR - Time Zero vs. LR - PBS	HR - Time Zero vs. HR - PBS
F value	*p*	F value	*p*	F value	*p*	*p*	*p*
0.359	0.554	4.963	**0.034**	0.491	0.489	0.704	0.187
0.082	0.777	0.956	0.337	0.026	0.873	-	-
0.099	0.755	0.392	0.536	0.203	0.656	-	-
0.034	0.856	0.989	0.328	0.001	0.971	-	-
0.207	0.653	0.259	0.615	0.001	0.984	-	-
2.438	0.130	5.320	**0.029**	0.139	0.712	0.253	0.529
0.007	0.933	12.319	**0.002**	0.414	0.525	**0.031**	0.203
0.002	0.963	12.446	**0.002**	0.086	0.772	0.125	0.053
1.379	0.250	20.794	**9.22E-05**	1.637	0.211	**0.002**	0.118
0.022	0.883	6.888	**0.014**	0.948	0.338	0.652	0.075
0.024	0.878	2.818	0.104	0.303	0.587	-	-
0.223	0.640	13.058	**0.001**	0.001	0.988	0.075	0.071
0.209	0.651	0.086	0.772	1.365	0.253	-	-
0.694	0.412	0.032	0.859	0.267	0.609	-	-
0.389	0.538	0.796	0.380	0.751	0.393	-	-
1.564	0.221	29.885	**7.76E-06**	0.013	0.911	0.004	0.003
0.028	0.868	56.436	**3.50E-08**	0.404	0.530	**1.99E-05**	**2.25E-04**
0.756	0.392	2.096	0.159	0.329	0.571	-	-
0.193	0.664	146.238	**1.24E-12**	0.454	0.506	**1.00E-08**	**1.00E-07**
0.079	0.780	6.847	**0.014**	1.651	0.209	**0.047**	0.783
0.196	0.661	4.744	**0.038**	0.065	0.800	0.534	0.332
0.113	0.740	4.019	0.055	3.852	0.056	-	-
1.334	0.258	4.163	0.051	0.357	0.555	-	-
0.219	0.644	0.488	0.490	0.239	0.629	-	-
0.554	0.463	1.673	0.206	0.016	0.899	-	-
0.025	0.876	0.498	0.486	0.001	0.983	-	-
0.001	0.990	15.876	**4.38E-04**	0.172	0.681	**0.021**	0.078

20°C - Time Zero vs. PBS Injection								**Fold Change - PBS vs. Forte Micro Injection**	
Two-Way ANOVA						*Tukey's HSD Post-Hoc*		*T-Test*	
Phenotype		Injection		Phenotype:Injection		LR - Time Zero vs. LR - PBS	HR - Time Zero vs. HR - PBS	12°C - LR vs. HR	20°C - LR vs. HR
F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
0.121	0.731	1.977	0.172	0.062	0.805	-	-	**0.036**	0.336
2.153	0.155	0.677	0.418	0.852	0.365	-	-	0.585	0.644
1.170	0.290	0.137	0.715	0.853	0.365	-	-	0.947	0.924
1.473	0.236	2.955	0.098	0.802	0.379	-	-	0.477	0.725
1.152	0.293	1.058	0.314	0.877	0.358	-	-	0.790	0.594
0.164	0.689	13.080	**0.001**	2.222	0.149	**0.007**	0.418	**0.010**	0.072
0.860	0.363	5.404	**0.029**	0.996	0.328	0.805	0.111	0.285	0.076
3.913	0.059	25.932	**2.94E-05**	0.786	0.384	**0.002**	**0.026**	0.261	0.476
0.044	0.835	12.927	**0.001**	0.066	0.800	0.057	0.102	0.989	0.134
0.002	0.968	0.046	0.831	1.654	0.210	-	-	0.253	**0.006**
0.001	0.988	4.957	**0.035**	3.484	0.074	**0.037**	0.990	0.680	**0.009**
4.374	**0.047**	8.230	**0.008**	0.048	0.829	0.279	0.147	0.426	0.056
0.493	0.489	3.811	0.062	0.144	0.708	-	-	0.411	0.698
0.047	0.831	0.516	0.479	0.033	0.858	-	-	0.123	0.982
1.034	0.319	1.339	0.258	0.835	0.370	-	-	0.696	0.940
0.003	0.957	11.946	**0.002**	1.374	0.252	0.449	**0.013**	**0.005**	0.275
0.647	0.429	20.742	**1.09E-04**	0.787	0.383	0.090	**0.003**	0.089	0.309
1.031	0.319	2.245	0.146	0.007	0.933	-	-	0.491	0.148
0.901	0.351	21.863	**7.91E-05**	0.395	0.535	**0.006**	**0.029**	0.974	0.264
0.284	0.599	5.425	**0.028**	0.841	0.368	0.134	0.708	0.398	0.252
0.065	0.801	4.841	**0.037**	1.581	0.220	0.098	0.877	0.129	0.166
0.005	0.942	3.806	0.062	3.837	0.061	-	-	**0.016**	0.611
0.938	0.342	3.936	0.058	2.978	0.096	-	-	0.626	0.174
0.099	0.755	0.286	0.598	0.047	0.830	-	-	0.994	0.523
0.501	0.486	1.734	0.199	0.204	0.655	-	-	0.268	0.408
0.407	0.529	1.481	0.235	0.135	0.716	-	-	0.162	**0.006**
0.765	0.390	2.407	0.133	0.866	0.361	-	-	0.910	0.362

Appendix B Table B-5. Permutational multivariate analysis of variance analysis (PERMANOVA) results obtained by comparing fold-change data across various transcripts and sampling time points between stress phenotypes. The *p*-values shown indicate whether a difference was found between LR and HR salmon; $p < 0.05$ indicating statistically significant differences, whereas $0.05 < p < 0.10$ indicates those that were suggestive of a difference. PERMANOVA results are given as *p*(perm), and scores on principal component axis 1 (PC1) and 2 (PC2) were compared using t-tests. The *p*-values for these analyses are also given.

Comparison	PERMANOVA $p_{(perm)}$	PC1 *p*-value	PC2 *p*-value
Head Kidney - All Transcripts at 24 hpi	0.268	-	-
Head Kidney - All Transcripts at 12 hpi	0.021	0.116	0.264
Head Kidney - 10 Immune-Related Transcripts at 24 hpi	0.266	-	-
Head Kidney - 10 Immune-Related Transcripts at 12 hpi	0.025	0.044	0.124
Head Kidney - 5 Corticosteroid Synthesis-Related Transcripts at 24 hpi	0.231	-	-
Head Kidney - 5 Corticosteroid Synthesis-Related Transcripts at 12 hpi	0.111	-	-
Liver - All Transcripts at 24 hpi	0.172	-	-
Liver - All Transcripts at 12 hpi	0.283	-	-
Liver - 7 Stress-Related and 3 Immune-Related Transcripts at 24 hpi	0.113	-	-
Liver - 7 Stress-Related and 3 Immune-Related Transcripts at 12 hpi	0.230	-	-
Liver - 7 Stress-Related Transcripts at 24 hpi	0.051	0.057	0.705
Liver - 7 Stress-Related Transcripts at 12 hpi	0.461	-	-



Appendix C Figure C-1. Feed intake (left y-axis, points connected by lines) of female triploid Atlantic salmon exposed to an incremental temperature increase (right y-axis, gradient line) and fed either the control diet, experimental diet #1 (ED1) or experimental diet #2. Points on the graph indicate the mean feed intake (± standard error) for each dietary treatment over the previous 5 days.

Appendix D Table D-1. Morphometrics and growth rates of fish organized by treatment and sex following the assessment that was performed as the warm treatment reached 20°C.

	Control		**Warm**	
	Male (n = 63)	**Female (n = 130)**	**Male (n = 54)**	**Female (n = 105)**
Weight (g)	406.7 ± 20.1	435.7 ± 15.2	463.5 ± 24.7	459.7 ± 16.4
Length (cm)	31.7 ± 0.4	32.5 ± 0.3	32.3 ± 0.5	32.7 ± 0.3
K	1.23 ± 0.02^{A}	1.21 ± 0.01^{A}	1.30 ± 0.02^{B}	1.27 ± 0.01^{B}
Weight Gain (g)	83.0 ± 8.2^{A}	80.3 ± 6.5^{A}	133.4 ± 11.2^{B}	123.9 ± 7.7^{B}
TGC [$g^{1/3}$ (°C d)$^{-1}$]	1.47 ± 0.13	1.34 ± 0.08	1.40 ± 0.10	1.32 ± 0.06
SGR (% body weight day^{-1})	0.61 ± 0.05^{A}	0.55 ± 0.03^{A}	0.92 ± 0.03^{B}	0.87 ± 0.04^{B}
HSI (%)	0.97 ± 0.03	0.94 ± 0.02	1.00 ± 0.03	0.94 ± 0.02

No significant differences ($p > 0.05$) between sexes within the same treatment were found. Upper case letters denote significant differences ($p < 0.05$) between treatments within the same sex. K, Fulton's condition factor; TGC, thermal growth coefficient; SGR, specific growth rate. Note: the control group was held at a constant temperature of 10°C.

Appendix D Table D-2. Comparison of incremental upper thermal tolerance (IT_{Max}), and morphometric data at IT_{Max}, for male (n = 126) and female (n = 138) Atlantic salmon (irrespective of family).

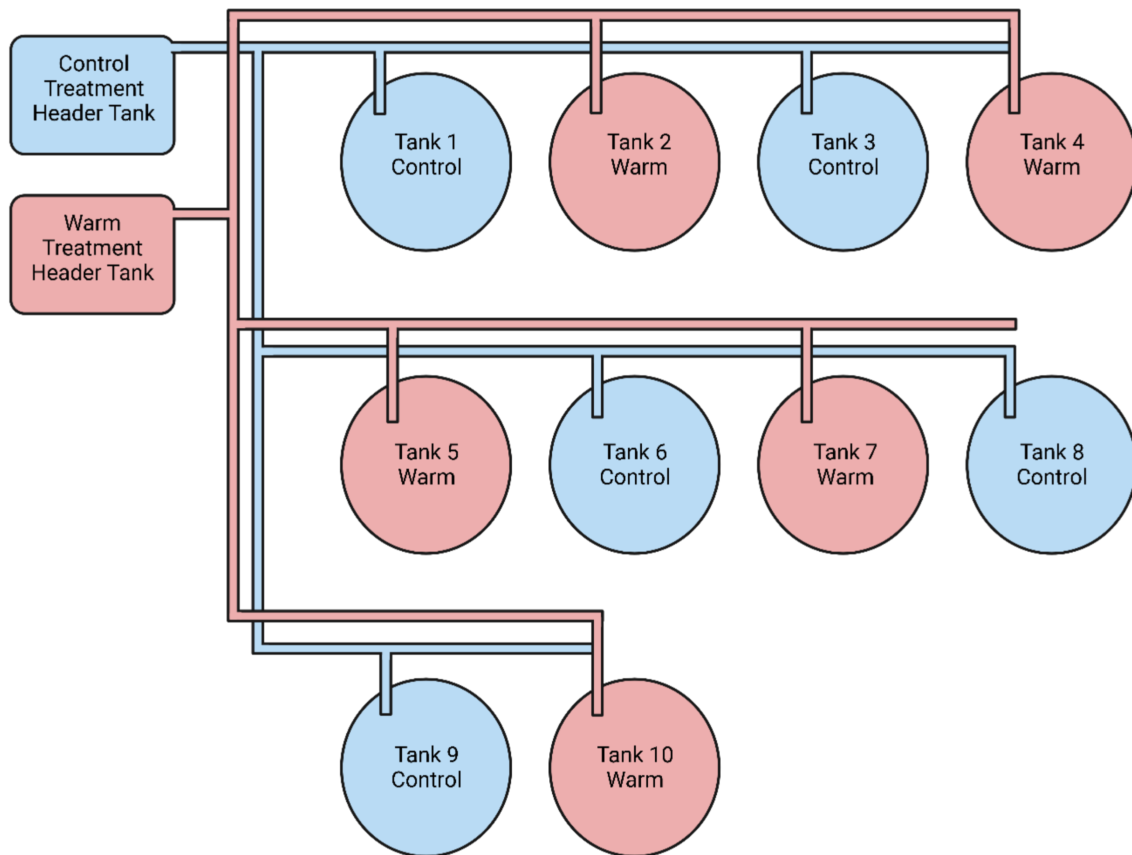
	Male	**Female**
IT_{Max} (°C)	24.1 ± 0.1	24.1 ± 0.1
Weight (g)	412.6 ± 17.3	439.3 ± 15.7
Weight Loss (g)	-48.0 ± 3.1	-47.9 ± 3.0
Length (cm)	32.4 ± 0.4[a]	33.5 ± 0.4[b]
K	1.13 ± 0.01[b]	1.10 ± 0.01[a]
HSI (%)	1.15 ± 0.02	1.21 ± 0.03
RVM (%)	0.084 ± 0.002	0.081 ± 0.001

Lower case letters denote significant differences ($p < 0.05$) between sexes. K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass

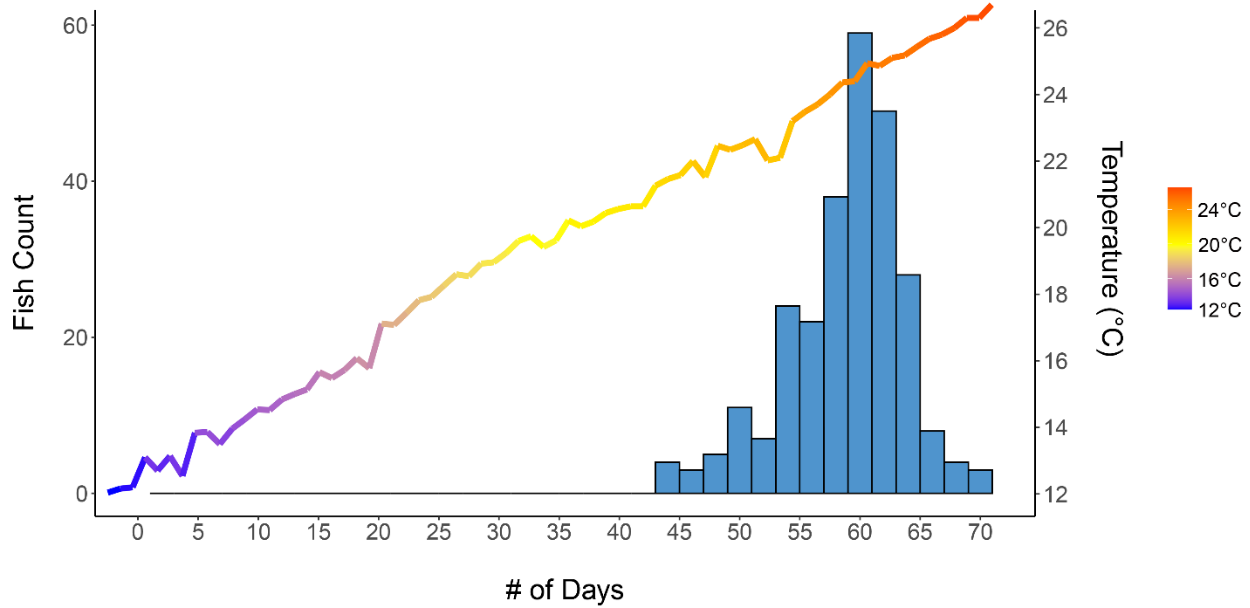
Appendix D Table D-3. Comparison of acute upper thermal tolerance (CT_{Max}) and morphometrics between male (n = 24) and female (n = 40) Atlantic salmon (irrespective of family).

	Male	**Female**
CT_{Max} (°C)	28.1 ± 0.1	28.0 ± 0.1
Weight (g)	687.3 ± 62.2	685.6 ± 37.3
Length (cm)	37.2 ± 0.9	37.7 ± 0.7
K	1.26 ± 0.03	1.27 ± 0.05
HSI (%)	1.13 ± 0.04[a]	1.27 ± 0.04[b]
RVM (%)	0.063 ± 0.003	0.065 ± 0.004

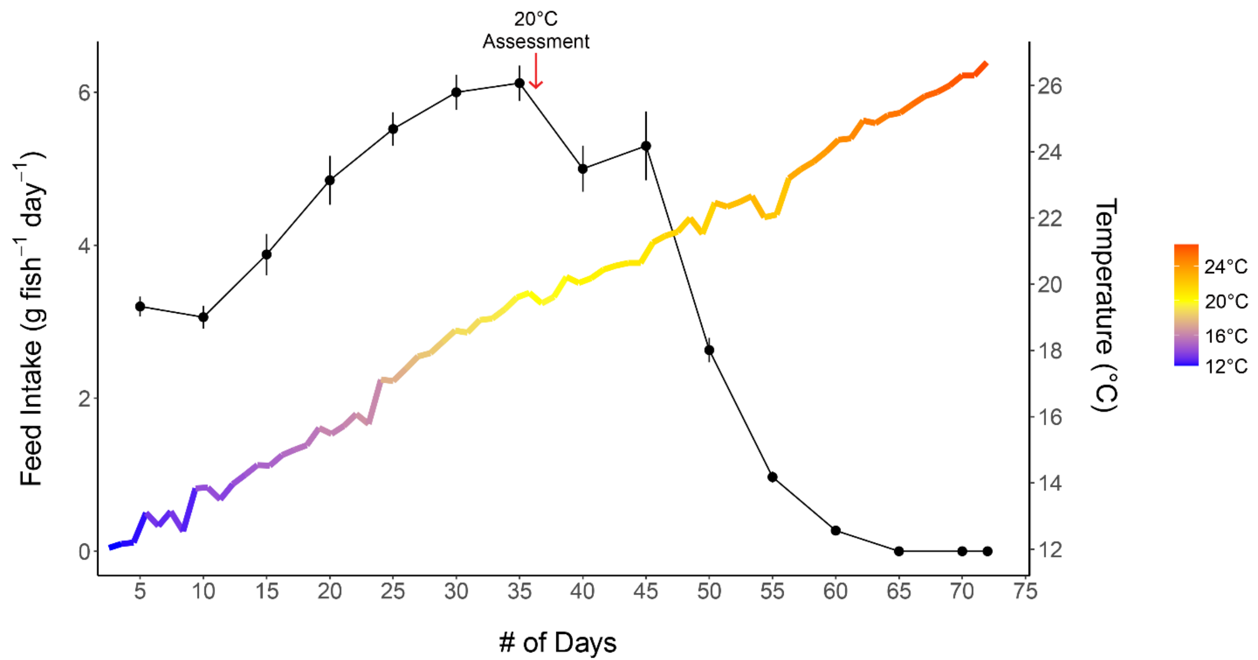
Lower case letters denote significant differences ($p < 0.05$) between sexes. K, Fulton's condition factor; HSI, hepatosomatic index; RVM, relative ventricular mass



Appendix D Figure D-1. Overview of the experimental tank layout. Temperature was controlled within each treatment's respective header tank via large plate heat-exchangers. This water was then supplied to each of the 5 tanks treatment^{-1}. The control treatment was maintained at 10°C for the majority of the experiment, while the warm treatment was exposed to an incremental temperature increase of 0.2°C day^{-1} from 12°C. Created with BioRender.com.



Appendix D Figure D-2. Histogram depicting the number of days before salmon reached their IT_{Max} (left y-axis; n = 265) when exposed to incremental temperature increase (+0.2°C day^{-1}) starting at 12°C (right y-axis, gradient line of the average temperature within the 5 tanks).



Appendix D Figure D-2. Feed intake (left y-axis, points connected by lines) of Atlantic salmon exposed to an incremental temperature increase starting at 12°C (right y-axis, gradient line of the average temperature within 5 tanks). Points on the graph indicate the mean feed intake (± standard error; n = 5) over the previous 5 days. Note: average feed intake dropped in the days following handling at the 20°C assessment.

