Low Temperatures Typical of Winter Cage-Site Conditions in Atlantic Canada Impact the Growth, Physiology, Health and Welfare of Atlantic Salmon (*Salmo salar*)

by

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Abstract

Limited research has been conducted on the physiology of Atlantic salmon (*Salmo salar*) at cold temperatures despite significant mortalities during the winter at sea-cages in Atlantic Canada. Thus, in this thesis, I exposed post-smolt cultured Atlantic salmon to a seasonal decline in temperature from 8 to 1°C (at 1°C week^{-1}), and a 'cold-shock' from 3 to 0°C for 4 or 24 hours. During the seasonal decline in temperature, feeding decreased starting at 6°C (and ceased by 1-2°C), osmoregulatory changes and increases in heat shock protein expression began at 4-5°C, and at 1°C elevated plasma cortisol levels indicative of mild stress were measured. The 'cold-shocks' resulted in a relatively small stress response (i.e., increases in plasma cortisol and glucose), but no other adverse effects or mortalities. Nonetheless, a number of mortalities/moribund fish were noted when various groups of Atlantic salmon were held for long periods at $\leq$ 8°C. Moribund fish were lethargic and swam erratically, had enlarged livers and plasma enzymes suggestive of liver damage, and developed ulcers to the head/jaw. The former symptoms are indicative of 'Winter Syndrome' described in gilthead sea bream (*Sparus aurata*) aquaculture, and these ulcers have previously been observed in Norway and associated with tenacibaculosis.

General Summary

Salmon aquaculture is an important industry in Atlantic Canada, and members of the sector are calling for increased research to limit the impacts of climate change. However, there has been limited focus on the effects of cold seawater temperatures associated with winter conditions and storms, despite the reports of large numbers of salmon cage-site mortalities after periods of intense cold. Thus, I held salmon in tanks and exposed them to both long-term and short-term decreases in temperature similar to what they would experience at Atlantic Canada's cage-sites to better understand how they respond to cold temperatures, and to determine if I could identify possible causes of mortality. I found that salmon can survive exposure to temperatures close to 0˚C. However, I also observed that a number of fish experienced health issues that were serious enough to result in mortalities, and performed analyses to identify/hypothesize the cause(s).

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Co-Authorship Statement

The research presented in this M.Sc. thesis was performed by Émile Vadboncoeur, under the supervision of Dr. A. Kurt Gamperl. Émile Vadboncoeur shared in the identification of the research topic and in the design of experiments, and was primarily responsible for: the rearing and care of experimental animals; conducting the described experiments; collecting and analyzing the data; and writing the first draft of all chapters and other sections of this thesis. This research was only possible through collaboration with many individuals.

Rebeccah M. Sandrelli[1] assisted in the running of all experiments, ensured access to materials for research and fish care, and provided edits for **Chapter 2**. Eric H. Ignatz[1] assisted in the experiments in **Chapters 3 and 4**. Kathy A. Clow[1] provided training on the biochemical analyses conducted in **Chapters 2 to 4,** and edited **Chapter 2**. Charlotte Nelson[2] conducted all chloride, osmolality and Na^+-K^+-ATPase activity measurements in Dr. Collin Brauner's lab, and Dr. Brauner[2] provided resources to conduct those analyses; they both provided edits/feedback on **Chapter 2**. Jennifer R. Hall[3] provided training in every facet of the qPCR assays and analysis, and provided edits on **Chapter 2**. Dr. Andrew K. Swanson[4] provided feedback throughout this M.Sc. thesis, and edited **Chapter 2**. Dr. A. Kurt Gamperl[1] provided supervision and input throughout the research program (including the identification of research topics and experimental design, while the experiments were being conducted, during data collection and analysis, and provided edits on all sections of this thesis).

From this thesis, I will be submitting three manuscripts for publication:

Authorship for the publication derived from **Chapter 2** is: Émile Vadboncoeur, Charlotte Nelson, Jennifer R. Hall, Kathy A. Clow, Rebeccah M. Sandrelli, Colin J. Brauner, Andrew K.

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List of Abbreviations and Symbols

°C	Degree centigrade
actb	Beta-actin
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP	Adenosine monophosphate
AST	Aspartate aminotransferase
BLAST	Basic local alignment search tool
bp	Base pair
CBC	Canadian Broadcast Company
cDNA	Complementary deoxyribonucleic acid
CF	Condition factor
cirbp – CIRBP	Cold inducible RNA-binding protein
CK	Creatine kinase
Cl^-	Chloride
CT	Cycle threshold
CT_{max}	Critical thermal maximum
CWV	Cold-water vibriosis
D	Day
DHA	Docosahexaenoic acid (22:6n-3)
e.g.	For example
ef1a	Elongation factor 1 alpha
ef1a1	Eukaryotic Translation Elongation Factor 1 Alpha 1
ef1a2	Eukaryotic Translation Elongation Factor 1 Alpha 2
eif3d	Eukaryotic translation initiation factor 3 subunit D
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid (20:5n-3)
FAO	Food and Agriculture Organization
FC	Feed consumption
g	Grams
h	Hours
hp	Horsepower
HCl	Hydrochloric acid
HCO_3	Bicarbonate
HSI	Hepatosomatic index
HSP	Heat-shock protein
hsp70	heat-shock protein 70kDa
hsp90	heat-shock protein 90kDa
I	Initial number of fish
i.e.	That is
IPCC	Intergovernmental Panel on Climate Change
ISAV	Infectious salmon anaemia virus
IT_{max}	Incremental thermal maximum