Appendix E Table E-1. List of the putative SERPINH1 proteins used in the multiple sequence alignment and phylogenetic tree analyses.

Scientific Name	Common Name	Nomenclature	GenBank Accession Protein ID
Salmo salar	Atlantic salmon	SERPINH1A-1	WIF20406.1
Salmo salar	Atlantic salmon	SERPINH1A-2	WIF20408.1
Salmo salar	Atlantic salmon	SERPINH1B-1	WIF20409.1
Salmo salar	Atlantic salmon	SERPINH1B-2	WIF20411.1
Salvelinus alpinus	Arctic charr	SERPINH1A-1	XP_023824265.1
Salvelinus alpinus	Arctic charr	SERPINH1A-2	XP_023869331.1
Salvelinus alpinus	Arctic charr	SERPINH1B-1	XP_023822328.1
Salvelinus alpinus	Arctic charr	SERPINH1B-2	XP_023838693.1
Esox lucius	Northern pike	SERPINH1A	XP_010868966.3
Esox lucius	Northern pike	SERPINH1B	XP_010875071.2
Gadus morhua	Atlantic cod	SERPINH1A	XP_030218157.1
Gadus morhua	Atlantic cod	SERPINH1B	XP_030237409.1
Oreochromis niloticus	Nile tilapia	SERPINH1A	XP_025766534.1
Oreochromis niloticus	Nile tilapia	SERPINH1B	XP_005474679.1
Thunnus maccoyii	Southern bluefin tuna	SERPINH1A	XP_042287329.1
Thunnus maccoyii	Southern bluefin tuna	SERPINH1B	XP_042269992.1
Xiphophorus maculatus	Southern platyfish	SERPINH1A	XP_005806743.1
Xiphophorus maculatus	Southern platyfish	SERPINH1B	XP_023207884.1
Danio rerio	Zebrafish	SERPINH1A	NP_001103844.1
Danio rerio	Zebrafish	SERPINH1B	NP_001296752.1
Anguilla anguilla	European eel	SERPINH1A	XP_035288101.1
Anguilla anguilla	European eel	SERPINH1B	XP_035240074.1
Lepisosteus oculatus	Spotted gar	SERPINH1_1	XP_006627785.1
Lepisosteus oculatus	Spotted gar	SERPINH1_2	XP_015206788.1
Acipenser ruthenus	Sterlet sturgeon	SERPINH1_1	XP_033865917.1
Acipenser ruthenus	Sterlet sturgeon	SERPINH1_2	XP_034779887.1
Acipenser ruthenus	Sterlet sturgeon	SERPINH1_3	XP_034759450.1
Acipenser ruthenus	Sterlet sturgeon	SERPINH1_4	XP_033875603.2
Erpetoichthys calabaricus	Reedfish	SERPINH1	XP_028655988.2
Polypterus senegalus	Senegal bichir	SERPINH1	XP_039600473.1
Latimeria chalumnae	West Indian Ocean coelacanth	SERPINH1	XP_006011241.1
Xenopus tropicalis	Tropical clawed frog	SERPINH1	XP_004912280.2
Anas platyrhynchos	Mallard duck	SERPINH1	XP_005022647.2
Gallus gallus	Chicken	SERPINH1	NP_990622.1
Alligator mississippiensis	American alligator	SERPINH1	BAF94140.1
Chelonia mydas	Green sea turtle	SERPINH1	EMP33479.1

Rattus norvegicus	Norway rat	SERPINH1	AAH86529.1
Mus musculus	Mouse	SERPINH1	NP_001399150.1
Homo sapiens	Human	SERPINH1	NP_001193943.1
Pan troglodytes	Chimpanzee	SERPINH1	NP_001233539.1
Felis catus	Domestic cat	SERPINH1	XP_006936976.1
Bos taurus	Cow	SERPINH1	AAI05339.1
Orcinus orca	Orca	SERPINH1	XP_004279854.1
Rhincodon typus	Whale shark	SERPINH1	XP_048461249.1
Petromyzon marinus	Sea lamprey	SERPINH1	XP_032831099.1

Appendix E Table E-2. qPCR primers used for the normalizer genes.

Gene Name (Gene Symbol) (GenBank Accession Number)	Nucleotide sequence (5'-3')	Efficiency (%)						Amplicon Size (bp)	Source
		Head Kidney[a]	Brain[a]	Gill[a]	Muscle[a]	Liver[b]	Head Kidney[b]		
60S ribosomal protein 32 (*rpl32*) (BT043656)[c]	F: AGGCGGTTTAAGGGTCAGAT	96.6	96.3	97.3	94.4	-	-	119	Xue et al. (2015)
	R: TCGAGCTCCTTGATGTTGTG								
eukaryotic translation initiation factor 3 subunit D (*eif3d*) (GE777139)[d]	F: CTCCTCCTCCTCGTCCTCTT	98.2	97.2	99.3	95.8	-	102.9	105	Caballero-Solares et al. (2017)
	R: GACCCCAACAAGCAAGTGAT								
polyadenylate-binding protein 1 (*pabpc1*) (EG908498)[e]	F: TGACCGTCTCGGGTTTTTAG	-	-	-	-	97.2	99.5	108	Xu et al. (2013)
	R: CCAAGGTGGATGAAGCTGTT								
elongation factor 1 alpha (*ef1a*) (NM_001141909)[e]	F: GTGGAGACTGGAACCCTGAA	-	-	-	-	90.1	-	155	Jones et al. (2007)
	R: CTTGACGGACACGTTCTTGA								

[a] Efficiencies were assessed in Crossman et al. (2023) for the multi-tissue study; the average efficiency for these 4 tissues was used to calculate the calibrated normalized relative quantities (CNRQ).

[b] Efficiencies were assessed in Chapter 3 for the stress phenotype study; the respective tissue-specific efficiency was used to calculate the normalized relative quantities (NRQs).

[c] Normalizer gene chosen for the multi-tissue study.

[d] Normalizer gene chosen for the multi-tissue and stress phenotype studies.

[e] Normalizer gene chosen for the stress phenotype study.

Appendix E Table E-3. Percent identity matrix of paired combinations of Atlantic salmon *serpinh1* paralogues presented with percentages at the nucleotide level above the diagonal (blue - OQ814177, OQ814179, OQ814180, OQ814182) and percentages at the protein level below the diagonal (green - WIF20406, WIF20408, WIF20409, WIF20411).

	serpinh1a-1	*serpinh1a-2*	*serpinh1b-1*	*serpinh1b-2*
serpinh1a-1		82.4	64.9	65.3
serpinh1a-2	92.0		64.9	65.9
serpinh1b-1	78.1	76.9		85.9
serpinh1b-2	77.3	75.6	95.1	

Appendix E Table E-4. Head kidney and liver gene expression statistical results compared between stress phenotypes. The table continues through pages 344-348.

			Time Zero									
			Two-Way ANOVA						*Tukey's HSD Post-Hoc*			
			Phenotype		Temperature		Phenotype:Temperature		LR vs. HR - 12°C	LR vs. HR - 20°C	LR - 12°C vs. LR - 20°C	HR - 12°C vs. HR - 20°C
			F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
Liver	Fig. 6-4A	*serpinh1a-1*	3.873	0.059	12.499	**0.002**	0.044	0.835	0..538	0.396	0.066	0.102
	Fig. 6-4B	*serpinh1a-2*	2.039	0.165	14.859	**0.001**	0.159	0.693	0.834	0.530	**0.030**	0.083
	Fig. 6-4C	*serpinh1b-1*	1.319	0.261	221.380	**1.57E-14**	0.632	0.434	0.739	0.999	**1.00E-08**	**1.00E-08**
	Fig. 6-4D	*serpinh1b-2*	0.180	0.675	164.980	**5.15E-13**	0.216	0.646	0.988	0.987	**1.00E-08**	**1.00E-08**
Head Kidney	Fig. 6-5A	*serpinh1a-1*	0.026	0.872	1.433	0.242	0.461	0.503	-	-	-	-
	Fig. 6-5B	*serpinh1a-2*	1.183	0.287	4.843	**0.037**	1.388	0.249	0.367	0.999	0.903	0.100
	Fig. 6-5C	*serpinh1b-1*	0.253	0.619	187.074	**1.18E-13**	2.002	0.169	0.741	0.759	**1.00E-08**	**1.00E-08**
	Fig. 6-5D	*serpinh1b-2*	0.005	0.943	127.984	**9.45E-12**	0.499	0.486	0.993	0.888	**1.00E-08**	**2.00E-07**

12°C (24 hpi) - PBS vs. Forte Micro Injection										
Two-Way ANOVA						*Tukey's HSD Post-Hoc*				
Phenotype		Injection		Phenotype:Injection		PBS LR vs. HR	Forte Micro LR vs. HR	LR - PBS vs. LR - Forte Micro	HR - PBS vs. HR - Forte Micro
F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
1.432	0.241	6.563	**0.016**	0.212	0.649	0.649	0.953	0.166	0.459
0.710	0.407	3.702	0.065	0.294	0.592	-	-	-	-
0.373	0.547	5.628	**0.025**	0.031	0.861	0.944	0.990	0.293	0.421
0.042	0.839	10.963	**0.003**	0.451	0.507	0.925	0.987	**0.041**	0.265
2.571	0.121	10.700	**0.003**	0.014	0.907	0.663	0.587	0.110	0.130
1.895	0.180	9.322	**0.005**	0.147	0.704	0.856	0.569	0.102	0.240
0.436	0.515	5.060	**0.033**	0.018	0.895	0.924	0.976	0.471	0.338
0.023	0.881	17.644	**2.60E-04**	1.177	0.288	0.947	0.760	**0.005**	0.131

20°C (12 hpi) - PBS vs. Forte Micro Injection									
Two-Way ANOVA						*Tukey's HSD Post-Hoc*			
Phenotype		Injection		Phenotype:Injection		PBS LR vs. HR	Forte Micro LR vs. HR	LR - PBS vs. LR - Forte Micro	HR - PBS vs. HR - Forte Micro
F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
1.233	0.277	6.922	**0.014**	0.045	0.834	0.849	0.956	0.339	0.210
1.300	0.265	4.930	**0.035**	0.794	0.381	0.553	0.999	0.784	0.150
0.274	0.605	2.852	0.103	0.700	0.410	-	-	-	-
1.314	0.262	10.595	**0.003**	0.009	0.926	0.935	0.887	0.109	0.140
3.070	0.092	3.251	0.083	1.944	0.175	-	-	-	-
0.001	0.982	5.585	**0.026**	0.900	0.352	0.941	0.860	0.800	0.107
1.219	0.280	1.727	0.200	1.131	0.297	-	-	-	-
1.632	0.213	4.279	**0.049**	0.001	0.983	0.761	0.735	0.498	0.450

12°C - Time Zero vs. PBS Injection							
Two-Way ANOVA						*Tukey's HSD Post-Hoc*	
Phenotype		Injection		Phenotype:Injection		LR - Time Zero vs. LR - PBS	HR - Time Zero vs. HR - PBS
F value	*p*	F value	*p*	F value	*p*	*p*	*p*
2.775	0.107	33.918	**2.94E-06**	0.004	0.950	**0.002**	**0.002**
1.944	0.174	40.100	**7.52E-07**	0.012	0.912	**0.001**	**0.001**
0.846	0.365	1.161	0.290	0.003	0.953	-	-
0.166	0.687	4.850	**0.036**	0.437	0.514	0.699	0.203
0.314	0.580	16.955	**3.06E-04**	8.371	**0.007**	0.822	**1.74E-04**
0.671	0.420	8.438	**0.007**	4.281	**0.048**	0.934	**0.008**
0.198	0.660	1.136	0.296	2.063	0.162	-	-
0.745	0.396	4.740	**0.038**	0.069	0.794	0.330	0.538

20°C - Time Zero vs. PBS Injection								**Fold Change - PBS vs. Forte Micro Injection**	
Two-Way ANOVA						*Tukey's HSD Post-Hoc*		*T-Test*	
Phenotype		Injection		Phenotype:Injection		LR - Time Zero vs. LR - PBS	HR - Time Zero vs. HR - PBS	12°C - LR vs. HR	20°C - LR vs. HR
F value	*p*	F value	*p*	F value	*p*	*p*	*p*	*p*	*p*
0.167	0.686	7.387	**0.012**	2.372	0.136	0.883	**0.026**	0.404	0.780
0.080	0.780	3.419	0.076	3.239	0.084	-	-	0.353	0.249
1.167	0.290	0.059	0.810	1.547	0.225	-	-	0.157	0.212
0.089	0.767	7.630	**0.010**	0.536	0.471	0.098	0.442	0.076	0.297
0.078	0.782	0.135	0.717	0.042	0.838	-	-	0.864	**0.049**
0.134	0.718	0.091	0.766	0.113	0.740	-	-	0.689	0.439
0.001	0.974	0.423	0.522	1.905	0.180	-	-	0.831	0.122
0.004	0.948	2.315	0.141	0.974	0.333	-	-	0.366	0.459

```
                                                                                    transcription start
                                                                                    1
                                                                                    acagtgggagggccca   16

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 107 ccagctgaagatctgtccttgtgggagctgagagagaaactagagtaaaactgctgaaagagcctggtctttaatgatcgttctgacact  196

 197 ATGTGGGTGACTAACCTGGTAGCCATGGCCTTACTGGCCACCGCAGCCTCTGCTGCTACTGCCGCCTCTGCTGCTACTGCTGCCTCTGCT  286
      M  W  V  T  N  L  V  A  M  A  L  L  A  T  A  A  S  A  A  T  A  A  S  A  A  T  A  A  S  A
 287 GCTACTGCCGCCTTGGCAGACAAGGTCCTAAGTAACCATGCCACCCTGCTGGCCAACAACAGCGCCAGCCTGGCCTTTAGCCTCTACCAG  376
      A  T  A  A  L  A  D  K  V  L  S  N  H  A  T  L  L  A  N  N  S  A  S  L  A  F  S  L  Y  Q
 377 AACATGGTCAAGGAGAAGGACCTGGAGAACATCCTCATCTCCCCCGTGGTGGTGGCCTCTTCCCTGGGCCTGGTGGCCCTCGGGGGCAAG  466
      N  M  V  K  E  K  D  L  E  N  I  L  I  S  P  V  V  V  A  S  S  L  G  L  V  A  L  G  G  K
 467 GCCTCCACCGCCTCCCAGGTGAAGACCGTGCTGAGTGCTGACAAGGTGAAGGACGAGCAGCTTCACGCCGGCCTGGCCGAGCTGCTAGAG  556
      A  S  T  A  S  Q  V  K  T  V  L  S  A  D  K  V  K  D  E  Q  L  H  A  G  L  A  E  L  L  E
 557 GAGGTCAGCGACTCCAAGACCCGCAACGTCACCTGGAAGATCAGCAACCGCATCTACAGCCCCAGCTCGGTCAACTTTGCAGATGCCTTT  646
      E  V  S  D  S  K  T  R  N  V  T  W  K  I  S  N  R  I  Y  S  P  S  S  V  N  F  A  D  A  F
 647 GTCAAGAGCAGCAAGAAGCACTACAACTATGACCACACTAAGATCAACTTTAAGGACAAGAAGAGCGCTGTGAAGTCCATCAACGATTGG  736
      V  K  S  S  K  K  H  Y  N  Y  D  H  T  K  I  N  F  K  D  K  K  S  A  V  K  S  I  N  D  W
 737 GCGGCCAAGTCCACCGACGGCAAGCTGCCCGAGGTCACCAAGGACGTGGAGAGGACCGATGGGGCCATGATCATCAACGCCATGTTCTTC  826
      A  A  K  S  T  D  G  K  L  P  E  V  T  K  D  V  E  R  T  D  G  A  M  I  I  N  A  M  F  F
         intron 1 = 124 bp
        gtgagt..gaacag

 827 AAACCTCATTGGGATGAACAGTTCCACCAAAAGATGGTGGACAACCGTGGCTTCCTGGTGTCCCGCTCTCACACTGTTGGTGTACCCATG  916
      K  P  H  W  D  E  Q  F  H  Q  K  M  V  D  N  R  G  F  L  V  S  R  S  H  T  V  G  V  P  M
                    intron 2 = 148 bp
                   gtaaat..ctgcag

 917 ATGCACCGCACAGGTCTCTATGGTTTCCACGAGGATACAGTGAATAAGCTTTTAATCCTGAGCATACCCCTGGCCCATAAAAAATCCAGC 1006
      M  H  R  T  G  L  Y  G  F  H  E  D  T  V  N  K  L  L  I  L  S  I  P  L  A  H  K  K  S  S
1007 CTGGTGTTCTTCATGCCCTACCACGTGGAGTCCCTGGAGAGGCTGGAGAAGCTGCTGACCTGCAAGCAGCTGGATGACTGGATGGGCAAG 1096
      L  V  F  F  M  P  Y  H  V  E  S  L  E  R  L  E  K  L  L  T  C  K  Q  L  D  D  W  M  G  K
                                                                 intron 3 = 316 bp
                                                                gtactg..acacag

1097 CTGAAGGAGACGGCTGTGGCTGTGTCTCTGCCCAAAGTCAGCATGGAAGTCAGCCACAACATCCAGAAACACCTTGGGGAGCTGGGTCTG 1186
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      T  E  A  V  D  K  T  K  A  D  L  S  N  I  S  G  K  K  D  L  Y  L  S  N  V  F  H  A  S  A
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1367 CCCTTCATCTTCCTAGTGAAGGACACCAAGACCAACTCCATCCTCTTCCTCGGCAGACTGGTCCGACCTAAGGGCGAGAAGATGAGAGAT 1456
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1457 GAATTAtaacaactttgagtcttaggtgaagtgtttttgtatgcacaggcgtttgtgtgtgtgcgtgtatgtgtttgtattttcctat 1546
      E  L  .
1547 gcaaaagagaggagagagagaaaatgaattgtacgtcttatacctcaagagtacattcccatttcatcatctatgtgccaacattcgtgc 1636

1637 ctgttgttcacaatgcatgattcagaaatatatttttgtgtattttgtcctgtgttgtaagaaacaaagcattgtgtactccaaaaaacc 1726

1727 attaacttcactttaacatggtatcatcaccaagcctgcaatccaaactgaatatcgccccatttcacaaaagagagcgatattcagttt 1816

1817 ggattccaggctagttagtaatcaccataccacgtggcatgtttgagtattttaattaagggcagatcacaggatggatacttacattaa 1906

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2177 gttttctttacaggttttgctatgattaccgtgtacccgtcaccacatatcccatttttagtttgtgaaattcgtgtgggttccagtatc 2266
                                         alternate polyA site
2267 tttaatatctaaattaaaatattttttttcacaagcttttcacaatcttactacctggtgctagcctattgttcactcccccgaaacac 2356
                polyA-signal
2357 attaatctatgaccaaggggacgcgcctctgtttgttactctgtttggctttttactgatccgcactgaaagcactgctctgtaatcttt 2446

2447 agctaactaacctctgaatgaaag                                                                   2470
```

Appendix E Figure E-1. Sequence (GenBank accessions: OQ814177 & OQ814178) and predicted structure of Atlantic salmon *serpinh1a-1*. The nucleotide sequences are numbered on both sides, and the inferred amino acids are shown below the coding sequence. The period shows the predicted stop codon. The lower-case letters indicate the non-coding nucleotide sequence, whereas the deduced protein coding sequence of *serpinh1a-1* is shown in upper-case letters. The intronic sequences were determined using Ensembl (assembly GCA_905237065.2, accession ENSSSAT00000123524.2). One polyadenylation signal (polyA-signal: AUUAAA) was found in the 3′-UTR.

```
              transcription start
              1
              actgtgcattgaaaatcactgtacatcattaacattttccattccccaacttccagaatatttcccccacggttt   75
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                                                                  gtaggt..cacaag
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 166 ATGTGGGTGACTAACCTGGTAGTCATGGCCCTATTGGCCACTGCAGCCTCCGCTGACACCGCCGCCTCGGCAGACAAGGTCCTGAGCAAC  255
      M  W  V  T  N  L  V  V  M  A  L  L  A  T  A  A  S  A  D  T  A  A  S  A  D  K  V  L  S  N
 256 CACGCCACCCTGCTGGCTGACAACAGCGCCAGCCTGGCCTTCAGCCTCTACCAGAACATAGCCAAGGAAAAGGACCTGGAGAACATCCTC  345
      H  A  T  L  L  A  D  N  S  A  S  L  A  F  S  L  Y  Q  N  I  A  K  E  K  D  L  E  N  I  L
 346 ATCTCCCCGGTGGTGGTGGCCTCTTCCCTGGGCCTGGTGGCACTCGGGGGCAAGGGCTCCACCACCTCCCAGATCAAGACTGTGCTGAAC  435
      I  S  P  V  V  V  A  S  S  L  G  L  V  A  L  G  G  K  G  S  T  T  S  Q  I  K  T  V  L  N
 436 GCTAACAAAGTGAAGGACGAGCAGCTGCACGCTGGCCTGGCCGAGCTCCTGGAGGAGGTCAGCAACTCCAAGGCCCGTAACGTCACCTGG  525
      A  N  K  V  K  D  E  Q  L  H  A  G  L  A  E  L  L  E  E  V  S  N  S  K  A  R  N  V  T  W
 526 AAGATCAGCAACCATCTATACAGCCCCAGCTCGGTCAACTTTGCAGATGCCTTTGTCAAGAGCAGCAAGAAGCACTACAAATATGACCAC  615
      K  I  S  N  H  L  Y  S  P  S  S  V  N  F  A  D  A  F  V  K  S  S  K  K  H  Y  K  Y  D  H
 616 ACTAAGATAAACTTCAAGAACAAGAAGAGCGCTCTGAAGTCCATCAACGATTGGGCGGCCAAATCCACCGATGGCAAGCTGCCCGAGGTA  705
      T  K  I  N  F  K  N  K  K  S  A  L  K  S  I  N  D  W  A  A  K  S  T  D  G  K  L  P  E  V
                                                                       intron 2 = 119 bp
                                                                       gtgagt..gaacag
 706 ACCAAGGATGTGGAGAAGACTGATGGGGCCATGATCATCAACGCCATGTTCTTCAAACCCCATTGGGATGAACAGTTCCATCATAAGATG  795
      T  K  D  V  E  K  T  D  G  A  M  I  I  N  A  M  F  F  K  P  H  W  D  E  Q  F  H  H  K  M
                                                                  intron 3 = 133 bp
                                                                  gtcagc..ctgcag
 796 GTGGACAACCGTGGCTTCCTGGTGTCTCACACTCACACTGTTGGTGTACCCATGATGCACCGCACAGGTATCTATGGCTTCCACGATGAC  885
      V  D  N  R  G  F  L  V  S  H  T  H  T  V  G  V  P  M  M  H  R  T  G  I  Y  G  F  H  D  D
 886 ACAGTGAATAAGCTGTTCATCCTGAGCATGCCCCTGGCCCATAAGAAGTCCAGCCTGGTGTTCTTCATGCCCTACCACGTGGAGCCCCTG  975
      T  V  N  K  L  F  I  L  S  M  P  L  A  H  K  K  S  S  L  V  F  F  M  P  Y  H  V  E  P  L
 976 GAGAGGCTGGAGAAGCTACTGACCCGTAAGCAGCTGGAAGACTGGATGGGCAAGCTAAAGGAGACAGCTGTTGCTGTGTCTCTGCCCAAA  1065
      E  R  L  E  K  L  L  T  R  K  Q  L  E  D  W  M  G  K  L  K  E  T  A  V  A  V  S  L  P  K
                                    intron 4 = 276 bp
                                    gtactg..tcacag
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1156 AACATCTCTGGGAAGAAGGACCTGTATCTGTCCAATGTCTTCCATGCCTCTGCCATGGAGTGGGACACCGATGGGAACCCCATAGACAGA  1245
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1246 AGCATCTTCGGCACAGACAAGCTGAAGAACCCCAAGCTGTTCTACGCTGACCATCCCTTCATCTTCCTGGTGAAGGACACCAAGACCAAC  1335
      S  I  F  G  T  D  K  L  K  N  P  K  L  F  Y  A  D  H  P  F  I  F  L  V  K  D  T  K  T  N
1336 TCCATCCTCTTCCTCGGCAGACTGGTGCGGCCTAAGGGCAAGAAGATGAGAGATGAATTAtaacactttggagtcttatgtgaagtgttt  1425
      S  I  L  F  L  G  R  L  V  R  P  K  G  K  K  M  R  D  E  L  .
1426 ttgtatgtgtgcgtgttatggtgtttgtgtgtgtgtttatgtgtttgtatttttctatgcaaagagaggaaagggagaaaatgaatttta  1515

1516 cgtctaatacctcaagagtacattcccatttgatcatcaatgtgccaacatttgtgcctgttgttcactatgtatgattcagaattatat  1605

1606 ttttgtgttgtaagaaacaaagcattgtgtactctaacttcaccttgacatggtattatcatctagcctggtttcaaaactgaatatcgc  1695

1696 cccatttcacaaaagagagtgatattgggtttggtcaccaccatgcttgagtatttaaattaagatgagatcacaggatggatacttaca  1785

1786 ttaagtatgatctaccaaactattgcttaaatgtaaaataagtgttcctctgttttcctctcctgtctccagttgggatcgcaactgatt  1875

1876 gcatactgtttcacttcactttggattataggctctttttctgtagtagttgtttatttgaatttcaccgaatcaacatcaacttgctat  1965

1966 tgcctgggtattcagtactctatattcaagaaataactaatttaacctttatttaactaggcaagccagttaagaaaaaggtattatttt  2055

2056 acaatgacggcctaccccggccaaacctaacccggacaacactgggacaattgtgcgccgccctatgggactcccgatcatggttggttg  2145

2146 tgacacagcccaggatcaaaccagggtctgtagtgacaactttagcactgaaatgcagtgccttcgaccactgcaccacttaggagccca  2235

2236 ctcatccatttgatagtcattcaaaggtgctgatcaaattgctactttttgtttttttgccatgattaccatgtccctttcaccaaatgt  2325

2326 ctaattgtgagtttgtgaaagggtcgcgccgctgtctgctactctgcatggctttttactgatccacactgaaggcactgcgcagcactc  2415

2416 tatagctaacttactagtgaaagaaaaaaagtatcagggatcaaggtaagacccagatgcaggctgtcgaagtaacaatgtttattgtag  2505

2506 caacaggggcaggcggactcagggtcagggacaggcagagttcagtaatccagaggtggagcaaaggtacaggacggcaggcaggctcag  2595

2596 ggtcgggcagagaggtcaggcaggcgggtacagggtcagtacaggcaaaggtcgaaaaccaggaggacgaggaaagagaggctgggaaac  2685

2686 gataggagcttacaggaaaaacgctggtaaacttgaacgaacaagatgaactggcaacaaacagacagagaaagcaggtataaatacaca  2775

2776 ggggataatagggaagataggcgacacctggagggggggtggagacaagcacaaggacaggtgaaacagatcagggtgtgacagaaaggcc  2865

2866 atgtctatggaaggcgtttgactaactaatcagtcttgtcgtgtctccaacactatcacgttttgccacaaaaaaaaagctttttgggaa  2955

2956 caatggaatggtatgagatatttacaagtttcctctttttccaataaaatgg                                      3007
                                                  polyA-signal
```

Appendix E Figure E-2. Sequence (GenBank accession: OQ814179) and predicted structure of Atlantic salmon *serpinh1a-2*. The nucleotide sequences are numbered on both sides, and the inferred amino acids are shown below the coding sequence. The period shows the predicted stop codon. The lower-case letters indicate the non-coding nucleotide sequence, whereas the deduced protein coding sequence of *serpinh1a-2* is shown in upper-case letters. The intronic sequences were determined using Ensembl (assembly GCA_905237065.2, accession ENSSSAT00000166397.1). One polyadenylation signal (polyA-signal: AAUAAA) was found in the 3′-UTR.

```
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 143 ATGTGGGTGACCAACGTTGTAGTTCTGTGCCTCCTGGCCGTTGCGGCCTCCGGAGAAGACAAGAAGAAGCTGAGCAGCCATGCCACCACC  232
      M  W  V  T  N  V  V  V  L  C  L  L  A  V  A  A  S  G  E  D  K  K  K  L  S  S  H  A  T  T
 233 ATGGCTGACAAGAGCGCCAACCTGGCCTTCAGCCTCTACCACACGGTGGCCAAGGAGAAGGACCTTGAAAACATCCTGATATCCCCTGTG  322
      M  A  D  K  S  A  N  L  A  F  S  L  Y  H  T  V  A  K  E  K  D  L  E  N  I  L  I  S  P  V
 323 GTGGTGGCCTCCTCCCTTGGCATGGTGGCTCTCGGGGGCAAGGCTTCCACCGCCTCCCAGGTCAAGTCTGTCCTCAGTGCTGACGCCCTG  412
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 413 AAGGATGAGCACCTGCACACAGGCCTGTCAGAGCTTCTGACTGAGGTTAGTGACCCCAAGACTCGTAACGTCACATGGAAGATCAGTAAC  502
      K  D  E  H  L  H  T  G  L  S  E  L  L  T  E  V  S  D  P  K  T  R  N  V  T  W  K  I  S  N
 503 CGCCTCTACGGCCCCAGCTCGGTCACCTTCGCCGACAACTTTGTGAAGAGCAGCAAGAAGCACTACAACTATGACCACTCGAAGATCAAC  592
      R  L  Y  G  P  S  S  V  T  F  A  D  N  F  V  K  S  S  K  K  H  Y  N  Y  D  H  S  K  I  N
 593 CTCAGGGACAAGAGGAGCGCAGTGAACTCCATCAACGAATGGGCGTCCAAGTCGACAGACGGCAAGCTGCCTGAGATCACCAAGGATGTG  682
      L  R  D  K  R  S  A  V  N  S  I  N  E  W  A  S  K  S  T  D  G  K  L  P  E  I  T  K  D  V
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 683 CAGAATGCCGACGGAGCAACGATCGCCAACGCTATGTTCTTCAAGCCTCACTGGGATGAGAAGTTCCATGAGAAGATGGTAGACAACCGT  772
      Q  N  A  D  G  A  T  I  A  N  A  M  F  F  K  P  H  W  D  E  K  F  H  E  K  M  V  D  N  R
                                          intron 4 = 124 bp
                                         gtaggt..ccacag
 773 GGCTTCCTGGTGACCCGTTCATTCACAGTCTCTGTTCCCATGATGCATCGCACCGGTCTCTATAAGTTCCATGATGACACAGAGAACAGG  862
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 863 TTGTTTGTGCTGGACATGCCACTGGGCCAGAAGCAGTCCAGCCTGGTGTTCATCATGCCCTATCACCTGGAACCCCTTGACAGGCTGGAG  952
      L  F  V  L  D  M  P  L  G  Q  K  Q  S  S  L  V  F  I  M  P  Y  H  L  E  P  L  D  R  L  E
 953 AAGCTGCTGACCCGCAAGCAGCTGGAAACCTGGATGGGAAAGATGGAGGAGAGGGCCGTGGCCATCTCTCTGCCCAAAGTCAGCATGGAG 1042
      K  L  L  T  R  K  Q  L  E  T  W  M  G  K  M  E  E  R  A  V  A  I  S  L  P  K  V  S  M  E
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1043 GTTAGCCACAACCTCCAGAAAACTCTTGGTGAACTTGGTCTGACCGACGCTGTGGACAAAACCAAGGCCGATCTGTCCAACATCTCCGGC 1132
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1133 AAGAAGGACCTGTACCTCTCCAACGTGTTCCACGCCTCTTCAATGGAGTGGGACATCGAGGGGAACCCCTTCGACACAAGCATCTTCGGA 1222
      K  K  D  L  Y  L  S  N  V  F  H  A  S  S  M  E  W  D  I  E  G  N  P  F  D  T  S  I  F  G
1223 AGCGAGAAGCTGAGGAACCCCAAGTTGTTCTATGCTGACCATCCCTTCATCTTCCTGGTGAAGGACAACAAGACCAACTCCATCCTCTTC 1312
      S  E  K  L  R  N  P  K  L  F  Y  A  D  H  P  F  I  F  L  V  K  D  N  K  T  N  S  I  L  F
1313 ATCGGCAGAATGGTGCGACCCAAAGGAGACAAGATGCGTGACGAGTTGtaatagttaatggacagtttgagagatactgttactgattta 1402
      I  G  R  M  V  R  P  K  G  D  K  M  R  D  E  L  .
1403 atcatgccaggaaatagtatgtgtattatggtatgactgttacccaaagaacacattccaataggtcatctgtggactgaatatctagac 1492

1493 ctactattgtatcacagacacattaagcactcccagggaacaggaacattcaaataaatgctgtttggcctttgcaatgcgccataatca 1582
                                                              polyA-signal
1583 ataacattgtcaaagtctgcatttcaatacatttcaggaaaaagccaaacaccacaagtgcctttttagagtacctgaacattgaatagc 1672

1673 agacgacccaacagcatgcttatattaagtatgatctctctgatttttgccttccataaaatcagaaattttccaccattcttttcacca 1762

1763 gtataggtttggcttctctctcttattgggtttgtgatatgtgtagttagttgcattatctttaatgtttaaagagaaaaagattgttgc 1852

1853 acaagctaatttcatgtcatactgttttccactttccaccctgcaaagtacatttctcatgctttcagttgtttttgcctcccaggtgca 1942

1943 ctgaaactgttcctctgaaactgttcctctgcagtctttgatttgattgagttatttatgccatttgccaaccaaactgtgtgactttgt 2032

2033 tgtaggcatttaccactaaaccttggaaagttgccataatgcagtgttgtgtcatttttaactcttttttttttcaataaaaagaaacct 2122
                                                                                 polyA-signal
2123 aaaatg                                                                                     2128
```

Appendix E Figure E-3. Sequence (GenBank accession: OQ814180 & OQ814181) and predicted structure of Atlantic salmon *serpinh1b-1*. The nucleotide sequences are numbered on both sides, and the inferred amino acids are shown below the coding sequence. The period shows the predicted stop codon. The lower-case letters indicate the non-coding nucleotide sequence, whereas the deduced protein coding sequence of *serpinh1b-1* is shown in upper-case letters. The intronic sequences were determined using Ensembl (assembly GCA_905237065.2, accession ENSSSAT00000109441.2). Two polyadenylation signals (polyA-signal: AAUAAA) were found in the 3′-UTR. The 44 bp section in grey between the first and second introns represents a potential splice variant site. This exon region was absent from 7 out of 8 clones that were sequenced.

```
                                transcription start
                                1
                                tccagaagtttcttttctgtgggatatataggccagagtaaaaataataggaacatcaatcgacag   66
                                          intron 1 = 1504 bp
                                          gtaagg..tttcag
  67 aggagctaacccaaagagtgatatctggaagaggtcgtgccaagctttgccaggcttctatagctgctcaagaaccagaagaaagacagg  156

 157 ATGTGGGTGACCGTCGTAGCTCTGTGCCTGCTGGCCATTGCGGCCTCCGGAGAAGACAAGAAGAAGCTGAGCAGCCACGCCACCACCATG  246
      M  W  V  T  V  V  A  L  C  L  L  A  I  A  A  S  G  E  D  K  K  K  L  S  S  H  A  T  T  M
 247 GCTGACCAGAGCGCCAACCTGGCCTTCAGACTCTACCTCACGGTGGCCAAGGAGAAGGGCCTTGACAACATCCTGATATCCCCTGTGGTG  336
      A  D  Q  S  A  N  L  A  F  R  L  Y  L  T  V  A  K  E  K  G  L  D  N  I  L  I  S  P  V  V
 337 GTGGCCTCCTCCCTGGGCATGGTGGCCCTCGGGGGCAAGGCTTCCACCGCCTCCCAGGTCAAGTCTGTCCTCAGCGCTGATGCCCTGAAG  426
      V  A  S  S  L  G  M  V  A  L  G  G  K  A  S  T  A  S  Q  V  K  S  V  L  S  A  D  A  L  K
 427 GATGAGCACCTGCACACAGGCCTGTCAGAGCTCCTGAATGAGGTCAGCGACCCCAAGACCCGTAACGTCACATGGAAGATCAGCAACCGC  516
      D  E  H  L  H  T  G  L  S  E  L  L  N  E  V  S  D  P  K  T  R  N  V  T  W  K  I  S  N  R
 517 CTCTACGGCCCCAGCTCGGTCACCTTTGCCGATGACTTTGTGAAGAGCAGCAAGAAGCACTACAACTATGACCATTCAAAAATCAACCTC  606
      L  Y  G  P  S  S  V  T  F  A  D  D  F  V  K  S  S  K  K  H  Y  N  Y  D  H  S  K  I  N  L
 607 AGGGACAAGCGGAGCGCAGTGAACTCCATCAATGAATGGGCGGCCAAGTCGACAAACGGCAAGCTGCCTGAGATCACCAAGGATGTGCAG  696
      R  D  K  R  S  A  V  N  S  I  N  E  W  A  A  K  S  T  N  G  K  L  P  E  I  T  K  D  V  Q
                               intron 2 = 146 bp
                              gtaagt..ctacag
 697 AATGCTGATGGAGCCATGATCGCCAACGCTATGTTCTTCAAGCCTCACTGGGATGAGAAGTTCCATGACGAGATGGTAGACAACCGTGGC  786
      N  A  D  G  A  M  I  A  N  A  M  F  F  K  P  H  W  D  E  K  F  H  D  E  M  V  D  N  R  G
                                          intron 3 = 112 bp
                                         gtaggt..ccacag
 787 TTTCTAGTGACCCGTTCATTCACAGTCTCTGTTCCCATGATGCATCGCACTGGTCTCTATAAGTTCCATGATGACACAGAGAACATGTTG  876
      F  L  V  T  R  S  F  T  V  S  V  P  M  M  H  R  T  G  L  Y  K  F  H  D  D  T  E  N  M  L
 877 TTTGTGCTGGACATGCCCCTGGGCCAGAAGCAGTCCAGCCTGGTGCTCATCATGCCCTACCACCTGGAGCCCCTGGACAGGCTGGAGAAA  966
      F  V  L  D  M  P  L  G  Q  K  Q  S  S  L  V  L  I  M  P  Y  H  L  E  P  L  D  R  L  E  K
 967 CTGCTGACCCGCAAGCAGCTGGAAACCTGGATGGGCAAGATGGAGGAGAGGGCCGTGGCCATCTCTCTGCCCAAAGTCAGTGTAGAAGTT 1056
      L  L  T  R  K  Q  L  E  T  W  M  G  K  M  E  E  R  A  V  A  I  S  L  P  K  V  S  V  E  V
       intron 4 = 164 bp
     gtaaga..ctccag
1057 AGCCACAACCTCCAGAAAACTCTTGGTGAGCTTGGTGTGACTGAAGCTGTGGACAAAACCAAGGCTGATCTGTCCAACATCTCTGGCAAG 1146
      S  H  N  L  Q  K  T  L  G  E  L  G  V  T  E  A  V  D  K  T  K  A  D  L  S  N  I  S  G  K
1147 AAGGACCTGTACCTCTCCAATGTGTTCCACGCCTCTGCCATGGAGTGGGACATTGAGGGAAACCCTTTCGACACCAGCATCTTCGGAAGC 1236
      K  D  L  Y  L  S  N  V  F  H  A  S  A  M  E  W  D  I  E  G  N  P  F  D  T  S  I  F  G  S
1237 GAGAAGCTGAGGAACCCCAAGTTATTCTACGCTGACCATCCCTTCATCTTCCTGGTGAAGGACAACAAGACCAACTCCATCCTCTTCATC 1326
      E  K  L  R  N  P  K  L  F  Y  A  D  H  P  F  I  F  L  V  K  D  N  K  T  N  S  I  L  F  I
1327 GGCAGAATGGTGCGACCCAAAGGAGACAAGATGCGTGATGAGTTAtaatagttaatggatgggagttaaggtttactgattgaatcatgc 1416
      G  R  M  V  R  P  K  G  D  K  M  R  D  E  L  .
1417 caggaaatggtacgtgtacagtatgtgtattatggtatcactgttacctcaagaacacattccaacaggtaacctgtggattgaatagct 1506

1507 aggcctagtaatgtatcacagacacattaagcactcccagggaacaggagtgtatacgtttgaatgaatgctgttggcctttgcaatgtg 1596

1597 ccataatgaataacattgttaaagtctgcatttcaacaaatttcaggaaatagtcaaacaccataagtgcctttatagagtacctgaaca 1686

1687 ttgaatagcagatgacctgacaggatgcttatattaattagctatgatctctcagatttctgccttccataaaatcagacattccaccat 1776

1777 tattttcaccagtacgggtttggcttctctcttattttgagtttgtgatctgtgtggttacttgcattatctttgtttaaagagaaatat 1866

1867 tgttgcacaagcaaatttcacatactatcttccattttccaaccagcaaagtacatttatcatgctttcagttgtttttgcctcccagtt 1956

1957 gcattgaaactgttcctctgtactctttggcaaatggcatagatatttaaattaaaacaaactgtgactttatgttgtaggcatttacca 2046
                                                                       polyA-signal
2047 caaaaccttgtaatgttgccataatgcaatgttgtgtcctttttactatttttcaataaaaagtaacac                      2116
                                                                           polyA-signal
```