k	Fulton's condition factor
K^+	Potassium
kg	Kilogram
L	Length
l	Litre
LDH	Lactate dehydrogenase
M	Cumulative mortality
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minute
µl	Microlitre
ml	Millilitre
mM	Millimolar
mOsm	Milliosmol
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
MT	Megaton
Na^+	Sodium
NADH - NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCBI	National Center for Biotechnology Information
ng	Nanogram
NKA	Na^+-K^+-ATPase
NL	Newfoundland and Labrador
nm	Nanometer
NS	Nova scotia
NTC	Non-template control
OB	Ouabain
OD	Optical density
p	P-value
pabpc1	Polyadenylate-binding protein 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
ppt	Parts per thousands
PUFA	Poly-unsaturated fatty acid
QC	Quality control
qPCR	Real-time quantitative polymerase chain reaction
r^2	Correlation coefficient
ROS	Reactive oxygen species
rpl32	60S ribosomal protein 32
RQ	Relative quantity
RVI	Regulatory volume increase

serpinh1	Serpin Family H Member 1
SGR	Specific growth ate
spp.	Several species
t	Tonne
TOI	Transcript of interest
U	Enzyme unit
U_{crit}	Critical swimming speed
UV	Ultra-violet
W_f	Final weight
W_i	Initial weight
W_l	Liver weight
xg	Times gravitational force
µg	Microgram
µl	Microlitre

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CHAPTER 1: General Introduction

1.1 Atlantic salmon culture and its challenges

Given the steady increase in the world's population, the demand for aquatic-based protein is on the rise. However, capture fisheries' landings (i.e., those based on wild fishes and shellfish) have not increased since the 1990's, and thus, aquaculture is seen as the solution to meet this demand for seafood (FAO, 2022). Aquaculture production currently provides just over one-half of the world's seafood (FAO, 2022). The Atlantic salmon (*Salmo salar*) represents 32.5% of global marine finfish aquaculture production, and is the fourth most economically important farmed species (FAO, 2022). In Canada, sea-cage reared Atlantic salmon accounted for 70% of all aquaculture production in 2020 (120,000 tonnes; t). This production mainly occurs in British Columbia and the Atlantic provinces (DFO, 2022), with salmon aquaculture production in Newfoundland restricted to the south coast of the island and involving major multi-national corporations (e.g., MOWI, Cooke Aquaculture Inc., and Grieg NL). Salmon production in the province of Newfoundland and Labrador (NL) increased from approximately 3,000 to 22,000 t between 2003 and 2013, and projections suggested that NL salmon production would soon reach approximatively 50,000 t ("Support Growth of the Aquaculture Industry to 50,000 MT Annually for Salmon and 10,750 MT Annually for Mussels - The Way Forward," 2018). However, production has actually decreased over the past several years due to a number of challenges that need to be addressed to ensure the growth and sustainability of the industry (Government of Newfoundland and Labrador, 2022).

These challenges are both biotic and abiotic. The two main biotic challenges facing the aquaculture industry in Atlantic Canada are infectious salmon anemia virus (ISAv) and sea lice (Gagné and LeBlanc, 2018; Medcalf et al., 2021). Development of

effective treatments for sea lice is ongoing (Jackson et al., 2018), and the use of lumpfish and the ballan wrasse as 'cleaner fish' has great potential as a method of biological control of this parasite; although there are several sustainability issues that have been identified (Geitung et al., 2020). However, there are currently no effective treatments or vaccines for ISAv, and laws require the culling of affected populations when the disease is identified (Gagné and LeBlanc, 2018). The major abiotic challenge to the aquaculture industry in Atlantic Canada and worldwide is climate change (Barange et al., 2018; Galappaththi et al., 2020; Maulu et al., 2021), and the combination of the above challenges can lead to major mortality events. For example, in 2019, a heat wave and high sea lice levels and their subsequent treatment led to a loss of 2.6 million salmon on the south coast of Newfoundland (Burke et al., 2020).

1.2 Temperature and Atlantic salmon

Temperature has often been described as the 'master' environmental factor with regards to the regulation of fish physiology (Brett, 1971). In the wild, Atlantic salmon can be found at a wide range of temperatures. For example, Strøm et al. (2020) reported Atlantic salmon at sea experienced temperatures from -0.5 to 12.9°C in northern Norway, with maximum daily fluctuations of 9.6°C. Whereas, in freshwater streams, they can experience temperatures from 0 to 27-30°C (Caissie et al., 2014; Gallant et al., 2017; Lund et al., 2002). The thermal biology of Atlantic salmon has also been studied in aquaculture settings. The temperature where Atlantic salmon growth is optimal is approximately 13°C for adult fish (Hevrøy et al., 2013), and the optimal temperature range for post-smolts is from 11.6 to 19.1°C (Elliott and Elliott, 2010). However, outside

of these temperatures, performance declines and temperature becomes a stressor, with mortalities at high temperatures beginning above 20˚C (Anttila et al., 2015; Elliott, 1991; Elliott and Elliott, 1995; Gallant et al., 2017; Gamperl et al., 2020; Wade et al., 2019).

Atlantic salmon reared for commercial production are maintained in tanks on land in relatively controlled environmental conditions from the egg until their transfer to sea-cages as smolts where they will grow until they reach market size (~4 to 6 kg). Site selection for these cages is a crucial step to ensure the growing fish will be protected from the harshest weather conditions. Given that Atlantic salmon are reared in several regions of the world, they are exposed to a variety of thermal ranges during the growout phase. Tasmania is the warmest region, where temperatures in Atlantic salmon sea-cages can exceed 20°C for several weeks and surface temperatures can reach 23˚C (Stehfest et al., 2017; Wade et al., 2019). Other regions with significant Atlantic salmon production at sea (Canada, Chile, and Northern Europe) have similar maximum temperatures, between 18 and 20°C (Gamperl et al., 2021; Madaro et al., 2021; Narváez et al., 2019). However, minimum temperatures are quite different. Fish reared in Tasmania and Chile only experience temperatures down to 6-9°C (Dempster et al., 2016; Narváez et al., 2019; Soto and Norambuena, 2004). Whereas, the lowest temperatures in Norway vary from 2 and 6°C given the large latitudinal range of the coastline (Falconer et al., 2020; Geitung et al., 2020; Korsøen et al., 2009; Madaro et al., 2021; Oppedal et al., 2011; Sandnes et al., 1988), and temperatures in Atlantic Canada and Iceland are below 5°C for 6 months, can be 1-2°C for several weeks, and can reach temperatures of 0˚C or lower [Björnsson et al., 2007; Sandrelli and Gamperl (In Prep; see Chapter 3)].