Appendix E Figure E-4. Sequence (GenBank accession: OQ814182) and predicted structure of Atlantic salmon *serpinh1b-2*. The nucleotide sequences are numbered on both sides, and the inferred amino acids are shown below the coding sequence. The period shows the predicted stop codon. The lower-case letters indicate the non-coding nucleotide sequence, whereas the deduced protein coding sequence of *serpinh1b-2* is shown in upper-case letters. The intronic sequences were determined using Ensembl (assembly GCA_905237065.2, accession ENSSSAT00000046979.2). Two polyadenylation signals (polyA-signal: AAUAAA/AUUAAA) were found in the 3′-UTR.

```
                      *        20         *        40         *        60         *        80         *
serpinh1a-1 : ACAGTGGGAGGGCCCAGCTCAGCTTACTGTGCATGGAAAATCACTGCACCTCATTGAAATACTTATTTCCCAACTTCCAGAAATTCATT :  90
serpinh1a-2 : -------------------------ACTGTGCATTGAAAATCACTGTACATCATTAACATTTTCCATTCCCCAACTTCCAGAA---TATT :  62
serpinh1b-1 : ------------------------------------------------------------------------------------------ :   -
serpinh1b-2 : ----------------------------------------------------------------------------TCCAGAAGTTTCTT :  14

                  100         *       120         *       140         *       160         *       180
serpinh1a-1 : TCC--CACCGGTTATAGACCAG-------------------------CTGAAGATCTGTCCT----TGTGGGAGCTGAGAGAGAAACT : 147
serpinh1a-2 : TCCCCCACCGGTTTGTAGACCAG-------------------------CTGAACGATCA--------TGTGGAAGCTGAGAGAGAAATT : 117
serpinh1b-1 : -----------------GTCAA--------------------------CAGACGAGCGAACCCAGAGAGTGAGGGCTGAGAGAGA---T :  43
serpinh1b-2 : TTCTGTGGCATATATAGGCCAGAGTAAAAATAATAGGAACATCAATCGACAGAGGAGCTAACCCAAAGAGTGATATCTGGAGAGAGG---T : 101

                      *       200         *       220         *       240         *       260         *
serpinh1a-1 : AGAGTAAAACT-----------------------------------------------------GCTGAAAGAGCCTGGTCTTTAATGATCG : 186
serpinh1a-2 : AGAGGAAACT------------------------------------------------------GCTGAAAGAGCCTG-TCTCTACTGATTT : 155
serpinh1b-1 : CGTGCCAAGCTCTGTCAGAATGAAAAACGAAAACCTTGACATCAGTATGAAAACATTTCAAGGCTTCTATAG-CTGCTCAAAAACCAGAA : 132
serpinh1b-2 : CGTGCCAAGCTTTGCCAG-----------------------------------------------GCTTCTATAG-CTGCTCAAGAACCAGAA : 146

               Start codon
                  280         *       300         *       320         *       340         *       360
serpinh1a-1 : TTCTGACACTATGTGGGTGACTAACCTGGTAGCCATGGCCTTACTGGCCACCGGCAGCCTCTGCTGCTACTGCCGCCTCTGCTGCTACTGC : 276
serpinh1a-2 : GGTTGACACCATGTGGGTGACTAACCTGGTAGTCATGGCCCTATTGGCCACTGCAGCCTCCGCTGACACCGCCGCCTC------------ : 233
serpinh1b-1 : GACAGAAAGGATGTGGGTGACCAACGTTGTAGTTCTGTGCCTCCTGGCCGTTGCGGCCTCCGG--------------------------- : 195
serpinh1b-2 : GAAAGACAGGATGTGGGTGAC---CGTCGTAGCTCTGTGCCTGCTGGCCATTGCGGCCTCCGG--------------------------- : 206

                      *       380         *       400         *       420         *       440         *
serpinh1a-1 : TGCCTCTGCTGCTACTGCCGCCTTGGCAGAC---AAGGTCCTAAGTAACCATGCCACCCTGCTGGCCAACAACAGCGCCAGCCTGGCCTT : 363
serpinh1a-2 : -------------------------GGCAGAC---AAGGTCCTGAGCAACCACGCCACCCTGCTGGCTGACAACAGCGCCAGCCTGGCCTT : 296
serpinh1b-1 : -------------------------AGAAGACAAGAAGAAGCTGAGCAGCCATGCCACCACCATGGCTGACAAGAGCGCCAACCTGGCCTT : 261
serpinh1b-2 : -------------------------AGAAGACAAGAAGAAGCTGAGCAGCCACGCCACCACCATGGCTGACCAGAGCGCCAACCTGGCCTT : 272

                  460         *       480         *       500         *       520         *       540
serpinh1a-1 : TAGCCTCTACCAGAACATGGTCAAGGAGAAGGACCTGGAGAACATCCTCATCTCCCCCGTGGTGGTGGCCTCTTCCCTGGGCCTGGTGGC : 453
serpinh1a-2 : CAGCCTCTACCAGAACATAGCCAAGGAAAAGGACCTGGAGAACATCCTCATCTCCCCGGTGGTGGTGGCCTCTTCCCTGGGCCTGGTGGC : 386
serpinh1b-1 : CAGCCTCTACCACACGGTGGCCAAGGAGAAGGACCTTGAAAACATCCTGATATCCCCTGTGGTGGTGGCCTCCTCCCTTGGCATGGTGGC : 351
serpinh1b-2 : CAGACTCTACCTCACGGTGGCCAAGGAGAAGGGCCTTGACAACATCCTGATATCCCCTGTGGTGGTGGCCTCCTCCCTGGGCATGGTGGC : 362

                      *       560         *       580         *       600         *       620         *
serpinh1a-1 : CCTCGGGGGCAAGGCCTCCACCGCCTCCCAGGTGAAGACCGTGCTGACTGCTGACAAGGTGAAGGACGAGCAGCTTCACGCCGGCCTGGC : 543
serpinh1a-2 : ACTCGGGGGCAAGGGCTCCACCACCTCCCAGATCAAGACTGTGCTGAACGCTAACAAAGTGAAGGACGAGCAGCTGCACGCTGGCCTGGC : 476
serpinh1b-1 : TCTCGGGGGCAAGGCTTCCACCGCCTCCCAGGTCAAGTCTGTCCTCAGTGCTGAGGCCCTGAAGGATGAGCACCTGCACACAGGCCTGTC : 441
serpinh1b-2 : CCTCGGGGGCAAGGCTTCCACCGCCTCCCAGGTCAAGTCTGTCCTCAGCGCTGATGCCCTGAAGGATGAGCACCTGCACACAGGCCTGTC : 452

                  640         *       660         *       680         *       700         *       720
serpinh1a-1 : CGAGCTGCTAGAGGAGGTCAGCGACTCCAAGACCCGCAACGTCACCTGGAAGATCAGCAACCGCCATCTACAGCCCCAGCTCGGTCAACTT : 633
serpinh1a-2 : CGAGCTCCTGGAGGAGGTCAGCAACTCCAAGGCCCGTAACGTCACCTGGAAGATCAGCAACCATCTATACAGCCCCAGCTCGGTCAACTT : 566
serpinh1b-1 : AGAGCTTCTGACTGAGGTTAGTGACCCCAAGACTCGTAACGTCACATGGAAGATCAGTAACCGCCTCTACGGCCCCAGCTCGGTCACCTT : 531
serpinh1b-2 : AGAGCTCCTGAATGAGGTCAGCGACCCCAAGACCCGTAACGTCACATGGAAGATCAGCAACCGCCTCTACGGCCCCAGCTCGGTCACCTT : 542

                      *       740         *       760         *       780         *       800         *
serpinh1a-1 : TGCAGATGCCTTTGTCAAGAGCAGCAAGAAGCACTACAACTATGACCACACTAAGATCAACTTTAAGGACAAGAAGAGCGCTGTGAAGTC : 723
serpinh1a-2 : TGCAGATGCCTTTGTCAAGAGCAGCAAGAAGCACTACAAATATGACCACACTAAGATAAACTTCAAGAACAAGAAGAGCGCTCTGAAGTC : 656
serpinh1b-1 : CGCCGACAACTTTGTGAAGAGCAGCAAGAAGCACTACAACTATGACCACTCGAAGATCAACCTCAGGGACAAGAGGAGCGCAGTGAACTC : 621
serpinh1b-2 : TGCCGATGACTTTGTGAAGAGCAGCAAGAAGCACTACAACTATGACCATTCAAAAATCAACCTCAGGGACAAGCGGAGCGCAGTGAACTC : 632

                  820         *       840         *       860         *       880         *       900
serpinh1a-1 : CATCAACGATTGGGCGGCCAAGTCCACCGACGGCAAGCTGCCCGAGGTCACCAAGGACGTGGAGAGGACCGATGGGGCCATGATCATCAA : 813
serpinh1a-2 : CATCAACGATTGGGCGGCCAAATCCACCGATGGCAAGCTGCCCGAGGTAACCAAGGATGTGGAGAAGACTGATGGGGCCATGATCATCAA : 746
serpinh1b-1 : CATCAACGAATGGGCGTCCAAGTCGACAGACGGCAAGCTGCCTGAGATCACCAAGGATGTGCAGAATGCCGACGGAGCAACGATCGCCAA : 711
serpinh1b-2 : CATCAATGAATGGGCGGCCAAGTCGACAAACGGCAAGCTGCCTGAGATCACCAAGGATGTGCAGAATGCTGATGGAGCCATGATCGCCAA : 722

                      *       920         *       940         *       960         *       980         *
serpinh1a-1 : CGCCATGTTCTTCAAACCTCATTGGGATGAACAGTTCCACCAAAAGATGGTGGACAACCGTGGCTTCCTGGTGTCCCGCTCTCACACTGT : 903
serpinh1a-2 : CGCCATGTTCTTCAAACCCCATTGGGATGAACAGTTCCATCATAAGATGGTGGACAACCGTGGCTTCCTGGTGTCTCACACTCACACTGT : 836
serpinh1b-1 : CGCTATGTTCTTCAAGCCTCACTGGGATGAGAAGTTCCATGAGAAGATGGTAGACAACCGTGGCTTCCTGGTGACCCGTTCATTCACAGT : 801
serpinh1b-2 : CGCTATGTTCTTCAAGCCTCACTGGGATGAGAAGTTCCATGACGAGATGGTAGACAACCGTGGCTTTCTAGTGACCCGTTCATTCACAGT : 812

                 1000         *      1020         *      1040         *      1060         *      1080
serpinh1a-1 : TGGTGTACCCATGATGCACCGCACAGGTCTCTATGGTTTCCACGAGGATACAGTGAATAAGCTTTTAATCCTGAGCATACCCCTGGCCCA : 993
serpinh1a-2 : TGGTGTACCCATGATGCACCGCACAGGTATCTATGGCTTCCACGATGACACAGTGAATAAGCTGTTCATCCTGAGCATGCCCCTGGCCCA : 926
serpinh1b-1 : CTCTGTTCCCATGATGCATCGCACCGGTCTCTATAAGTTCCATGATGACACAGAGAACAGGTTGTTTGTGCTGGACATGCCACTGGGCCA : 891
serpinh1b-2 : CTCTGTTCCCATGATGCATCGCACTGGTCTCTATAAGTTCCATGATGACACAGAGAACATGTTGTTTGTGCTGGACATGCCCCTGGGCCA : 902
```

```
                                    serpinh1b-2 F
                      *      1100         *      1120         *      1140         *      1160         *
serpinh1a-1  : TAAAAAATCCAGCCTGGTGTTCTTCATGCCCTACCACGTGGAGTCCCTGGAGAGGCTGGAGAAGCTGCTGACCTGCAAGCAGCTGGATGA : 1083
serpinh1a-2  : TAAGAAGTCCAGCCTGGTGTTCTTCATGCCCTACCACGTGGAGCCCCTGGAGAGGCTGGAGAAGCTACTGACCCGTAAGCAGCTGGAAGA : 1016
serpinh1b-1  : GAAGCAGTCCAGCCTGGTGTTCATCATGCCCTATCACCTGGAACCCCTTGACAGGCTGGAGAAGCTGCTGACCCGCAAGCAGCTGGAAAC :  981
serpinh1b-2  : GAAGCAGTCCAGCCTGGTGCTCATCATGCCCTACCACCTGGAGCCCCTGGACAGGCTGGAGAAACTGCTGACCCGCAAGCAGCTGGAAAC :  992

                                                              serpinh1b-1 F
                  1180         *      1200         *      1220         *      1240         *      1260
serpinh1a-1  : CTGGATGGGCAAGCTGAAGGAGACGGCTGTGGCTGTGTCTCTGCCCAAAGTCAGCATGGAAGTCAGCCACAACATCCAGAAACACCTTGG : 1173
serpinh1a-2  : CTGGATGGGCAAGCTAAAGGAGACAGCTGTTGCTGTGTCTCTGCCCAAAGTCTGCATGGAAGTCAGCCACAACATCCAGAAACACCTTGG : 1106
serpinh1b-1  : CTGGATGGGAAAGATGGAGGAGAGGGCCGTGGCCATCTCTCTGCCCAAAGTCAGCATGGAGGTTAGCCACAACCTCCAGAAAACTCTTGG : 1071
serpinh1b-2  : CTGGATGGGCAAGATGGAGGAGAGGGCCGTGGCCATCTCTCTGCCCAAAGTCAGTGTAGAAGTTAGCCACAACCTCCAGAAAACTCTTGG : 1082

                       serpinh1b-2 R                                                         serpinh1a-2 F
                      *      1280         *      1300         *      1320         *      1340         *
serpinh1a-1  : GGAGCTGGGTCTGACAGAGGCTGTGGATAAGACCAAGGCGGACCTGTCCAACATCTCTGGGAAGAAGGACCTGTACCTGTCCAACGTCTT : 1263
serpinh1a-2  : GGAGCTGGGTCTGACTGAGGCTGTGGATAAGACCAAGGCGGACCTGTCCAACATCTCTGGGAAGAAGGACCTGTATCTGTCCAATGTCTT : 1196
serpinh1b-1  : TGAACTTGGTCTGACCGACGCTGTGGACAAAACCAAGGCCGATCTGTCCAACATCTCCGGCAAGAAGGACCTGTACCTCTCCAACGTGTT : 1161
serpinh1b-2  : TGAGCTTGGTGTGACTGAAGCTGTGGACAAAACCAAGGCTGATCTGTCCAACATCTCTGGCAAGAAGGACCTGTACCTCTCCAATGTGTT : 1172

                         serpinh1b-1 R                                              serpinh1a-2 R
                  1360         *      1380         *      1400         *      1420         *      1440
serpinh1a-1  : CCATGCCTCTGCCATGGAGTGGGACACTGATGGGAACCCCATCAATACCAGCATCTTTGGCACCGATAAACTGAAAAACCCCAAGTTGTT : 1353
serpinh1a-2  : CCATGCCTCTGCCATGGAGTGGGACACCGATGGGAACCCCATAGACAGAAGCATCTTCGGCACAGACAAGCTGAAGAACCCCAAGCTGTT : 1286
serpinh1b-1  : CCACGCCTCTTCAATGGAGTGGGACATCGAGGGGAACCCCTTCGACACAAGCATCTTCGGAAGCGAGAAGCTGAGGAACCCCAAGTTGTT : 1251
serpinh1b-2  : CCACGCCTCTGCCATGGAGTGGGACATTGAGGGAAACCCTTTCGACACCAGCATCTTCGGAAGCGAGAAGCTGAGGAACCCCAAGTTATT : 1262

                                                                                             serpinh1a-1 F
                      *      1460         *      1480         *      1500         *      1520         *
serpinh1a-1  : CTACGCTGACCATCCCTTCATCTTCCTAGTGAAGGACACCAAGACCAACTCCATCCTCTTCCTCGGCAGACTGGTCCGACCTAAGGGCGA : 1443
serpinh1a-2  : CTACGCTGACCATCCCTTCATCTTCCTGGTGAAGGACACCAAGACCAACTCCATCCTCTTCCTCGGCAGACTGGTGCGGCCTAAGGGCAA : 1376
serpinh1b-1  : CTATGCTGACCATCCCTTCATCTTCCTGGTGAAGGACAACAAGACCAACTCCATCCTCTTCATCGGCAGAATGGTGCGACCCAAAGGAGA : 1341
serpinh1b-2  : CTACGCTGACCATCCCTTCATCTTCCTGGTGAAGGACAACAAGACCAACTCCATCCTCTTCATCGGCAGAATGGTGCGACCCAAAGGAGA : 1352

                                  Stop codon
                  1540         *      1560         *      1580         *      1600         *      1620
serpinh1a-1  : GAAGATGAGAGATGAATTATAACAACTTTGAGTCTTAGGTGAAGTGTTTTTGTATGCACAGGCGTT----TGTGTGTGTGTGCGTGTATG : 1529
serpinh1a-2  : GAAGATGAGAGATGAATTATAACACTTTGGAGTCTTATGTGAAGTGTTTTTGTATGTGTGCGTGTTATGGTGTTTGTGTGTGTGTTTATG : 1466
serpinh1b-1  : CAAGATGCGTGACGAGTTGTAATA---------------------GTTAATGGACA-------------------GTTTGAGAGATACTG : 1391
serpinh1b-2  : CAAGATGCGTGATGAGTTATAATA---------------------GTTAATGGA----------------------TGGCAGTTAAGG : 1397

                      *      1640         *      1660         *      1680         *      1700         *
serpinh1a-1  : TGTTTGTATTTTCCTATGCAAAAGAGAGGAGAGAG----AGAAAATGAATT---GTACGTCTTATACCTCAAGAGTACATTCCCATTTCA : 1612
serpinh1a-2  : TGTTTGTATTTTTCTATGC-AAAGAGAGGAAAGGG----AGAAAATGAATT---TTACGTCTAATACCTCAAGAGTACATTCCCATTTGA : 1548
serpinh1b-1  : T-TACTGATTTAATCATGC-CAGGAAAT-----------AGTATGTGTATTATGGTATCACTGTTACCCAAAGAACACATTCCAA-TAGG : 1467
serpinh1b-2  : TTTACTGATTGAATCATGC-CAGGAAATGGTACGTGTACAGTATGTGTATTATGGTATCACTGTTACCTCAAGAACACATTCCAA-CAGG : 1485

                      serpinh1a-1 R
                  1720         *      1740         *      1760         *      1780         *      1800
serpinh1a-1  : TCATCTATGTGCC-AACATTCGTGCCTGTTGTTCACAATGCATGATTCAGAAATATATTTTTGTGTATTTTGTCCTGTGTTGTAAGAAAC : 1701
serpinh1a-2  : TCATCAATGTGCC-AACATTTGTGCCTGTTGTTCACTATGTATGATTCAGAATTATATTTTTGTG--------------TTGTAAGAAAC : 1623
serpinh1b-1  : TCATCTGTGGACTGAATATCTAGACCTACT------ATTGTATCA--CAGACACAT-----TAAGCAC-----------TCCCAGGGAAC : 1533
serpinh1b-2  : TAACCTGTGGATTGAATAGCTAGGCCTAGT------AATGTATCA--CAGACACAT-----TAAGCAC-----------TCCCAGGGAAC : 1551

                      *      1820         *      1840         *      1860         *      1880         *
serpinh1a-1  : AAAGCATTGTGTACTCCAAAAAACCATTAACTTCACTTTAACATGGTATCATCACCAAGCCTGCAATCCAAA-CTGAATATCGCCCCATT : 1790
serpinh1a-2  : AAAGCATTGTGTACTCTAA-----------CTTCACCTTGACATGGTATTATCATCTAGCCTGGTTTCAAAA-CTGAATATCGCCCCATT : 1701
serpinh1b-1  : AGGA------ACATTCAAATAAATGCT--GTTTGGCCTTTGCAATGCGCCATAATCAATAACATTGTCAAAGTCTGCATTTCAATACATT : 1615
serpinh1b-2  : AGGAGTGTATACGTTTGAATGAATGCT---GTTGGCCTTTGCAATGTGCCATAATGAATAACATTGTTAAAGTCTGCATTTCAACAAATT : 1638

                  1900         *      1920         *      1940         *      1960         *      1980
serpinh1a-1  : TCACAAAGAGAGCGATATTCAGTTTGGATTCCAGGCTAGTTAGTAATCACCATACCACGTGGCATGTTTGAGTATTTTAA---TTAAGG : 1877
serpinh1a-2  : TCACAAAGAGAGTGATATTGGGTTTGGTCACCA-----------------------------CCATGCTTGAGTATTTAAA---TTAAGA : 1760
serpinh1b-1  : TCAGGAAAAAG-------------CCAAACACCA----------------------CAAGTGCCTTTTTAGAGTACCTGAACATTGAATA : 1670
serpinh1b-2  : TCAGGAAATAG-------------TCAAACACCA----------------------TAAGTGCCTTTATAGAGTACCTGAACATTGAATA : 1693

                      *      2000         *      2020         *      2040         *      2060         *
serpinh1a-1  : GCAGATCACAGGAT-GGATACTTACATTAAG----TATGATCTCCCAGACTATTGCCTTAAATATAAAATCAGCGTTACTCTCAGGGTTT : 1962
serpinh1a-2  : TGAGATCACAGGAT-GGATACTTACATTAAG----TATGATCTACCAAACTATTG-CTTAAATGTAAAATAAGTGTTCCTCTGTTTTCCT : 1844
serpinh1b-1  : GCAGACGACCCAACAGCATGCTTATATTAAG----TATGATCTCTCTGATTTTTGCCTT--CCATAAAATCAGAAATTTTCC----ACCA : 1750
serpinh1b-2  : GCAGATGACCTGACAGGATGCTTATATTAATTAGCTATGATCTCTCAGATTTCTGCCTT--CCATAAAATCAGACATTCC------ACCA : 1775

                  2080         *      2100         *      2120         *      2140         *      2160
serpinh1a-1  : CCTCTCCTGTTAGTCTGCGATCCCAACTGAATGCACACTCTGTTTCACTATTGTTTCACTTCACTTTGAATTCCAGACTATTTGTTTCTA : 2052
serpinh1a-2  : CTCCTGTCTCCAGT-TGGGATCGCAACTGATTGCATAC-------------TGTTTCACTTCACTTTGGATTATAGGCTCTTTTTCTGTA : 1920
serpinh1b-1  : TTCTTTTCACCAGTATAG-------------------------------GTTTGGCTTCTCTCTCTTATTGGG--------TTTGTG : 1798
serpinh1b-2  : TTATTTTCACCAGTACGG-------------------------------GTTTGGCTTCTCTCTTATTTTGAG--------TTTGTG : 1823
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                      *     2180         *     2200         *     2220         *     2240         *
serpinh1a-1 : ACAGT-----TTACTTGAGATT----AAGTCAACATCAAATTATTATTGTCGGGGTATTCA-TACTCTTTATTCCAAAAATAACTAATTG : 2132
serpinh1a-2 : GTAGTTGT--TTATTTGAATTTCACCGAATCAACATCAACTTGCTATTGCCTGGGTATTCAGTACTCTATATTCAAGAAATAACTAATTT : 2008
serpinh1b-1 : ATATGTGTAGTTAGTTGCATTA----------------------------------------TCTTTAATGTTTAAAGAGAAAAAGATT- : 1847
serpinh1b-2 : ATCTGTGTGGTTACTTGCATTA-------------------------------------------TCTTTGTTTAAAGAGAAAT--ATT- : 1867

                  2260         *     2280         *     2300         *     2320         *     2340
serpinh1a-1 : TGTGATAATGTCATTCAAATGTGCTGAGCAAGTAAACTGTTTCTGTTTTCTTTACAGGTTTTGCTATGATTACCGTGTACCCGTCACCAC : 2222
serpinh1a-2 : AACCTTTATTTAACT-AGGCAAGCCAGTTAAGAAAAAGGTATTATTTTACAATGACGGCCTACCCCGGCCAAACCTAACCCGGACAACAC : 2097
serpinh1b-1 : ----------------------GTTGCACAAGCTAA---TTTCATGTCATACT----GTTTTCCACTTTCCACCCTGCAAAGTACATTTC : 1908
serpinh1b-2 : ----------------------GTTGCACAAGCAAA---TTTCA---CATACT----ATCTTCCATTTTCCAACCAGCAAAGTACATTTA : 1925

                      *     2360         *     2380         *     2400         *     2420         *
serpinh1a-1 : --------------------------ATATCCCATTTTTAGTTTGTGAAATTCGTGTGGGTT--------------CCAGTATCTTTAAT : 2272
serpinh1a-2 : TGGGACAATTGTGCGCCGCCCTATGGGACTCCCGATCATGGTTGGT--------TGTGACACAGCCCAGGATCAAACCAGGGTCTGTAGT : 2179
serpinh1b-1 : -----------------------------TCATGCTTTCAGTTGTT--------TTTGCCTC--------------CCAGGT-------- : 1939
serpinh1b-2 : -----------------------------TCATGCTTTCAGTTGTT--------TTTGCCTC--------------CCAGTT-------- : 1956

                  2440         *     2460         *     2480         *     2500         *     2520
serpinh1a-1 : ATCT------AAATTAAAATATTTTTTT-----------CACAAGCT----------------TTTTCACAATC---------------- : 2314
serpinh1a-2 : GACAACTTTAGCACTGAAATGCAGTGCCTTCGACCACTGCACCACTTAGGAGCCCACTCATCCATTTGATAGTCATTCAAAGGTGCTGAT : 2269
serpinh1b-1 : ----------GCACTGAAA--CTGTTCCT-------CTGAAACTGTT----------------CCTCTGCAGTC---------------- : 1978
serpinh1b-2 : ----------GCATTGAAA--CTGTTCCT-------CTGTA----CT----------------CTTTGGCAA------------------ : 1989

                      *     2540         *     2560         *     2580         *     2600         *
serpinh1a-1 : --------------TTACTACCTGGTGCTAGCCTAT-TGTTCACTCCCCCGAA-----ACACATTAATCTATGACCAAGGGGACGCGCCT : 2384
serpinh1a-2 : CAAATTGCTACTTTTTGTTTTTTTGCCATGATTACCATGTCCCTTTCACCAAATGTCTAATTGTGAGTTTGTG---AAAGGGTCGCGCCG : 2356
serpinh1b-1 : -----------------TTTGATTTGATTGAGTTATTTATGCCATTTGCCAAC---CAAACTGTGTGACTTTGTTGTAGG---------- : 2038
serpinh1b-2 : ---------------------------ATGGCATAGATATTTAAATTA--AAA---CAAACTGTGACTTTATGTTGTAGG---------- : 2037

                  2620         *     2640         *     2660         *     2680         *     2700
serpinh1a-1 : CTGTTTGTTACTCTGTTTGGCTTTTTACTGATCCGCACTGAAAGCACTGCTCTGTAATCTTTAGCTAACTAACCTCTGAATGAAAG---- : 2470
serpinh1a-2 : CTGTCTGCTACTCTGCATGGCTTTTTACTGATCCACACTGAAGGCACTGCGCAGCACTCTATAGCTAACTTACTAGTGAAAGAAAAAAAG : 2446
serpinh1b-1 : -CATTTACCACTA-----AACCTTGGAAAGTTGC---CATAATGCAGTGTTGTGTCATTTTTAACTCTTTTTTTTTTCAATAAAAAGAAA : 2119
serpinh1b-2 : -CATTTACCACAA-----AACCTTGTAATGTTGC---CATAATGCAATGTTGTGTCCTTTTTTACTATTTT-----TCAATAAAAAGTAA : 2113

                      *     2720         *     2740         *     2760         *     2780         *
serpinh1a-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1a-2 : TATCAGGGATCAAGGTAAGACCCAGATGCAGGCTGTCGAAGTAACAATGTTTATTGTAGCAACAGGGGCAGGCGGACTCAGGGTCAGGGA : 2536
serpinh1b-1 : CCTAAAATG--------------------------------------------------------------------------------- : 2128
serpinh1b-2 : CAC--------------------------------------------------------------------------------------- : 2116

                  2800         *     2820         *     2840         *     2860         *     2880
serpinh1a-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1a-2 : CAGGCAGAGTTCAGTAATCCAGAGGTGGAGCAAAGGTACAGGACGGCAGGCAGGCTCAGGGTCGGGCAGAGAGGTCAGGCAGGCGGGTAC : 2626
serpinh1b-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1b-2 : ------------------------------------------------------------------------------------------ :    -

                      *     2900         *     2920         *     2940         *     2960         *
serpinh1a-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1a-2 : AGGGTCAGTACAGGCAAAGGTCGAAAACCAGGAGGACGAGGAAAGAGAGGCTGGGAAACGATAGGAGCTTACAGGAAAAACGCTGGTAAA : 2716
serpinh1b-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1b-2 : ------------------------------------------------------------------------------------------ :    -

                  2980         *     3000         *     3020         *     3040         *     3060
serpinh1a-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1a-2 : CTTGAACGAACAAGATGAACTGGCAACAAACAGACAGAGAAAGCAGGTATAAATACACAGGGGATAATAGGGAAGATAGGCGACACCTGG : 2806
serpinh1b-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1b-2 : ------------------------------------------------------------------------------------------ :    -

                      *     3080         *     3100         *     3120         *     3140         *
serpinh1a-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1a-2 : AGGGGGGTGGAGACAAGCACAAGGACAGGTGAAACAGATCAGGGTGTGACAGAAAGGCCATGTCTATGGAAGGCGTTTGACTAACTAATC : 2896
serpinh1b-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1b-2 : ------------------------------------------------------------------------------------------ :    -

                  3160         *     3180         *     3200         *     3220         *     3240
serpinh1a-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1a-2 : AGTCTTGTCGTGTCTCCAACACTATCACGTTTTGCCACAAAAAAAAAGCTTTTTGGGAACAATGGAATGGTATGAGATATTTACAAGTTT : 2986
serpinh1b-1 : ------------------------------------------------------------------------------------------ :    -
serpinh1b-2 : ------------------------------------------------------------------------------------------ :    -

                      *     3260
serpinh1a-1 : --------------------- :    -
serpinh1a-2 : CCTCTTTTTCCAATAAAATGG : 3007
serpinh1b-1 : --------------------- :    -
serpinh1b-2 : --------------------- :    -
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Appendix E Figure E-5. Alignment of the four Atlantic salmon *serpinh1* paralogues (GenBank accession numbers OQ814177, OQ814179, OQ814180 and OQ814182). As identified by GeneDoc, black shading with white font shows the 100% conserved residues amongst the paralogues. Dark gray shading with white font, and light gray with black font, denote 80 and 60% conservation amongst the residues, respectively. The numbers above the sequences show the relative positions of the nucleotides. The green line and font show the start codon, and similarly, orange shows the stop codon. Different shades of blue for the lines and font represent the forward (F) and reverse (R) primers used in qPCR for each of the four paralogues.

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                                          *        20         *        40         *        60         *        80         *
             Salmo salar SERPINH1A-1 : ---------MWVTNLVAMALLATAASAATAASAATAA------------------------------SAATAALAD-KVLSNHATLLANN :  50
             Salmo salar SERPINH1A-2 : ---------MWVTNLVVMALLATAA------------------------------------------SADTAASAD-KVLSNHATLLADN :  38
             Salmo salar SERPINH1B-1 : ---------MWVTNVVVICLLAVA---------------------------------------------ASGE--DKKKLSSHATTMADK :  34
             Salmo salar SERPINH1B-2 : ---------MWVT-VVAICLLAIA---------------------------------------------ASGE--DKKKLSSHATTMADQ :  33
        Salvelinus alpinus SERPINH1A-1 : ---------MWVTNLVAMALLATAA------------------------------------------SAATAALAD-KVLSNHATLLANN :  38
        Salvelinus alpinus SERPINH1A-2 : ---------MWVTNLVAMALLATAA------------------------------------------SXATAASAD-KVLSNHATLLADN :  38
        Salvelinus alpinus SERPINH1B-1 : ---------MWVTNVVVICLLAVA---------------------------------------------ASEE--DKKKLSSHATTMADK :  34
        Salvelinus alpinus SERPINH1B-2 : ---------MWVT-VVAICLLAVA---------------------------------------------ASGE--DKKKLSSHATTMADQ :  33
               Esox lucius SERPINH1A : ---MVTNLLEIVTNLVAMAFLATAA------------------------------------------SAATATSAD-KVLSNHATLLADN :  44
               Esox lucius SERPINH1B : ---------MWMTNIVAICLLALV---------------------------------------------ASAE--D-KKLSSHATTMADQ :  33
       Xiphophorus maculatus SERPINH1A : ---------MWLTLLVSIALLAPAI------------------------------------------SAATATTAD-KVLSNHATILADN :  38
       Xiphophorus maculatus SERPINH1B : ---------MWMTTAVAICLLAFV---------------------------------------------ASAE--D-KKLSSHANTLADN :  33
       Oreochromis niloticus SERPINH1A : ---------MWVTNLVSLVLLTTAA------------------------------------------SAATSTSKN-KVLSNHATILADS :  38
       Oreochromis niloticus SERPINH1B : ---------MWMTNIVGICLLALV---------------------------------------------ASAE--D-VKLSTHATTLADK :  33
            Thunnus maccoyii SERPINH1A : ---------MWVTNLVALVLLATAA------------------------------------------SAATSASPD-KVLSNHATILADN :  38
            Thunnus maccoyii SERPINH1B : ---------MWVTKVAALCLLALV---------------------------------------------ASAE--D-KKLSSHATTLADN :  33