1.3 Culture of Atlantic salmon at low temperature

High temperatures have been the focus of research with the goal of understanding and mitigating the effects of climate change on finfish production (Barange et al., 2018; Galappaththi et al., 2020; Maulu et al., 2021). Climate change is generally thought of as an increase in average temperatures, and/or in the frequency and intensity of heat waves (IPCC, 2022), and both of these phenomenon have been the focus of many studies on cultured Atlantic salmon over the last decade (Beemelmanns et al., 2021c, 2021b, 2021a; Gamperl et al., 2020; Gerber et al., 2021; Stehfest et al., 2017; Wade et al., 2019). However, low temperatures can have significant negative impacts on aquaculture production, including reduced growth, the loss of appetite, and mortalities; all of which can result in economic losses (Handeland et al., 2008; Reid et al., 2022; Spilsberg et al., 2022). Even though the industry plans to grow in regions where low (winter) temperatures are a challenge (e.g., Newfoundland), there is limited knowledge on the physiological responses of salmon to cold (< 5˚C) temperatures, and the causes of mortality at cage-sites at low temperatures. In contrast to high temperatures, there is an absolute lower limit at which salmon can survive. Indeed, the freezing point of a salmon's body fluids ranges between -0.5 and -1.0°C (Fletcher et al., 1988; Hurst, 2007). Despite the industry's best efforts over the past few years, several winter mortality events have been recorded in Atlantic Canada and Iceland. The explanations given for these winter mortality events were temperature, weather, and poor fish and site management. Some of these winter mortality events clearly report temperatures of approximately the freezing point of Atlantic salmon plasma and extracellular fluids (Huffman, 2019; Pennell, 2014; Willick, 2019). However, others report mortalities related to severe winter

storms (CBC News, 2020), and farmers in Iceland reported that cold sea temperatures in 2020 caused salmon to move to the bottom of their sea-cages, and that rubbing against the netting eventually caused wounds that led to the fish's death (Ćirić, 2020). Similarly, the loss of 1500 – 2000 t of adult Atlantic salmon was due to low temperatures and 'reduced fish heath' (Moore, 2022); moribund salmon from the latter event floated to the surface, and mortalities removed from the cages had 'winter sores' and 'wounds' to the head so severe that the 'head was gone' (Stundin, 2022).

1.4 Low temperatures and salmon: gaps in current knowledge

It was established almost 5 decades ago that the lower temperature limit for salmonids (including Atlantic salmon) in seawater is ~1°C based on poor growth and the risk of mortality (Saunders et al., 1975). However, there has been surprisingly limited effort to date to understand: i) the temperature at which sub-lethal effects occur, and their subsequent severity; and ii) the most effective/appropriate genetic, genomic or protein biomarkers for assessing cold effects / cold tolerance in salmon, as well as for use in broodstock selection programs to increase cold tolerance. Further, the only two studies that have examined such questions have provided contradictory information. Sandnes et al. (1988) sampled Atlantic salmon at a cage-site in Norway between October (7.7°C) and May (6.9°C), with temperatures decreasing to 2°C in February. Blood analyses showed that alkaline phosphorylase, aspartate aminotransferase and creatinine (markers of tissue damage and organ failure) actually decreased slightly at the coldest temperatures, while all other measured blood parameters were unchanged over the course of the study and were within the range considered normal for healthy fish. In contrast,

Liu et al. (2020) performed a lab-based study on Atlantic salmon parr in China where water temperature was lowered from 16°C to 1°C over 6 weeks, and reported a significant increase in all parameters measured (glucose, lactate dehydrogenase, creatine kinase, alkaline phosphatase and aspartate transaminase) between 6°C and 4°C, and that they continued to increase as temperature declined. It is unclear whether these disparate data are due to population or age (size) differences in how Atlantic salmon respond to cold temperatures, or the quite different environments/experimental protocols under which these samples were taken. Furthermore, the reported plasma parameters in these publications are indices of tissue damage, and thus, provide no information on when the fish become stressed by declining temperatures, and/or whether cold exposure is concomitant with a disturbance in the fish's osmotic/ionoregulatory balance.

1.5 Effects of low temperatures on cultured fish

While information on the physiological impacts of gradual declines in temperature on Atlantic salmon is still ambiguous, low temperatures have been identified as a problem for the production of many other species including Nile tilapia (*Oreochromis niloticus*; Hassaan et al., 2019; Panase et al., 2018), European seabass (*Dicentrarchus labrax*; Islam et al., 2021, 2020; Zhang et al., 2021), orange-spotted grouper (*Epinephelus coloides*; Sun et al., 2019), yellow drum (*Nibea albiflora*; Jiao et al., 2020; Song et al., 2019; Zhu et al., 2020) and gilthead sea bream (*Sparus aurata*; Gallardo et al., 2003; Ibarz et al., 2010; Melis et al., 2017). In general, these studies show that low temperatures reduce feeding and growth, and that significant stress can lead to difficulties in meeting energy demands at low temperature and that this can result in increased