               Gadus morhua SERPINH1A : ---------MLVTSLVALALLAHTA------------------------------------------SSATSASPD-KVLSTHASLLADN :  38
               Gadus morhua SERPINH1B : ---------MWMTSVLALCLLAPL---------------------------------------------ASAD--G-RKLSSHATLLADN :  33
                Danio rerio SERPINH1A : ---------MLASNVLLICLLATV---------------------------------------------SAN-----KTLSSIATTLADN :  31
                Danio rerio SERPINH1B : ---------MWVSSLIAICLLAVA---------------------------------------------VSGE--D-KKLSTHATSMADT :  33
            Anguilla anguilla SERPINH1A : MLLLTYLETMSLTNVVAFCLVFAT---------------------------------------------VTNG--D-KQL------LADK :  36
            Anguilla anguilla SERPINH1B : ---------MRATNMAAICLLAILA------------------------------------------GMAAGE--E-GKLSSHATALADN :  36
        Lepisosteus oculatus SERPINH1_1 : ---------MWVTKLVALSLLAAL---------------------------------------------ATAE--E-KKLSSHASMLADT :  33
        Lepisosteus oculatus SERPINH1_2 : ----------MAFKAAVVSFLIGI---------------------------------------------VVA---EPVALKAPTSVLSES :  32
         Acipenser ruthenus SERPINH1_1 : ---------MWLTKLVAFSLLVAV---------------------------------------------ATAE--Q-KKLSPHASTLADS :  33
         Acipenser ruthenus SERPINH1_2 : ---------MWLTKLVAFSLLVAV---------------------------------------------ATVE--Q-KKLSPHASTLADS :  33
         Acipenser ruthenus SERPINH1_3 : ----------MALKLAIVCIIFSL---------------------------------------------AR-A--DPVALKDHASVLADS :  32
         Acipenser ruthenus SERPINH1_4 : ----------MALKLAVVCIIFSL---------------------------------------------AR-A--DPVALKDHASVLADS :  32
     Erpetoichthys calabaricus SERPINH1 : ---------MWAIKIAAIGICVAL---------------------------------------------ATAE--D-KKLSSHATSLADH :  33
        Polypterus senegalus SERPINH1 : ---------MWAIKIAAIGICVAL---------------------------------------------ATAE--D-KKLSSHAATLADH :  33
        Latimeria chalumnae SERPINH1 : ---------MWMTRILSISIIAAV---------------------------------------------AICE--E-KKLSEHATTLADR :  33
         Xenopus tropicalis SERPINH1 : ---------MWMIKLLALSIILVVDAAVDKKPVVADKKPVAVDKKPVVADKKPVVADKKPVAADKKPVAEPPV--E-KKISQHANVLADK :  78
        Anas platyrhynchos SERPINH1 : ---------MWIIVVLALCGLAMA---------------------------------------------VPSE--D-RKLSDKATTLADR :  33
             Gallus gallus SERPINH1 : ---------MQIFLVLALCGLAAA---------------------------------------------VPSE--D-RKLSDKATTLADR :  33
   Alligator mississippiensis SERPINH1 : ---------MWAIQLLAMGVIVAA---------------------------------------------VPSE--D-KKLSDKANALADR :  33
            Chelonia mydas SERPINH1 : ---------MWVTNLLALCALVAA---------------------------------------------VPSE--D-KKLSDKAAALADR :  33
          Ratus norvegicus SERPINH1 : ---------MRSLLLGTLCLLAVALAAEVKKPVE---------------------------------ATAPGT--A-EKLSSKATTLAER :  45
             Mus musculus SERPINH1 : ---------MRSLLLGTLCLLAVALAAEVKKPLE---------------------------------AAAPGT--A-EKLSSKATTLAER :  45
             Homo sapiens SERPINH1 : --------MRSLLLLSAFCLLEAALAAEVKKPAA---------------------------------AAAPGT--A-EKLSPKAATLAER :  46
          Pan troglodytes SERPINH1 : --------MRSLLLLSAFCLLEAALAAEVKKPAA---------------------------------AAAPGT--A-EKLSPKAATLAER :  46
              Felis catus SERPINH1 : --------MRLLALLGACCLLAVALAAEVKKPAA---------------------------------AAAPGT--A-EKLSPKAATLAER :  46
               Bos taurus SERPINH1 : --------MRALLLLSTLCLLARALAAEVKKPAA---------------------------------AAAPGT--A-EKLSPKAATLAER :  46
              Orcinus orca SERPINH1 : --------MRALLLLSTFCLLARALAAEVKKPAA---------------------------------TAAPGP--A-EKLSPKAATFAER :  46
           Rhincodon typus SERPINH1 : --------MNRCRREGNHHVLHNIKRNSNSSRLFGLS------------------------------RFMEQDGTHSPQPGEHGSMLADS :  52
        Petromyzon marinus SERPINH1 : ---MASTLRMLLLAALASGALAAAAAADGKKATVSKADA----------------------------AAANNATAPPKNLSEHAKKVGEG :  59
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Serpin Superfamily

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                                            100         *       120         *       140         *       160         *       180
             Salmo salar SERPINH1A-1 : SASLAFSLYQNMVKE-KDL--ENILISPVVVASSLGLVALGGKASTASQVKTVLSADKVKDEQLHAGLAELLEEVSDSKT---------R : 128
             Salmo salar SERPINH1A-2 : SASLAFSLYQNIAKE-KDL--ENILISPVVVASSLGLVALGGKGSTTSQIKTVLNANKVKDEQLHAGLAELLEEVSNSKA---------R : 116
             Salmo salar SERPINH1B-1 : SANLAFSLYHTVAKE-KDL--ENILISPVVVASSLGMVALGGKASTASQVKSVLSADALKDEHLHTGLSELLTEVSDPKT---------R : 112
             Salmo salar SERPINH1B-2 : SANLAFRLYLTVAKE-KGL--DNILISPVVVASSLGMVALGGKASTASQVKSVLSADALKDEHLHTGLSELLNEVSDPKT---------R : 111
        Salvelinus alpinus SERPINH1A-1 : SASLAFSLYQNMVKE-KDL--ENILISPVVVASSLGLVALGGKASTASQVKMVLSADKVKDEQLHAGLAELLEEVSDSKT---------R : 116
        Salvelinus alpinus SERPINH1A-2 : SASLAFSLYQNIAKE-KDL--ENILISPVVVASSLGLVALGGKASTASQVKTVLSANKVKDEQLHAGLAELLEEVSDSKA---------R : 116
        Salvelinus alpinus SERPINH1B-1 : SANLAFSLYHMVAKE-KGL--DNILISPVVVASSLGMVALGGKASTASQVKSVLSADALKDEHLHTGLSELLTEVSDPKT---------R : 112
        Salvelinus alpinus SERPINH1B-2 : STNLAFRLYHTVAKE-KGL--DNILISPVVVASSLGMVALGGKASTASQVKSVLSADALKDEHLHTGLSELLNEVSDPKT---------R : 111
               Esox lucius SERPINH1A : SASLAFSLYQNIAKE-KDM--ENILISPVVVASSLGLVALGGKASTASQVKTVLSADKVKDEQLHAGLAELLEEVTDSKA---------R : 122
               Esox lucius SERPINH1B : SAKLAFSLYHSVAKE-KGL--ENILISPVVVASSLGMVALGGKASTVSQAKSVLSADALKDEHLHTGLSELMSEVSDPKN---------R : 111
       Xiphophorus maculatus SERPINH1A : SANLAISLYQNMAKE-KGA--ENILISPVVVASSLGLVALGGKGSTASQVKTILNAAKVQDEQLHSGLAELLTEVSDPKE---------R : 116
       Xiphophorus maculatus SERPINH1B : SANLAFSLYHKMAQD-KNT--DNIVVSPVVVASSLGLVALGGKATTASQVKTVLSADKLKDEHLHAGLSELLSVVSNQKT---------R : 111
       Oreochromis niloticus SERPINH1A : TANLAFSLYQSMVKD-KNV--ENIIISPVVVASSLGLVALGGKASTASQVKTILNVAKVKDEHLHSSLAELLIEMSDPKT---------R : 116
       Oreochromis niloticus SERPINH1B : SANLAFSLYHTMAKE-KDT--ENIIISPVVVASSLGLVALGGKASTASQVKTVLSADKLKDEHLHAGLSELLTEVSNAKT---------R : 111
            Thunnus maccoyii SERPINH1A : SADFAFSLYQNMAKE-KDV--ENIIISPVVVATSLGLVALGGKASTASQVKTILNAAKVKDEQLHSGLAELLTEVSDPKT---------R : 116
            Thunnus maccoyii SERPINH1B : SANLAFSLYHNMAKE-KDT--ENILISPVVVASSLGMVALGGKASTASQVKTVLSADKLKDEHLHAGLSELLSEVSDAKK---------R : 111
               Gadus morhua SERPINH1A : SASLAFSLYQRVAQE-KDT--ENILISPVVVASALGLVALGGKASTASQVKSLLKADKLKDEQLHAGLGELLSEVSDPAV---------R : 116
               Gadus morhua SERPINH1B : SADLAFSLYHNMALD-KGT-GDNILVSPVVVASSLGVIALGGKAATASQVKTVLRADALKDEHLHAGLSELMSEVSDAKT---------R : 112
                Danio rerio SERPINH1A : SATLAFNLYQNMAKD-KDL--ENILISPVVVASSLGLVALGGKSNTASQVKTVLSAASVKDEQLHSGLSELLTEVSNPKA---------R : 109
                Danio rerio SERPINH1B : SANLAFNLYHNVAKE-KGL--ENILISPVVVASSLGMVAMGSKSSTASQVKSVLKADALKDEHLHTGLSELLTEVSDPQT---------R : 111
            Anguilla anguilla SERPINH1A : STSLALNLYHNMVKE-KNQ--GNILISPVVVASSLGLVALGGKASTASQVKTVLSADKVKDAQLHASLAELLTEVSNSTA---------R : 114
            Anguilla anguilla SERPINH1B : SANLAFNLYHNVAKE-KDL--ENIAISPVVVASSLGLVALGGKASTASQVKTVLAAGAVKDEHLHAGLAELLTEVSNSTA---------R : 114
        Lepisosteus oculatus SERPINH1_1 : SASLAFNLYHNMAKD-KNA--ENILFSPVVVASSLGLVHLGGKSSTASQAKTVLSADKVKDDQLHTGLSELLTEVSNSTA---------R : 111
        Lepisosteus oculatus SERPINH1_2 : TVNLGLNLYHTMVKD-PSLKSENILFSPVVVATSLGVMSLGAKDKTASQVKSLLRVN-LHEDKLHPAFSELFNDVSNETA---------R : 111
         Acipenser ruthenus SERPINH1_1 : SARLAFDLYHSMAKE-KDL--DNLLISPVVVSSSLGLIHLGGQSSTAAQVKKVLHVDKVSDEQLHAGLSELLSEVSNSSA---------R : 111
         Acipenser ruthenus SERPINH1_2 : SARLAFDLYHSMAKE-KDL--DNLLISPVVVSSSLGLIHLGGQSSTAAQVKKVLHVDKVSDEQLHAGLSELLSEVSNSSA---------R : 111
         Acipenser ruthenus SERPINH1_3 : TLSLGLNLYQTMVKD-QNLRSENILFSPVVLASSLGVMTMGAKENTASQVKSVLNVD-LQEDKLHPAFSALLSDVSDEKA---------R : 111
         Acipenser ruthenus SERPINH1_4 : TLSLGLNLYQTMVKD-QNLRSENILFSPVVLASSLGVMTMGAKENTASQVKSVLNVD-LQEDKLHPAFSALLSEVSDEKA---------R : 111
     Erpetoichthys calabaricus SERPINH1 : SSNFAFSLYHNMAKE-KNM--ENILFSPVVVASSLGLVHLGGQSNTASQVKKVLAAEKVNLDHLHNGLSELLTEVSNSTA---------R : 111
        Polypterus senegalus SERPINH1 : SSNLAFSLYHNMAKE-KNI--ENILFSPVVVASSLGLVHLGGQSNTASQAKKVLSAEKVNLDHLHNGLSELLTEVSNSTA---------R : 111
        Latimeria chalumnae SERPINH1 : SSTLAFNLYHTMAKE-KNM--ENILISPVVVASSLGLVSLAGKASTASQAKAVLSVDKLKDEHLHAGLSELLNEVSNSTA---------R : 111
         Xenopus tropicalis SERPINH1 : SAGLAFNLYQTMAKD-KNV--ENILLSPVVVASSLGLVSLGGQASTAAQAKAVLSADKLSDEHIHSGLAELLNEVSNSTA---------R : 156
        Anas platyrhynchos SERPINH1 : STTLAFNLYHAMAKD-KDM--ENILLSPVVVASSLGLVSLGGKAATASQAKAVLSADKLNLDYVHSGLSELLNEVSNSTA---------R : 111
             Gallus gallus SERPINH1 : STTLAFNLYHAMAKD-KNM--ENILLSPVVVASSLGLVSLGGKATTASQAKAVLSADKLNLDYVHSGLSELLNEVSNSTA---------R : 111
   Alligator mississippiensis SERPINH1 : SATLAFNLYHAMAKD-KNM--ENILVSPVVVASSLGLVSLGGKAATASQAKAVLSADKLNLDYVHSGLSELLNEVSNSTA---------R : 111
            Chelonia mydas SERPINH1 : SATLAFNLYHTMAKD-KNM--ENILVSPVVVASSLGLVSLGGKATTASQAKAVLSADKLNLDYIHGGLSELLNEVSNSTA---------R : 111
          Ratus norvegicus SERPINH1 : STGLAFSLYQAMAKD-QAV--ENILLSPLVVASSLGLVSLGGKATTASQAKAVLSAEKLRDEEVHTGLGELLRSLSNSTA---------R : 123
             Mus musculus SERPINH1 : STGLAFSLYQAMAKD-QAV--ENILLSPLVVASSLGLVSLGGKATTASQAKAVLSAEKLRDEEVHTGLGELLRSLSNSTA---------R : 123
             Homo sapiens SERPINH1 : SAGLAFSLYQAMAKD-QAV--ENILVSPVVVASSLGLVSLGGKATTASQAKAVLSAEQLRDEEVHAGLGELLRSLSNSTA---------R : 124
          Pan troglodytes SERPINH1 : SAGLAFSLYQAMAKD-QAV--ENILVSPVVVASSLGLVSLGGKATTASQAKAVLSAEQLRDEEVHAGLGELLRSLSNSTA---------R : 124
              Felis catus SERPINH1 : SAGLAFSLYQAMAKD-QAV--ENILLSPVVVASSLGLVSLGGKAATASQAKAVLSAEQLRDEEVHAGLGELLRALSNSTA---------R : 124
               Bos taurus SERPINH1 : SAGLAFSLYQAMAKD-QAV--ENILLSPVVVASSLGLVSLGGKAATASQAKAVLSAEQLRDDEVHAGLGELLRSLSNSTA---------R : 124
              Orcinus orca SERPINH1 : SAGLAFSLYQAMAKD-QAV--ENILLSPVVVASSLGLVSLGGKAATASQAKAVLSAEQLRDEEVHAGLGELLRSLSNSTA---------R : 124
           Rhincodon typus SERPINH1 : TINLALNLYKTMVKD-RKLSSDNILFSPLVVASSLGVMSLGAKDKTANQVKSLLNIN-LRLDVFHPTFSELFNEVSNETT---------R : 131
        Petromyzon marinus SERPINH1 : NWAFAIDLYQSVAKAVPAM--ENVVLSPVLVASALGAAQLGASSGTASRLLKAINPSGLPGEGFHSGLAEVLGDLASQEEEAAAAAATWR : 147
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                                              *      200         *       220         *       240         *       260         *
Salmo salar SERPINH1A-1              : NVTWKISNRIYSPSSVNFADAFVKSSKKHYNYDHTKINFKDKKSAVKSINDWAAKSTDGKLPEVTKDVERTDGAMIINAMFFKPHWDEQF : 218
Salmo salar SERPINH1A-2              : NVTWKISNHLYSPSSVNFADAFVKSSKKHYKYDHTKINFKNKKSALKSINDWAAKSTDGKLPEVTKDVEKTDGAMIINAMFFKPHWDEQF : 206
Salmo salar SERPINH1B-1              : NVTWKISNRLYGPSSVTFADNFVKSSKKHYNYDHSKINLRDKRSAVNSINEWASKSTDGKLPEITKDVQNADGATIANAMFFKPHWDEKF : 202
Salmo salar SERPINH1B-2              : NVTWKISNRLYGPSSVTFADDFVKSSKKHYNYDHSKINLRDKRSAVNSINEWAAKSTNGKLPEITKDVQNADGAMIANAMFFKPHWDEKF : 201
Salvelinus alpinus SERPINH1A-1       : NVTWKISNRIYSPSSVNFADAFVKSSKKHYNYDHTKINFKDKKSAVKSINDWAAKSTDGKLPEVTKDVERTDGAMIINAMFFKPHWDEQF : 206
Salvelinus alpinus SERPINH1A-2       : NVTWKISNRLYTPSSVNFADAFVKSSKKHYKYDHTKINFKNKKSAVKSINAWAAKFTDGKLPEVTKDVEKTDGAMIINAMFFKPHWDEQF : 206
Salvelinus alpinus SERPINH1B-1       : NVTWKISNRLYGPSSVTFADNFVKSSKKHYNYDHSKINLRDKRSAVNSINEWAAKSTDGKLPEITKDVQNADGATIANAMFFKPHWDEKF : 202
Salvelinus alpinus SERPINH1B-2       : NVTWKISNRLYGPSLVTFADDFVKSSKKHYNYDHSKINLRDKRRAVNSIKEWAAKSTNGKLLEITKDVQNADGAMIANAMFFKPHWDEKF : 201
Esox lucius SERPINH1A                : NVTWKISNRLYGPSTVNFIDAFVKNSKKHYNYDHSKINFKDKKSAVKSINDWAAKSTDGKLPEVTKDVEKTDGAMIINAMFFKPHWDEQF : 212
Esox lucius SERPINH1B                : NVTWKISNRLYGPSSVNFDDNFVKSSKKHYNYDHSKINLRDKRSILNSINEWAAKSTDGKLPEITKDVPNADGATIANAMFFKPHWDEKF : 201
Xiphophorus maculatus SERPINH1A      : NVTWKISNRLYGPSSVTFAENFVKSSKKHYKCDHSKINFRDKKSAVNSINEWAAKSTGGKLPEVTKDVENTDGAMIINAMFFKPHWDEQF : 206
Xiphophorus maculatus SERPINH1B      : NSTWKINNRLYGPSSVSFADDFVKTSKKHYNYDHSKINFRDKRSAVNSINEWAAKSTDGKLPEITKDVQNPDGAMIVNAMFFKPHWDERF : 201
Oreochromis niloticus SERPINH1A      : NITWKISNRLYGPSSVTFVDDFVKSSKKHYNCEHSKINIRDKKSAVKSINEWAANSTDGKLPEVTKNVEKTDGAMIISAMFFKCHWDEQF : 206
Oreochromis niloticus SERPINH1B      : NTTWKINNRLYGPSSVSFADEFVKNSKKHYNYDHSKINFRDKRSAVNSINEWAAKSTGGKLPEVTKDVQNPDGAMIVNAMFFKPHWEEKF : 201
Thunnus maccoyii SERPINH1A           : NVTWKISNRLYGPSSVNFVDDFVKSSKKHYNYDHSKINFSDKKSAVNSINEWAAKSTDGKLPEVTKDVEKTDGAMIINAMFFKPHWDEQF : 206
Thunnus maccoyii SERPINH1B           : NTTWMINNRLYGPSSVSFADDFVKTSKKHYNYDHSKINFRDKRSAVNSINEWAAKSTGGKLPEITKDVQNADGAMIVNAMFFKPHWDEKF : 201
Gadus morhua SERPINH1A               : NVTWKIRSRLYGPSSITFTESFLKKSKKLYNCEHSKINFKDKKSAMKAINEWGVKSTDGKLPEVAKNVEKTDGAMIINAMFFKPHWNEQF : 206
Gadus morhua SERPINH1B               : NTTWKISNRLYGPSSVSFADAFVKDSKKRYNYDHSKINLKDKKSAVNSINEWAAASTNGKLPEVTKDVPNADGAMLINAMFFKPHWDEMF : 202
Danio rerio SERPINH1A                : NVTWKISNRFYGPSSVSFVDDFLKSSKKHYNYDHSKINFRDKRSAVKAINDWASKSTDGKLPEVTKDVEKTDGAMIINAMFYKPHWNEQF : 199
Danio rerio SERPINH1B                : NVTWKISNRLYGPSSVSFAEDFVKNSKKHYNYEHSKINFRDKRSAINSINEWAAKTTDGKLPEITKDVKNTDGAMIVNAMFFKPHWDEKF : 201
Anguilla anguilla SERPINH1A          : NVTWKISTHLYGPSSVKFANNFIKSSKKHYNCEHSKINFRDKRSAIKSINEWASKSTDGKVPEVTKDMDKTDGATIVNAMFFKPHWDEQF : 204
Anguilla anguilla SERPINH1B          : NVTWKISGRLYGPSSVNFADDFVKSSKKHYNYEHSKINFRDKRSALKSINEWAAKSTDGKLPEVTKDVDKTDGAMIVNAMFFKPHWDERF : 204
Lepisosteus oculatus SERPINH1_1      : NVTWKIGSRLYGPSSVNFADEFVKSSKKHYNYEHSKINFRDKRSAVKSINEWAAQTTDGKLPEVTKDVEKTDGAMIVNAMFFKPHWDEKF : 201
Lepisosteus oculatus SERPINH1_2      : NTTWKVGSRLYGPTSAELRQEFVEKSRKHYRHDHSKINFRDKRNALKSINEWAAENTGGRLSEITRDLPGSDGAMFVNAMHFKPHWGEAF : 201
Acipenser ruthenus SERPINH1_1        : NVTWKIGNRLYGPSAVTFTDAFVQSSKKHYNCEHAKINMRDKQGAVKALNEWAAHTTDGKLPEITKDVEETDGAIIANAMFFKPHWDEKF : 201
Acipenser ruthenus SERPINH1_2        : NVTWKIGSRLYGPSAVTFTDAFVQSSKKHYNCEHAKINMRDKQGAVKALNEWAAHTTDGKLPEITKDVEETDGAIIANAMFFKPHWDEKF : 201
Acipenser ruthenus SERPINH1_3        : NTTWKMGSRLYGPTSVNLRPQFVESSRKLYNHEHAKVNFRDKRRALQSINEWASQSTMGRVAEVTRELPGADGALFINAMYFKPHWEESF : 201
Acipenser ruthenus SERPINH1_4        : NTTWKMGSRLYGPTSVNLRPQFVESSRKLYNHEHAKVNFRDKRRALQSINEWASQSTMGRVAEVTRELPGADGALFINAMYFKPHWEESF : 201
Erpetoichthys calabaricus SERPINH1   : NVTWKIGNRLYGPSSVNFVDNFVQSSKKHYNCDHSKINFRDKRSALKSINEWAAKTTDGKLSEITKDVENTDGAMIVNAMYFKPHWHERF : 201
Polypterus senegalus SERPINH1        : NVTWKIGNRLYGPSSVNFVDNFVQSSKKHYNCDHSKINFRDKRSALKSINEWAAKTTDGKLSEITKDVENTDGAMIVNAMYFKPHWHERF : 201
Latimeria chalumnae SERPINH1         : NVTWKMGSRLYGPSSVTFADDFVKRSKKHYSIDHSKINFRDKRSALKSINDWAAKTTDGKLPEITKDVEKTDGAMIINAMFYKPHWDEKF : 201
Xenopus tropicalis SERPINH1          : NVTWKIGNRLYGPSSISFTDDFVKNSKKHYNYEHSKINFRDKRSTLRSINEWASQATDGKLPEVTSDMERTDGALIVNAMFFKPHWDERF : 246
Anas platyrhynchos SERPINH1          : NVTWKIGNRLYGPASINFADDFVKNSKKHYNYEHSKINFRDKRSALKSINEWAAQTTDGKLPEVTKDVEKTDGALIVNAMFFKPHWDEKF : 201
Gallus gallus SERPINH1               : NVTWKIGNRLYGPASINFADDFVKNSKKHYNYEHSKINFRDKRSALKSINEWAAQTTDGKLPEVTKDVEKTDGALIVNAMFFKPHWDEKF : 201
Alligator mississippiensis SERPINH1  : NVTWKIGNRLYGPSSISFADDFVKNSKKHYNYEHSKINFRDKRSALKSINEWASQTTNGKLPEVTKDVEKTDGALIVNAMFFKPHWDEKF : 201
Chelonia mydas SERPINH1              : NVTWKLGNRLYGPSSISFAEDFVKSSKKHYNYEHSKINFRDKRSALKSINEWASQTTNGKLPEVTTNVEKTDGALIVNAMFFKPHWEERF : 201
Ratus norvegicus SERPINH1            : NVTWKLGSRLYGPSSVSFADDFVRSSKQHYNCEHSKINFRDKRSALQSINEWASQTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDEKF : 213
Mus musculus SERPINH1                : NVTWKLGSRLYGPSSVSFADDFVRSSKQHYNCEHSKINFRDKRSALQSINEWASQTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDEKF : 213
Homo sapiens SERPINH1                : NVTWKLGSRLYGPSSVSFADDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAAQTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDEKF : 214
Pan troglodytes SERPINH1             : NVTWKLGSRLYGPSSVSFADDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAAQTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDEKF : 214
Felis catus SERPINH1                 : NVTWKLGSRLYGPSSVSFAEDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAAQTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDERF : 214
Bos taurus SERPINH1                  : NVTWKLGSRLYGPSSVSFAEDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAAQTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDERF : 214
Orcinus orca SERPINH1                : NVTWKLGSRLYGPSSVSFAEDFVRSSKQHYNCEHSKINFRDKRSALQSINEWAAQTTDGKLPEVTKDVERTDGALLVNAMFFKPHWDERF : 214
Rhincodon typus SERPINH1             : NTTWKIGSRLYGPNSVKFRDDFVQKSKMHYKHDHSKINLRDKRSTIQSINEWASQTTDGKLKEIVKDMSSVDGALFVNAMYFKPHWDEKF : 221
Petromyzon marinus SERPINH1          : NHTWKAASRVYAPSGVTFSQGFVSSSKARYGLQHDKVNLKDKRGALKALNEWAAQNTGGKVKEVAKELDGADGAVFVNALFFKGRWNEKF : 237
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                                            280         *       300         *       320         *       340         *       360
Salmo salar SERPINH1A-1              : HQKMVDNRGFLVSRSHTVGVPMMHRTGLYGFHEDTVNKILIILSIPLAHKKSSLVFFMPYHVESLERLEKLLTCKQLDDWMGKLKETAVAV : 308
Salmo salar SERPINH1A-2              : HHKMVDNRGFLVSHTHTVGVPMMHRTGIYGFHDDTVNKIFIILSMPLAHKKSSLVFFMPYHVEPLERLEKLLTRKQLEDWMGKLKETAVAV : 296
Salmo salar SERPINH1B-1              : HEKMVDNRGFLVTRSFTVSVPMMHRTGLYKFHDDTENRIFVIDMPLGQKQSSLVFIMPYHLEPLDRLEKLLTRKQLETWMGKMEERAVAI : 292
Salmo salar SERPINH1B-2              : HDEMVDNRGFLVTRSFTVSVPMMHRTGLYKFHDDTENMLFVIDMPLGQKQSSLVLIMPYHLEPLDRLEKLLTRKQLETWMGKMEERAVAI : 291
Salvelinus alpinus SERPINH1A-1       : HQKMVDNRGFLVSRSHTVGVPMMHRTGLYGFHEDTVNKILIILSIPLAHKKSSLVFFMPYHVESLERLEKLLTCKQLDDWMGKLKETAVAV : 296
Salvelinus alpinus SERPINH1A-2       : HHKMVDNRGFLVSHTHTVGVPMMHRTGIYGFHEDTVNKIFIILSIPLAHKKSSMVFFMPYHVEPLERLEKLLTRKQLEDWMGKLKETAVAV : 296
Salvelinus alpinus SERPINH1B-1       : HEKMVDNRGFLVTRSFTVSVPMMHRTGLYKFHDDTENRIFVIDMPLGQKQSSLVFIMPYHLEPLDRLEKLLTRKQLETWMGKMEERAVAI : 292
Salvelinus alpinus SERPINH1B-2       : HEKMVDNRGFLVTRSFTVSVPMMHRTGLYKFHDDTENMIFVIXMPLGQKQSSLVFIMPYHLEPLDRLEKLLTRKQLETWMGKMEERAVAI : 291
Esox lucius SERPINH1A                : HHAMVDNRGFLVPRSHTIGVPMMHRTGLYGFHEDTVNKIFIILSIPLAHKKSSLVFFMPYNVEPLERLEKLLTSKQLDTWMGKLKETAVAV : 302
Esox lucius SERPINH1B                : HEKMVDNRGFLVTRSFTISVPMMHRTGLYKFFDDTENRIFVIDMPLGQKQSSLVYIMPYHLEPLERLEKLLTRKQLDTWMGKLEERAVAI : 291
Xiphophorus maculatus SERPINH1A      : HPTMVDNRGFLVSGSYTVALQMMHRTGLFDFYDDTTNKLYLLSMPLAHKKSTVVFVMPHHMEPLDRVEKMLSKKQLDTWMGKMKQTAVAV : 296
Xiphophorus maculatus SERPINH1B      : HEKMVDNRGFLVTRSFTVGVSMMHRTGLYDFYEDKENNIYIINMPLGKKEASMILIMPYHLEPLDRLEKLLTKKQVDTWISKMENKAVAI : 291
Oreochromis niloticus SERPINH1A      : HREMVDNRGFMVSRSYTVSVQMMHRTGLFGFYDDKDNKLSILSMPLSHKKSTVVFIMPYHVEPLDRLEKMLTKKQVDRWIGLLKETAIAV : 296
Oreochromis niloticus SERPINH1B      : HDKMVDTRGFLVTRSFTVGVPMMHRTGLYDFYEDTENKIFVINMPLGQKQASMILIMPYHLEPLERLEKLLTRKQVDTWISKMENRAVAI : 291
Thunnus maccoyii SERPINH1A           : HHKMVDQRGFLVPHSYTVSVQMMHRTGLYNFYDDKTNKIYILSMPLAHKKSTVVFLMPYHVEPLERLEKMLTKKQLDTWMGKLQQTAIAV : 296
Thunnus maccoyii SERPINH1B           : HEKMVDNRGFLVSRSFTVGVPMMHRTGLYDFYEDTENRIFVINMPLGQKQASMILIMPYHLEPLERLEKLLTRKQVDTWISKMENKAVAI : 291
Gadus morhua SERPINH1A               : HPKMVDGRVFLASRGLTISVEMMHRTGLYDYYEDVDNKITVLSIPLAHKKSSVVFVMPLHVEPLERVEKLLSGKQLDTWVAKMEEKAVAV : 296
Gadus morhua SERPINH1B               : HESMVDSRSFMVTRSFTVGIPMMHRTGLYDFHEDTENRIFAINMPLGKNQASMILIMPYYLEPLERLEKLLTREQVDTWVGKMEKRAVAV : 292
Danio rerio SERPINH1A                : HHKMVDNRGFLVHRSFTVSVPMMHRTGIYGFLDDTTNKILVIEMPLAHKMSSLVLIMPYHVESLERVEKLLTRQQLNTWVSAMEQKAVAI : 289
Danio rerio SERPINH1B                : HHKMVDNRGFLVTRSHTVSVPMMHRTGIYGFYEDTENRFLIVSMPLAHKKSSMIFIMPYHVEPLDRLENLLTRQQLDTWISKLEERAVAI : 291
Anguilla anguilla SERPINH1A          : HHKLVDDRVFLVSPSFTVNIPMMHRTGIYSFHEDTKNSIYLLSMPLAHKMSSMIFIMPYHVEPLDRLEKLLTQEQLDTWIGSLEEKAVAI : 294
Anguilla anguilla SERPINH1B          : HHKMVDNRAFLVTRSFTISVPMMHRTGLYHFHEDTENKIYVINMPLAHKKSSMILIMPYHVEPLERLEKLLTPKQLDTWISKTEERAVAV : 294
Lepisosteus oculatus SERPINH1_1      : HHKMVDNRGFLVSRSYTVSVPMMHRTGLYMFHEDSEKRIYVLSMPLAHKMSSMIIIMPYHVEPLERLEKLLTRKQLDTWISKMEERAVAV : 291
Lepisosteus oculatus SERPINH1_2      : HHKMVDNRGFLMSKTHSVSVPMMHRTGFYKYYEDEVNQIQIVEMPLGHRQTSMMIIMPFHVEPLERLEKMLTKEQLSVWSNKLQERAVAV : 291
Acipenser ruthenus SERPINH1_1        : HHQMVDNRAFLVSRSFTVSVPMMHRTGFYRFHEDKENKIYVINMPLAHKKSSMILIMPFHVEPLERLEKLLSRKQLDTWISKLEETAIAL : 291
Acipenser ruthenus SERPINH1_2        : HHKMVDNRGFMVSRSFTVSVPMMHRTGFYRFHEDKENKIYVINMPLAHKKSSMILIMPFHVEPLERLEKLLSRKQLDTWISKLEETAIAL : 291
Acipenser ruthenus SERPINH1_3        : HDKMVDKRGFMTSRTHTVSVLMMHRTGYYKFYEDNDNRIYIIEMQLAHKHSSMVFIMPFYVEPLERVEKMLTKEQLNRWFSKLENRAVAL : 291
Acipenser ruthenus SERPINH1_4        : HDKMVDKRGFMTSRTHTVSVLMMHRTGYYKFYEDNDNRIYIIEMQLAHKHSSMVFIMPFYVEPLERVEKLLTKEQLNRWFSKLENRAVAL : 291
Erpetoichthys calabaricus SERPINH1   : HHKMVDNRGFLVSRSFTVSVPMMHRTGIYGFYEDTENHIYVIKIDLAHKMSSMIFVMPYHVEPLDRLEKLLTRKQVDIWISKLEEKAVAV : 291
Polypterus senegalus SERPINH1        : HHKMVDNRGFMVSRSFTVSVPMMHRTGIYGFYEDTENQIYVFKMDLAHKMSSMIFLMPYHVEPLDRLEKLLTRKQVDIWISKLEEKAVAV : 291
Latimeria chalumnae SERPINH1         : HEKMVDNRGFMVSRSYTVAVPMMHRTGLYHFYDDEVNKIQVIEMPLAHKLSSMLFVMPYHVEQLDRLEKLLTREQVNTWSSKLQQVAVAV : 291
Xenopus tropicalis SERPINH1          : HHQMVDNRGFMVTRSFTVSVPMMHRTGLYNYLEDEKNGIQIIEMPLAHKLSSMLFIMPHHVEPLERVEKLLTREQVKTWVGKMTKKAVAV : 336
Anas platyrhynchos SERPINH1          : HHKMVDNRGFMVSRSYTVGVPMMHRTGLYNYYDDETEKIQVVEMPLAHKLSSMIFIMPNHVEPLERVEKLLNREQLKTWAGKMKKRSVAI : 291
Gallus gallus SERPINH1               : HHKMVDNRGFMVTRSYTVGVPMMHRTGLYNYYDDEAEKIQVVEMPLAHKLSSMIFIMPNHVEPLERVEKLLNREQLKTWASKMKKRSVAI : 291
Alligator mississippiensis SERPINH1  : HHTMVDNRGFMVTRSFTVGVPMMHRTGLYNYFDDETEKIQIVEMPLAHKLSSMIFIMPNHVEPLERVEKLLTREQLKTWISKLKKRSVAI : 291
Chelonia mydas SERPINH1              : HHKMVDNRGFMVTRSYTVGVPMMHRTGLYNYFDDEAEKIQIVEMPLAHKLSSMIFIMPNHVEPLERVEKLLTREQLKTWIGKLKKRAVAI : 291
Ratus norvegicus SERPINH1            : HHKMVDNRGFMVTRSYTVGVTMMHRTGLYNYYDDEKEKLQLVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKTWMGKMQKKAVAI : 303
Mus musculus SERPINH1                : HHKMVDNRGFMVTRSYTVGVTMMHRTGLYNYYDDEKEKLQMVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKAWMGKMQKKAVAI : 303
Homo sapiens SERPINH1                : HHKMVDNRGFMVTRSYTVGVMMMHRTGLYNYYDDEKEKLQIVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKIWMGKMQKKAVAI : 304
Pan troglodytes SERPINH1             : HHKMVDNRGFMVTRSYTVGVMMMHRTGLYNYYDDEKEKLQIVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKIWMGKMQKKAVAI : 304
Felis catus SERPINH1                 : HHKMVDNRGFMVTRSYTVGVTMMHRTGLYNYYDDEKEKLQIVEMPLAHKLSSLIILMPHHVEPLERLEKLLTKEQLKIWMGKMQKKAVAI : 304
Bos taurus SERPINH1                  : HHKMVDNRGFMVTRSYTVGVTMMHRTGLYNYYDDEKEKLQMVEMPLAHKLSSLIIIMPHHVEPLERLEKLLTKEQLKVWMGKMQKKAVAI : 304
Orcinus orca SERPINH1                : HHKMVDNRGFMVTRSYTVGVTMMHRTGLYNYYDDEKEKLQIVEMPLAHKLSSLIIIMPHHVEPLERLEKLLTEEQLKTWMGKIQKKAVAI : 304
Rhincodon typus SERPINH1             : HDPLVDKRGFMVTRTHTTSVPMMHRTGLYNYYEDEVNKVQIIEMPLAHKFSSMILIMSRHVEPLERIEKLLTKEQLNNWFSKLQKRAMSV : 311
Petromyzon marinus SERPINH1          : HHQMVDTRGFLTSRSHTISIQMMHRTGFYNFYHDEKAQVQLIEMQLKGNLESLLIALPLHTESLERLEKLLTKQQLEEWTSKLQKKTIAV : 327
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                                              *       380       *       400       *       420       *       440       *
Salmo salar SERPINH1A-1                : SLPKVSMEVSHNIQKHLGELGLTEAVDKTKADLSNISGKKDLYLSNVFHASAMEWDTDGNPINTSIFGTDKLKNPKLFYADHPFIFLVKD : 398
Salmo salar SERPINH1A-2                : SLPKVCMEVSHNIQKHLGELGLTEAVDKTKADLSNISGKKDLYLSNVFHASAMEWDTDGNPIDRSIFGTDKLKNPKLFYADHPFIFLVKD : 386
Salmo salar SERPINH1B-1                : SLPKVSMEVSHNLQKTLGELGLTDAVDKTKADLSNISGKKDLYLSNVFHASSMEWDIEGNPFDTSIFGSEKLRNPKLFYADHPFIFLVKD : 382
Salmo salar SERPINH1B-2                : SLPKVSVEVSHNLQKTLGELGVTEAVDKTKADLSNISGKKDLYLSNVFHASAMEWDIEGNPFDTSIFGSEKLRNPKLFYADHPFIFLVKD : 381