mortalities. Further, there is accumulating data which shows that low temperatures cause lipid accumulation in the liver, increased liver size (hepatosomatic index, HSI) and liver dysfunction. The most extensive volume of research has been conducted on gilthead sea bream where the impacts on production in the northern Mediterranean have been significant, and this metabolic condition at low temperatures is referred to as 'Winter Syndrome' [see Ibarz et al., (2010) for a review]. With regards to Atlantic salmon, they have been reported to suffer from a condition formerly called 'Hitra disease' and now known as 'cold water vibriosis' that results in enlarged livers at low temperature. However, research on this disease was primarily focused on the causative infectious agents and not on the physiological response of the Atlantic salmon when reared at low temperatures. *Aliivibrio salmonicida* was identified as the causative agent of the disease (Egidius et al., 1986; Holm et al., 1985; Urbanczyk et al., 2007) and, in addition to an increase in HSI, it causes hemorrhages to most internal organs (Egidius et al., 1984, 1981; Poppe et al., 1985).

Another condition at low temperature that has been increasingly problematic for salmon production is skin ulcers, which have received considerable research attention since the 1990's (Lunder et al., 1995). Two main diseases have been associated with the appearance of skin ulcers. The first one is 'Winter ulcer' in which *Moritella viscosa* is believed to be the primary infectious agent (Karlsen et al., 2014). A vaccine has been developed for this disease, and is administered to Atlantic salmon prior to moving them into sea-cages (Karlsen et al., 2017). The second disease is 'Tenacibaculosis' caused by *Tenacibaculum spp.* (Nowlan et al., 2021; Småge et al., 2018; Spilsberg et al., 2022). These two conditions have been associated with temperatures below 8-10°C in

Norwegian salmon aquaculture (Lunder et al., 1995; Olsen et al., 2011). However, the appearance of skin ulcers has also been reported in Atlantic Canada in the summer and fall when water temperatures are above 10-12˚C (MacKinnon et al., 2020, 2019); although the pathogen responsible for this condition has not been definitively identified to date. With many pathogens potentially affecting Atlantic salmon in winter, it becomes important to understand how low temperature may negatively impact a fish's capacity to maintain homeostasis, and cause chronic stress and suppress immune functions.

Finally, winter mortalities at cage-sites are also reported in association with harsh winter conditions and winter storms (CBC News, 2020), with the latter expected to increase in frequency and intensity, and result in episodes of rapid cooling as deeper waters mix with surface waters in contact with cold air (i.e., episodes of 'cold-shock' or 'super chill') (Szekeres et al., 2016). We know that fish experience stress when exposed to rapid declines in water temperature (Donaldson et al., 2008; Reid et al., 2022). However, there is little information on the impacts of 'cold-shock' (i.e., exposure to temperatures near 0˚C) or its duration on Atlantic salmon physiology or survival, or on what the most appropriate and effective biomarkers are for assessing fish health or welfare at cold temperatures.

1.6 Research goals

There is much to understand about the physiology of Atlantic salmon reared at low water temperatures, especially in the context of aquaculture. In this thesis, I examine two temperature regimes that emulate winter conditions in Newfoundland sea-cages. First, in Chapter 2, salmon were exposed to a seasonally relevant cold-exposure (8 to 1°C

in 7 weeks) to observe the physiological changes that occur when seawater temperatures are slowly lowered. Second, in Chapter 3, I exposed salmon to an acute 'cold-shock' (of 4 and 24 h in duration) so that I could measure physiological changes when temperature is rapidly lowered to close to 0°C. These two regimes were specifically chosen with the goal(s) of identifying if such conditions result in mortalities, and to examine the physiological changes associated with these two different decreases in water temperature. The salmon's plasma and liver were sampled throughout the two cold-exposures, with the plasma analyzed for several biomarkers that have been shown to be good indicators of cold-stress in multiple species (Donaldson et al., 2008; Reid et al., 2022). The plasma was analyzed for changes in ion (Cl^-, Na^+ and Cl^-) concentration and osmolality since cold-stressed fish are known to experience changes in plasma ion levels (Islam et al., 2021; Staurnes et al., 1994). Low temperatures have also been shown to impact stress-related hormones and metabolites (i.e., cortisol, glucose and lactate) (Islam et al., 2020; C. Liu et al., 2020; Melis et al., 2017), and so these parameters were also measured. When tissues experience severe stress, cells begin to release their contents (a process called 'cell leakage') and/or undergo apoptosis, and enzymes usually only found inside the cells of certain tissues make their way into the plasma. By measuring the activity of these enzymes, it is possible to measure the impacts of an environmental or physiological condition on cell and tissue damage. Such measurements of plasma enzymes are a common tool in human and animal biomedicine (Boyd, 1983; Brewster, 2018; Klein et al., 2020). They have also previously been used to identify cold-stress in fish (Liu et al., 2020a; Panase et al., 2018; Sandnes et al., 1988). Finally, in Chapter 2, samples of the liver were used to measure the transcript (mRNA) expression levels of several stress-

related genes that are considered to be amongst the best indicators of temperature-related stress in fishes (Beemelmanns et al., 2021b, 2021a; Liu et al., 2020b; Vergauwen et al., 2010; Xu et al., 2018).

In the above two experiments, and one conducted by my colleague Emma Porter (also an M.Sc. student in Dr. Gamperl's lab), a number of salmon became moribund or died. This provided me with the opportunity to examine the aetiology of these cold-related mortalities in affected fish in detail, and the physiological disturbances and other cold-related effects that might be the cause of, and/or be associated with, cold-related mortalities in Atlantic salmon in countries such as Iceland and Eastern Canada. This research is the topic of Chapter 4 of my thesis.

Collectively, this thesis provides the Atlantic Canada salmon aquaculture industry with key information on how to better manage their fish during the cold months, and valuable data on which physiological indices ('biomarkers') are most appropriate for evaluating the effects of low temperatures on salmon physiology, health and welfare. Blood was one of the main targets for sampling as collecting blood from fish via caudal puncture is a simple, and non-lethal, procedure which makes it a suitable choice for the aquaculture industry. Such information is critical to the aquaculture industry in Atlantic Canada given the recent adverse effects of winter temperatures on Atlantic salmon production and survival (i.e., 'winter kill') (Pennell, 2014; Undercurrent News, 2020; Willick, 2019). The liver was also sampled given evidence that fish (including salmon) exposed to cold temperatures can develop 'fatty liver disease' (Dessen et al., 2021; Ibarz et al., 2010; Pastorino et al., 2019; Weber and Bosworth, 2005) that is often associated with tissue damage, and metabolic and other forms of dysfunction; these symptoms are

often associated with a condition called ‘Winter Disease’ or ‘Winter Syndrome’ in a number of fish species (Ibarz et al., 2010; Islam et al., 2020; Song et al., 2019). The ultimate goal of this latter research was to provide preliminary evidence as to whether Atlantic salmon can also suffer from this condition as suggested by the recent data of Dessen et al. (2021).

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CHAPTER 2: Lowering Temperature to 1°C Results in Physiological Changes, Stress and Mortality in Cultured Atlantic Salmon (*Salmo salar*)

This chapter is in revision with the journal Aquaculture as: Émile Vadboncoeur, Charlotte Nelson, Jennifer R. Hall, Kathy A. Clow, Rebeccah M. Sandrelli, Colin J. Brauner, Andrew K. Swanson, A. Kurt Gamperl. Lowering Temperature to 1°C Results in Physiological Changes, Stress and Mortality in Cultured Atlantic Salmon (*Salmo salar*).

Abstract

Atlantic salmon (*Salmo salar*) reared in sea-cages in Atlantic Canada and Iceland can be exposed to temperatures as low as 0-1°C during the winter months, and this can result in large-scale mortalities. Despite this threat to salmon aquaculture, most of our knowledge of this species' thermal biology is at temperatures above 10°C. In this laboratory experiment, Atlantic salmon post-smolts (175 ± 24 g) were exposed to a temperature decline of 1°C week^{-1} (from 8 to 1°C) with an additional week at 1°C (to simulate temperature changes in sea-cages in Atlantic Canada during winter months) or a constant temperature of 8°C (control), and sampled weekly. Growth was reduced by 58% in the experimental group and was associated with a decrease in appetite beginning at 6°C. Starting at 4-5°C, an osmoregulatory disturbance ensued characterized by higher plasma [Cl^-], lower plasma [K^+] and increased osmolality. *Hsp70* and *hsp90* transcript expression also increased as temperature was reduced from 5 to 1°C. However, plasma cortisol levels did not increase (to ~18 ng ml^{-1}) until the fish reached 1°C. *Serpinh1* and *cirbp* expression were not good biomarkers of decreasing temperature. Symptoms suggestive of liver dysfunction (changes in plasma [aspartate aminotransferase] and an increase in liver size) were also evident at 1°C. Finally, at 1°C, 5% of the fish died, and these mortalities were typically associated with snout ulceration/erosion and fin abrasions/rot. In summary, Atlantic salmon held under controlled laboratory conditions at temperatures < 5°C suffered from decreased growth performance, osmoregulatory disturbance, liver dysfunction, stress, opportunistic infections, and increased mortality.

2.1 Introduction

In the wild, Atlantic salmon (*Salmo salar*) can be found at a wide range of temperatures. For example, Strøm et al. (2020) reported that Atlantic salmon at sea experienced temperatures from -0.5 to 12.9°C in northern Norway, with maximum daily fluctuations of 9.6°C. Whereas, in freshwater streams, they can experience temperatures from 0 to 27-30°C (Caissie et al., 2014; Gallant et al., 2017; Lund et al., 2011). The thermal niche / biology of Atlantic salmon has also been studied in both laboratory and aquaculture settings as this species is cultured in many countries [e.g., Norway, Chile, Scotland, Australia (Tasmania) and Canada] and worldwide production and market values in 2020 were approximately 2.7 million metric tons and $15 billion (USD), respectively (FAO, 2021). The temperature at which Atlantic salmon growth is optimal is ~12 to 19°C for post-smolts (Elliott and Elliott, 2010) and 13°C for adult salmon (Hevrøy et al., 2013). However, beyond these values, performance declines and temperature becomes a stressor (Anttila et al., 2015; Beemelmanns et al., 2021c, 2021a; Elliott, 1991; Elliott and Elliott, 1995; Gallant et al., 2017; Gamperl et al., 2020; Wade et al., 2019). For instance, Beemelmanns et al. (2021b) reported that stress-related gene expression in adult Atlantic salmon of Saint John River (Canada) origin begins to increase when temperatures reach 16°C during an incremental (increase at 1°C week^{-1}) challenge to their upper temperature maximum (IT_{max}), and long-term exposure of salmon to temperatures above 22-23°C results in significant mortalities (Gamperl et al., 2020; Hvas et al., 2017).