Salvelinus alpinus SERPINH1A-1         : SLPKVSMEVSHNIQKHLGELGLTEAVDKTKADLSNISGKKDLYLSNVFHASAMEWDTDGNPINTSIFGTDKLKNPKLFYADHPFIFLVKD : 386
Salvelinus alpinus SERPINH1A-2         : SLPKVCMEVSHNIQKHLGELGLTEAVDKTKADLSNISGKKDLYLSNVFHASAMEWDTDGNPIDRSIFGTDKLKNPKLFYADHPFIFLVKD : 386
Salvelinus alpinus SERPINH1B-1         : SLPKVSMEVSHNLQKTLGELGLTDAVDKTKADLSNISGKKDLYLSNVFHASSMEWDIEGNPFDTSIFGSEKLRNPKLFYADHPFIFLVKD : 382
Salvelinus alpinus SERPINH1B-2         : SLPKVSVEVSHNLQKTLGELGLTEAVDKTKADLSNISGKKDLYLSNVFHASAMEWDIEGNPFDTSIFGSEKLRNPKLFYADHPFIFLVKD : 381
Esox lucius SERPINH1A                  : SLPKVSMEVSHNIQKHLGALGLTEAVDKKKADLSNISGKKDLYLSNVFHASAMEWGTDGNPIDTSVFGTDKLKNPKLFYADHPFIFLVKD : 392
Esox lucius SERPINH1B                  : SLPKINVEVSHNLQKTLGELGLTDAVDKTKADLSNISGKKDLYLSNVFHASAMEWDIDGNPYDTSIFGTEKLRNPKLFYADHPFIFLVKD : 381
Xiphophorus maculatus SERPINH1A        : SLPKVSMEVSHNLQKHLQDLGLTEAVDKSKADLSNVSGKKDLYLSGVLHAAALEWDTHGNEIDTSIFGTEKLKNPRLFYADHPFIFLIKD : 386
Xiphophorus maculatus SERPINH1B        : SLPKVSLEVSHNLQKYLAELGLTEAVDKSKADLSNISGKKDLYLSNVFHASALELDVDGNPYDTSIFGTDKLKNPKLFYVDHPFIFLVKD : 381
Oreochromis niloticus SERPINH1A        : SLPKVSMEVSHDLQKHLQELGLTEAVDKSKADFSNISGKKDLYLSSVFHASALEWDTDGNEIDTSIFGTDKLKNPKLFYADHPFIFLVKD : 386
Oreochromis niloticus SERPINH1B        : SLPKISVEVSHNLQKHLAELGLTEAVDKAKADLSNISGKKDLYLSSVFHASALELDIEGNPYDTSIFGTEKLRNPKLFYVDHPFIFLVKD : 381
Thunnus maccoyii SERPINH1A             : TLPKVSMEVSHNLQKHLEELGLTEAVNKSKADLSKISGKKDLYLSNVFHASAMEWDTAGNEMDPSIFGTDKLKNPRLFYADHPFIFLVKD : 386
Thunnus maccoyii SERPINH1B             : SLPKISVEVSHNLQKHLAELGLTEAVDKAKADLSNISGKKDLYLSNVFHASALELNIEGNPYDTSIFGSEKLRNPKLFYVDHPFIFLVKD : 381
Gadus morhua SERPINH1A                 : SLPKISMEVSHNLQKHLGELGLTDAVDKAKADLSNISGKKDLYLANVFHASAVEWATEGNPIDPTVYSSDKLKNPKLFYADHPFLFLVKD : 386
Gadus morhua SERPINH1B                 : SMPKVSMEVSHNLQKHLATLGLTEAVDKAKADLSNITGKKNLYLSNVFHASALELDTLGNPFDTSIFGSEKLRNPRLFYVDHPFIFLVKD : 382
Danio rerio SERPINH1A                  : SLPKVSMEVSHNLQKHLAELGLTEAVDKAKADLSNISGKKDLYLSNVFHASAMEWDTEGNPPDTSIFGTDQLKNPKLFYADHPFVFLVKD : 379
Danio rerio SERPINH1B                  : SLPKVSMEVSHDLQKHLGELGLTEAVDKSKADLSNISGKKDLYLSNVFHASSLEWDTEGNPFDPSIFGSEKMRNPKLFYADHPFIFLVKD : 381
Anguilla anguilla SERPINH1A            : SLPKVSMEVSHNLQKHLGELGLTEAVDKTKADLSNISGKKDLYLANVFHASALEWDTEGNPFDVSIFNTEKLKNPKLFYADHPFIFLVKD : 384
Anguilla anguilla SERPINH1B            : SLPKVSMEVSHNLQKNIAELGLTEAVDKAKADLSNISGKKDLYLANVFHASALEWDTEGNPFDTSIFGSDKLKNPKLFYADHPFIFVVKD : 384
Lepisosteus oculatus SERPINH1_1        : SLPKVCMEVSHNLQKHLGELGLTEAVDKAKADLSNISGKKDLYLSNVFHASAMELDTEGNPFDTNIYGSEKLRNPKLFYADHPFIFVIKD : 381
Lepisosteus oculatus SERPINH1_2        : SLPKINVDVNHELQKHLQELGLAEAVDKSKADFSGMTGKKDLHLSNFLHATAFDLDTDGNPFDQDIYGREEMRSPKLFYVDHPFIFLIQD : 381
Acipenser ruthenus SERPINH1_1          : SLPKVGMEVSHKLQKHLGELGLTEALDKSKADLSKVSGKKGLYVSNVFHASALEWDTDGNPFDTSIFGSEKMKNPRLFYADHPFIFLVKD : 381
Acipenser ruthenus SERPINH1_2          : SLPKVGMEVSHKLQKHLGELGLTEALDKSKADLSKVSGKKGLYVSNVFHASALEWDTDGNPFDTSIFGSEKMKNPRLFYADHPFIFLVKD : 381
Acipenser ruthenus SERPINH1_3          : SLPKINLDVSHELQKHLQELGLAEAVDKTKADFSGMTGKKDLHVSNVLHAAVLEWDTEGKPFTQDIYGQPEFKSPKLFYADHPFIFLVRD : 381
Acipenser ruthenus SERPINH1_4          : SLPKINLDVSHELQKHLQELGLAEAVDKTKADFSGMTGKKDLHVSNVLHAAVLEWDTEGNPFTQDIYGQLEFKSPKLFYADHPFIFLVRD : 381
Erpetoichthys calabaricus SERPINH1     : SLPKVELDVSHDLKKHLAELGMSEAVDKSKADFSKISGKKDLYLTNVFHASALEWDTEGNEYDASIYGTEKLRNPKLFYADHPFIFLVKD : 381
Polypterus senegalus SERPINH1          : SLPKVELDVSHDLKKHLAELGMSEAVDKSKADFSKISGKKDLYLTNVFHASALEWDTEGNEYDAGIYGTEKLRNPKLFYADHPFIFLVKD : 381
Latimeria chalumnae SERPINH1           : SLPKVSMEVSHELQKYLATLGLTEAVDKAKADLSTISGKKDLYLANVFHAAALEWDTEGNPFDSSIYSREEMRNPKLFYVDHPFVFLIKD : 381
Xenopus tropicalis SERPINH1            : SLPKVSLEVSHDLQKHLGDLGLTEAIDKTKADLSKISGKKDLYLASMFHAAALEWDTDGNPFDSDIYSREELRAPKLFYVDHPFVFLIKD : 426
Anas platyrhynchos SERPINH1            : SLPKVVLEVSHDLQKHLAGLGLTEAIDKSKADLSKISGKKDLYLSNVFHAAALEWDTEGNPYDADIYGREEMRNPKLFYADHPFVFLIKD : 381
Gallus gallus SERPINH1                 : SLPKVVLEVSHDLQKHLADLGLTEAIDKTKADLSKISGKKDLYLSNVFHAAALEWDTDGNPYDADIYGREEMRNPKLFYADHPFIFMIKD : 381
Alligator mississippiensis SERPINH1    : SLPKVSLEVSHDLQKHLADLGLTEAIDKNKADLSKISGKKDLYLSNVFHAAALEWDTEGNPFDADIYGREEIRNPRLFYADHPFIFLIKD : 381
Chelonia mydas SERPINH1                : SLPKVSLEVSHDLQKHLADLGLTEAMDKNKADLSKISGKKDLYLSNVFHAAALEWDTEGNPFDADIYGREEMRNPKLFYADHPFVFVIKD : 381
Ratus norvegicus SERPINH1              : SLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFEWDTEGNPFDQDIYGREELRSPKLFYADHPFIFLVRD : 393
Mus musculus SERPINH1                  : SLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFEWDTEGNPFDQDIYGREELRSPKLFYADHPFIFLVRD : 393
Homo sapiens SERPINH1                  : SLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFELDTDGNPFDQDIYGREELRSPKLFYADHPFIFLVRD : 394
Pan troglodytes SERPINH1               : SLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFELDTDGNPFDQDIYGREELRSPKLFYADHPFIFLVRD : 394
Felis catus SERPINH1                   : SLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFEWDTEGNPFDQDIYGREELRSPKLFYADHPFIFLVRD : 394
Bos taurus SERPINH1                    : SLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFEWDTDGNPFDQDIYGREELRSPKLFYADHPFIFLVRD : 394
Orcinus orca SERPINH1                  : SLPKGVVEVTHDLQKHLAGLGLTEAIDKNKADLSRMSGKKDLYLASVFHATAFEWDTDGNPFDQDIYGREELRSPKLFYADHPFIFLVRD : 394
Rhincodon typus SERPINH1               : SLPKVNIEVSHELQKYMQELGLTEAVDKDKADFSGITGKKDLHLSNMLHVTAIEWNTEGNPYDTDLHSHESMKTPKLFYADHPYIFLVRD : 401
Petromyzon marinus SERPINH1            : SMPKGLLQGSADIKNSLADLGLAEVGDKAKADFSGMTGGREIHIGSILHTAALEFDTEGEEYDMSVHGHPDMRNPHLFYIDHPFFFLVRD : 417
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                                              460       *
Salmo salar SERPINH1A-1                : TKTNSILFLGRLVRPKGEKMRDEL : 422
Salmo salar SERPINH1A-2                : TKTNSILFLGRLVRPKGKKMRDEL : 410
Salmo salar SERPINH1B-1                : NKTNSILFIGRMVRPKGDKMRDEL : 406
Salmo salar SERPINH1B-2                : NKTNSILFIGRMVRPKGDKMRDEL : 405
Salvelinus alpinus SERPINH1A-1         : TKTNSILFLGRLVRPKGEKMRDEL : 410
Salvelinus alpinus SERPINH1A-2         : TKTNSILFLGRLVRPKGKKMRDEL : 410
Salvelinus alpinus SERPINH1B-1         : NKTNSILFIGRMVRPKGDKMRDEL : 406
Salvelinus alpinus SERPINH1B-2         : NKTNSILFIGRMVRPKGDKMRDEL : 405
Esox lucius SERPINH1A                  : TKTNSILFLGRLVRPKGDKIRDEL : 416
Esox lucius SERPINH1B                  : NKTNSILFIGRIVRPKGDKMRDEL : 405
Xiphophorus maculatus SERPINH1A        : KKTNSILFIGRMVRPKGEKMRDEL : 410
Xiphophorus maculatus SERPINH1B        : NKTNSILYIGRVVRPKGDKMRDEL : 405
Oreochromis niloticus SERPINH1A        : QKTSSILFIGRMVRPKGEKMRDEL : 410
Oreochromis niloticus SERPINH1B        : NKTNSILYIGRVVKPKGDKMRDEL : 405
Thunnus maccoyii SERPINH1A             : QKTNSILFIGRMVRPKGEKMRDEL : 410
Thunnus maccoyii SERPINH1B             : NKTNSIMYIGRVVRPKGDKMRDEL : 405
Gadus morhua SERPINH1A                 : LKTKSILYMGRMVRPKGDKIRDEL : 410
Gadus morhua SERPINH1B                 : NKSGAIMYIGRMVKPNGDKMRDEL : 406
Danio rerio SERPINH1A                  : NKTNSILFMGRLIRPKGDKMRDEL : 403
Danio rerio SERPINH1B                  : NKTNSILFIGRLVRPKGDKMRDEL : 405
Anguilla anguilla SERPINH1A            : NKTKSILLIGRLVRPKGEKMRDEL : 408
Anguilla anguilla SERPINH1B            : NKTNSILFIGRLMRPKGDKMRDEL : 408
Lepisosteus oculatus SERPINH1_1        : NKTNSILYIGRLVRPKGDKMRDEL : 405
Lepisosteus oculatus SERPINH1_2        : KKTNSILLIGRLVKPNGDGNHDEL : 405
Acipenser ruthenus SERPINH1_1          : NESDSILFIGRLVRPKGEKMRDEL : 405
Acipenser ruthenus SERPINH1_2          : NESNSILFIGRLVRPKGEKMRDEL : 405
Acipenser ruthenus SERPINH1_3          : NKTNSILLIGRLVKPKGEN-HDEL : 404
Acipenser ruthenus SERPINH1_4          : NKTNSILLIGRLVKPKGEN-HDEL : 404
Erpetoichthys calabaricus SERPINH1     : NKSNSILFIGRLQRPKGDKMRDEL : 405
Polypterus senegalus SERPINH1          : NKSNSILYIGRLQRPKGDKMRDEL : 405
Latimeria chalumnae SERPINH1           : NKTNSILFIGRLVKPKGEKMRDEL : 405
Xenopus tropicalis SERPINH1            : EKTDSILFIGRLVRPKGDKIRDEL : 450
Anas platyrhynchos SERPINH1            : SKTNSILFIGRLVRPKGDKMRDEL : 405
Gallus gallus SERPINH1                 : SKTNSILFIGRLVRPKGDKMRDEL : 405
Alligator mississippiensis SERPINH1    : NKTNSILFIGRLVRPKGDKMRDEL : 405
Chelonia mydas SERPINH1                : NKTNSILFIGRLVKPKGDKMRDEL : 405
Ratus norvegicus SERPINH1              : NQSGSILFIGRLVRPKGDKMRDEL : 417
Mus musculus SERPINH1                  : NQSGSILFIGRLVRPKGDKMRDEL : 417
Homo sapiens SERPINH1                  : TQSGSILFIGRLVRPKGDKMRDEL : 418
Pan troglodytes SERPINH1               : TQSGSILFIGRLVRPKGDKMRDEL : 418
Felis catus SERPINH1                   : TQSGSILFIGRLVRPKGDKMRDEL : 418
Bos taurus SERPINH1                    : TQSGSILFIGRLVRPKGDKMRDEL : 418
Orcinus orca SERPINH1                  : TQSGSILFIGRLVRPKGDKMRDEL : 418
Rhincodon typus SERPINH1               : KKTNSILLIGKLVKPKGNN-HDEL : 424
Petromyzon marinus SERPINH1            : ARSGATLLIGRCMRPMGSGRHDEL : 441
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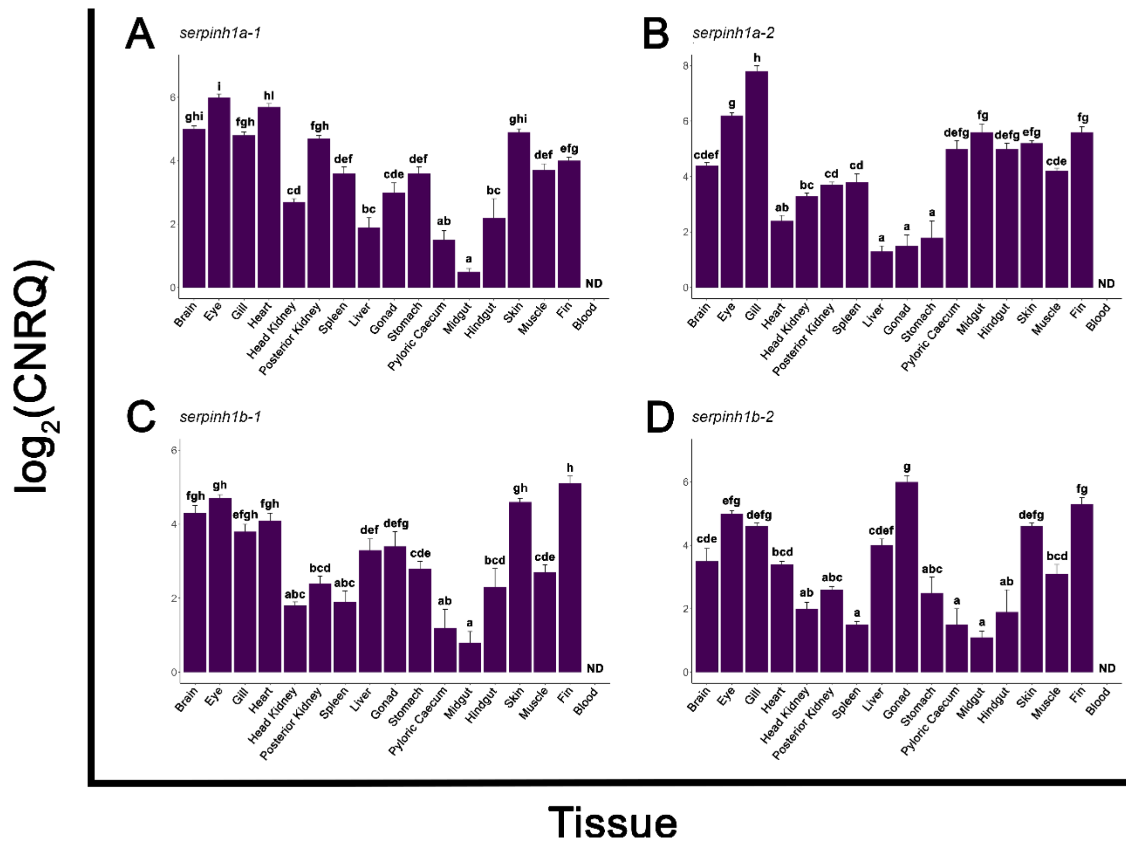
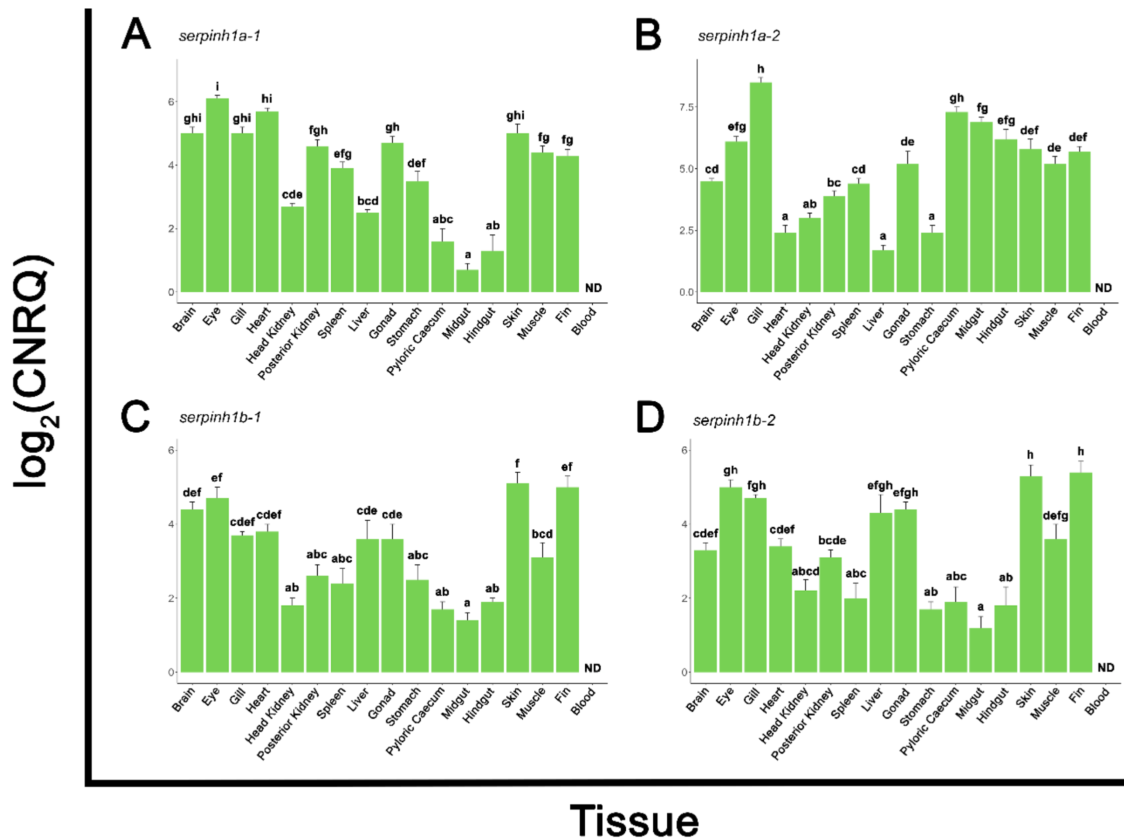
Appendix E Figure E-6. Multiple sequence alignment of Atlantic salmon SERPINH1 paralogue amino acid sequences with putative orthologous sequences. Sequences were obtained from the NCBI non-redundant protein database (see Appendix E Table E-1). As identified by GeneDoc, black shading with white font shows the 90-100% conserved residues amongst the putative orthologous sequences. Dark gray shading with white font, and light gray with black font, denote 80 and 60% conservation amongst the residues, respectively. Conservative substitutions are treated as identical based on GeneDoc similarity scores. The numbers above the sequences show the relative positions of the amino acid residues of SERPINH1 putative orthologues. The Serpin domain was predicted using the InterPro database (https://www.ebi.ac.uk/interpro/) and is indicated by the red line. *Salmo salar* (Atlantic salmon), *Salvelinus alpinus* (Arctic charr), *Esox lucius* (northern pike), *Xiphophorus maculatus* (southern platyfish), *Oreochromis niloticus* (Nile tilapia), *Thunnus maccoyii* (southern bluefin tuna), *Gadus morhua* (Atlantic cod), *Danio rerio* (zebrafish), *Anguilla anguilla* (European eel), *Lepisosteus oculatus* (spotted gar), *Acipenser ruthenus* (sterlet sturgeon), *Erpetoichthys calabaricus* (reedfish), *Polypterus senegalus* (Senegal bichir), *Latimeria chalumnae* (West Indian Ocean coelacanth), *Xenopus tropicalis* (tropical clawed frog), *Anas platyrhynchos* (mallard duck), *Gallus gallus* (chicken), *Alligator mississippiensis* (American alligator), *Chelonia mydas* (green sea turtle), *Rattus norvegicus* (Norway rat), *Mus musculus* (mouse), *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Felis catus* (domestic cat), *Bos taurus* (cow), *Orcinus orca* (orca), *Rhincodon typus* (whale shark), *Petromyzon marinus* (sea lamprey).



Appendix E Figure E-7. Constitutive expression of A) *serpinh1a-1*, B) *serpinh1a-2*, C) *serpinh1b-1* and D) *serpinh1b-2* in different tissues of adult female Atlantic salmon (n=4). Calibrated normalized relative quantities (CNRQ) are presented as means ± 1 standard error. Values without a letter in common are significantly ($p < 0.05$) different between tissues (1-way ANOVA). In all cases, the letter ‘a’ signifies the lowest value within a comparison. ND = not detected.



Appendix E Figure E-8. Constitutive expression of A) *serpinh1a-1*, B) *serpinh1a-2*, C) *serpinh1b-1* and D) *serpinh1b-2* in different tissues of adult male Atlantic salmon (n=4). Calibrated normalized relative quantities (CNRQ) are presented as means ± 1 standard error. Values without a letter in common are significantly ($p < 0.05$) different between tissues (1-way ANOVA). In all cases, the letter ‘a’ signifies the lowest value within a comparison. ND = not detected.