There is also significant variation in winter temperatures in different regions where Atlantic salmon are reared at sea. While water temperatures at cage-sites in winter only decline to 6-9°C in Chile and Tasmania (Dempster et al., 2016; Narváez et al., 2019; Soto

and Norambuena, 2004; Wade et al., 2019), and 2-6°C in Norway given this countries' wide latitudinal range (Falconer et al., 2020; Geitung et al., 2020), on the east coast of Canada, temperatures at cage-sites will typically decline to ≤ 2°C for months (Cold Ocean Salmon, Pers. Comm.; Sandrelli et al., unpubl.). Furthermore, on the east coast of Canada and Iceland, so-called 'super-chill' events (to ≤ 0°C) in the winter months have recently been associated with large scale mortalities (Ćirić, 2020; Huffman, 2019; Pennell, 2014; The Fish Site, 2022; Undercurrent News, 2020; Willick, 2019), and it is expected that climate change will result in more frequent winter storms and episodes of upwelling that will bring colder deeper waters to the surface and result in rapid decreases in ocean temperatures from increased contact with cold air (i.e., episodes of 'cold-shock' or 'super-chill') (Environmental Law and Policy Center, 2019; Reid et al., 2022; Szekeres et al., 2016; U.S. Global Change Research Program et al., 2017).

It was established almost 5 decades ago that the lower temperature limit for salmonid culture (including Atlantic salmon) in seawater is 1°C based on evidence of poor growth and the risk of mortality (Saunders et al., 1975). However, there has been surprisingly little effort to understand the temperature at which sub-lethal effects occur in Atlantic salmon and their severity, and the only two studies that have examined such questions have provided contradictory information. Sandnes et al. (1988) collected blood samples from adult Atlantic salmon at a cage-site in northern Norway between October (7.7°C) and May (6.9°C), with temperatures dropping to 2°C in February. These authors showed that alkaline phosphorylase (AP), aspartate aminotransferase (AST) and creatinine (markers of tissue damage or organ failure) decreased slightly at the coldest temperatures, but that all other measured blood parameters were unchanged over the

course of the study and within the range considered normal for healthy fish. In contrast, Liu et al. (2020) performed a laboratory-based study on salmon parr where temperature was lowered from 16°C to 1°C over 6 weeks, and reported that similar parameters [plasma glucose, lactate dehydrogenase (LDH), creatine kinase (CK), AP and AST] increased as temperature was reduced from 6°C and 4°C, and continued to rise as temperature declined further.

It is unclear whether these disparate data are due to population or age (size) differences in how Atlantic salmon respond to cold temperatures, to the different environments/experimental protocols under which these samples were taken. Further, the reported plasma parameters in these publications are all indices of tissue damage, and thus, provide no information on when salmon become stressed by declining temperatures, and/or whether cold exposure is concomitant with a disturbance in the salmon's osmotic/ionoregulatory balance. In the current study, post-smolt Atlantic salmon were subjected to a temperature decrease from 8 to 1°C (at a rate of 1°C $week^{-1}$) over 7 weeks and then maintained for an additional week at 1°C (i.e., a typical temperature change seen between December and February in Atlantic Canada; see **Figure S1**). We measured production characteristics (i.e., feed consumption, growth rate, etc.), plasma indices of tissue damage (AST, LDH and CK) and energy metabolism (glucose and lactate), plasma ions and osmolality, plasma cortisol, and liver transcript expression of key stress-related genes as temperature was lowered, and compared these data with another group held at a constant temperature of 8°C over the same period. These data significantly enhance our understanding of how cold temperatures impact the physiology of Atlantic salmon,

identify particular temperatures where Atlantic salmon health and welfare may be compromised, and identify potential means to mitigate this industry challenge.

2.2 Materials and Methods

This study was approved by the Animal Care Committee of Memorial University of Newfoundland and Labrador (Protocol #21-02-KG). All procedures conducted on the salmon were performed in accordance with the Canadian Council on Animal Care's Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (Canadian Council on Animal Care, 2005).

2.2.1 Experimental animals

The Atlantic salmon used in this experiment were reared in freshwater at a Cooke Aquaculture Inc. commercial hatchery in New Brunswick (Canada) and transferred directly to saltwater to the Dr. Joe Brown Aquatic Research Building (JBARB; Department of Ocean Sciences, Memorial University of Newfoundland and Labrador) in January of 2021 without any significant mortalities (< 1%). These fish were initially held in a 3000 L tank for 3 weeks in this facility, and were fed to apparent satiation daily with commercial pellets (Signature Salmon Ration, Northeast Nutrition, NS. Canada). Then, 280 fish (175 ± 24 g) were distributed into eight 500 L tanks (35 fish $tank^{-1}$) supplied with 10 L min^{-1} of seawater (32 ppt salinity) at ~8°C and ~100% air saturation, and a 12h light : 12h dark photoperiod. The fish were acclimated to the tanks for two weeks before the start of the experiment.

2.2.2 Experimental protocol

The 8 tanks were randomly separated into control and experimental treatment groups (4 tanks per treatment) (**Figure S2)**. Fish in the control tanks were maintained at a constant temperature of 8°C throughout the study; whereas fish in the experimental tanks were subjected to an incremental decrease in temperature of 1°C per week from 8 to 1°C, followed by an additional week at 1°C (**Figure S3; Table S1**). Temperature in the experimental tanks was controlled by mixing 8°C and ambient (~1°C) seawater in different proportions as the experiment proceeded. The weekly temperature change was normally spread out over 2-3 days, and the resulting temperature was maintained for 4-5 days before the fish were sampled. The exception was when the experimental fish were maintained at 6°C for 10 days because of technical issues within the JBARB; however, this had no noticeable impact on the experiment. Oxygen levels in the tanks were maintained between 100 and 115% air saturation, with temperature and dissolved oxygen levels recorded every morning using a handheld probe and meter (YSI, ProODO, OH, USA). The fish were fed to satiation twice daily (at 10:00 and 14:00), and feeding was suspended when pellets began to accumulate at the bottom of the tanks. Feed consumption (FC) was calculated by dividing feed provided to each tank by the average weight of fish in that tank. Fish from the control and experimental tanks (2 fish per tank; n=8) were haphazardly netted and sampled for morphometric measurements, blood and liver (detailed below) when the fish were at 8°C, and when fish in the decreasing temperature regime reached 6, 5, 4, 3, 2 and 1°C. An additional set of samples was taken after 2 weeks at 1°C. Finally, morphometric measurements were taken from all animals

remaining after 2 weeks at 1°C [n=54 (control) and 59 (cold-exposure) per treatment, see **Table 2-1**].

2.2.3 Morphometric measurements

The morphometric measurements consisted of body weight, fork length and liver weight.

Specific growth rate (SGR) was calculated according to the formula:

$$SGR \text{ (in \% weight day}^{-1}) = 100 (\ln W_f - \ln W_i) / t$$

Where W_f was final weight (g) of the fish measured after 2 weeks of acclimation at 1°C, W_i was initial weight (g) at the first sampling point at 8°C, and t was the time (in days) between initial and final sampling (63 days).

Condition factor (CF) and hepatosomatic index (HSI) were calculated using the following formulas:

$$CF = 100 [W_t / L^3]$$

$$HSI = 100 [W_L / W_t]$$

where W_t was body weight (g), W_L was liver weight (g) and L was fork length (cm).

Survival was calculated daily using the formula:

$$Survival (\%) = 100 - (M / 35 * 100)$$

Where M was the cumulative number of mortalities (mortalities and culls) in a tank, and 35 was the initial number of fish in each tank. Average survival was calculated daily for each treatment (n=4).

The fish remaining in the tanks after 2 weeks at 1°C were scored based on external condition using a system adapted from Noble et al. (2018). The animals were examined for four characteristics: snout damage, fin damage, skin lesions and scale loss. Each characteristics was scored from 0-3 with 0 being indicative of an absence of the trait, and 1 to 3 indicating least severe to most severe as adapted from Noble et al. (2018) (**Figure S4)**. Further, if the fin damage was active, as opposed to healed, the fin damage score was doubled. The four scores for each fish were summed to give a total score for each individual.

2.2.4 Blood and liver sample collection

Fish (n=2 per tank) were haphazardly, but carefully netted and anesthetized with 0.4 g l^{-1} of tricaine methanesulfonate (Syndel Laboratories Ltd, Vancouver, BC. Canada) and bled by caudal puncture in < 3 min. of netting using heparinized 1 ml syringes and 21 gauge needles. Blood samples were transferred to a 2 ml Eppendorf® tube containing 75 µl of saline containing lithium heparin (Sigma Chemical Co., Oakville, ON, Canada; 1000 units per ml) and centrifuged at 1,100 *xg* for 1 min. The plasma was then aliquoted into 1.5 ml Eppendorf® tubes for the various analyses, immediately flash frozen in liquid nitrogen, and stored at -80°C. Using Rnase AWAY® (Sigma Chemical Co., Oakville, ON, Canada) cleaned surgical instruments, the liver was removed, weighed, and a subsample

(~100 mg) placed in a 1.5 ml Rnase-free Eppendorf® tube, flash frozen in liquid nitrogen, and then stored at -80°C.

To comply with COVID-19 protocols, a change in the sampling protocol was made during the experiment. At 8°C (first sampling point), fish were sampled at 9 AM, 22 hours after feeding. At all other temperatures (sampling points), fish were sampled at 5 PM, 8 hours after feeding. However, this appeared to have had limited impact on the measured temperature-dependent parameters (see Section 2.3..5).

2.2.5 Biochemical analyses

2.2.5.1 Spectrophotometry

All enzyme activities, and glucose and lactate concentrations were measured at 340 nm, whereas plasma cortisol concentrations were measured at 580 nm, at 25°C, using a SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA. USA). These assays were modified from established protocols developed for human plasma (Bergmeyer et al., 1978), fish plasma (Casillas et al., 1982), fish liver (Treberg et al., 2002) and fish muscle (Treberg et al., 2003). All chemicals were purchased from Sigma chemicals unless otherwise stated.

Assay conditions were as follows:

Aspartate aminotransferase (AST; E.C. 2.6.1.1). 50 mM of imidazole (pH 7.4), 200 mM aspartate, 0.2 mM NADH, 0.05 mM pyridoxal–5–phosphate and 14.5 U/ml malate dehydrogenase. The reaction was initiated by the addition of α-ketoglutarate to 7 mM.

Lactate dehydrogenase (LDH; E.C. 1.1.1.27). 50 mM imidazole (pH 7.4) and 0.2 mM NADH. The reaction was initiated by the addition of pyruvate to 1 mM.

Creatine kinase (CK; E.C. 2.7.3.2). 50 mM Imidazole (pH 7.4), 5 mM $MgCl_2$, 2 mM ADP, 0.8 mM NADP, 5 mM glucose, 10 mM AMP, 2 U/ml hexokinase, 2 U/ml glucose–6–phosphate dehydrogenase, and 10 µM of P1,P5-Di(adenosine-5') pentaphosphate. The reaction was initiated by the addition of phosphocreatine to 50 mM.

Glucose. Plasma samples were deproteinized in 1:4 0.6% perchloric acid. The assay medium contained 250 mM Imidazole (pH 7.8), 5 mM $MgSO_4$, 10 mM ATP, 0.8 mM $NADP^+$ and 10 U/mlglucose-6-phosphate dehydrogenase. hexokinase to 10 U/ml.

Lactate. Plasma samples were deproteinized in 1:4 0.6% perchloric acid. The assay medium contained 200 mM of glycine buffer solution and 25 mM NAD. The reaction was initiated by the addition of lactate dehydrogenase to 400 U/ml.

Plasma cortisol levels were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (NEOGEN Corp. Lexington, KY. USA).

2.2.6 Analyses of transcript expression levels of putative stress-responsive genes in liver

2.2.6.1 RNA extraction, Dnase treatment and column purification

For RNA extraction, ~100 mg of liver was homogenized in a 2 ml Rnase-free Eppendorf® tube containing 400 µl of TRIzol Reagent (Invitrogen/Thermo Fisher Scientific, Burlington, ON, Canada) and a 5 mm stainless steel bead (QIAGEN, Mississauga, ON, Canada) using a TissueLyser II (QIAGEN) set at 25 Hz for 2.5 min. An additional 400 µl of TRIzol Reagent was then added to the tube and the sample further homogenized at 25 Hz for 2.5 min. The samples were then stored at -80°C. To

increase tissue disruption, the samples were thawed on wet ice and passed through QIAshredder (QIAGEN) spin columns. 200 µl of Trizol was then added to bring the total volume of the homogenate to 1 ml, and TRIzol total RNA extractions were completed following the manufacturer's instructions. Finally, RNA pellets were resuspended in nuclease-free UltraPure distilled water (Invitrogen/Thermo Fisher Scientific).

High levels of lipid in fish liver often interfere with column-purification. Thus, an additional step, adapted from Xu et al. (2013), was performed to further purify the samples. Briefly, TRIzol-extracted RNA subsamples (150 µg) were combined with nuclease-free water to a final volume of 300 µl in a 1.5 ml RNAse-free Eppendorf® tube. An equal volume of acid phenol:chloroform:isoamyl alcohol (125:24:1) (pH 4.5) (AM9720; Ambion/Thermo Fisher Scientific) was then added, vortexed for 30 sec. and centrifuged at 21,000 *xg* at 4°C for 20 min. Then, 260 µl of the aqueous phase was transferred to a clean tube and the RNA precipitated with 0.1 volumes of 3M sodium acetate (pH 5.5) (AM9740; Ambion/Thermo Fisher Scientific) and 2.5 volumes of ice-cold anhydrous ethyl alcohol (Commercial Alcohols Inc., Brampton, ON) at – 80°C for 12 h, and centrifuged at 21,000 *xg* at 4°C for 30 min. The RNA pellets were then washed with 1 ml of 75% ethanol and centrifuged at 21,000 *xg* at 4°C for 20 min. Finally, the ethanol was removed, the RNA pellet was air dried at room temperature for 10 min, and resuspended in 100 µl of nuclease-free water (Invitrogen/Thermo Fisher Scientific), with heating at 55°C for 10 min.

The acid phenol:chloroform:isoamyl alcohol extracted RNA samples (45 µg) were then each treated with 6.8 Kunitz units of DnaseI (Rnase-Free Dnase Set, QIAGEN) with the manufacturer's buffer (1X final concentration) at room temperature for 10 min. to

degrade any residual genomic DNA. Dnase-treated RNA samples were column-purified using the Rneasy Mini Kit (QIAGEN) following the manufacturer's instructions. RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity was assessed by A260/280 and A260/230 NanoDrop UV spectrophotometry (NanoDrop™ One UV-Vis spectrophotometer, Thermo Scientific) after each of the three extraction/clean-up steps. The column-purified liver RNA samples were of high integrity (tight 28S and 18S ribosomal RNA bands, with 28S being approximately twice as intense as 18S) (**Figure S5**), and purity; with A260/280 ratios between 2.08 and 2.15 and A260/230 ratios between 1.77 and 2.38.

2.2.6.2 Real-time quantitative polymerase chain reaction (qPCR) overview

The transcript expression levels of 10 genes with functional annotations related to the stress response were measured in liver of the control and cold-exposure fish (n=8 per group) at 8, 6, 5, 4, 3, 2 and 1°C using qPCR. These genes included cold-inducible RNA binding protein (*cirbp*), serpin peptidase inhibitor, clade H, member 1 (*serpinh1*) [alias heat shock protein 47 (*hsp47*)], 4 heat shock protein 70-like genes *(hsp70a, hsp70b, hsp70c* and *hsp70d)* and 4 cytosolic heat shock protein 90 (*hsp90a*) genes, including 2 *hsp90a* alpha paralogues (*hsp90aa1a* and *hsp90aa1b*) and 2 *hsp90a* beta paralogues *(hsp90ab1a* and *hsp90ab1b).* These genes are commonly used to measure thermal stress in fish (Akbarzadeh et al., 2018; Beemelmanns et al., 2021b; Morgan et al., 2022; Swirplies et al., 2019). The qPCR analyses were designed and performed based on the Minimum Information for Publication of qPCR Experiments (MIQE) guidelines (Bustin et al., 2009).

2.2.6.3 cDNA synthesis and qPCR parameters

First-strand cDNA templates for qPCR were synthesized in 20 µl reactions from 1 µg of DnaseI-treated, column purified, total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific) following the manufacturer's instructions.

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. Amplifications were performed using the QuantStudio 6 Flex Real Time PCR system (384-well format) (Applied Biosystems/Thermo Fisher Scientific). 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 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.

2.2.6.4 Primer design and quality control testing

Details on GenBank accession numbers, qPCR primer sequences, amplicon sizes and amplification efficiencies are presented in **Table S4**. Many of these primers were designed previously (see references in **Table S4**). Primers new to this study (*hsp90ab1a* and *hsp90ab1b*) were manually designed with the aid of Primer3 (Kõressaar et al., 2018; Koressaar and Remm, 2007; Untergasser et al., 2012) to ensure specificity. The four *hsp70* and the four *hsp90* genes analyzed in this study are isoforms/paralogues; the percentage identity at the nucleotide level is reported in **Table S2 and S3**, respectively.

The primer pairs for these genes are located in areas where the nucleotide sequences have a minimum of 3 bp difference between each sequence to ensure specificity (see **Figure S6 and S7**).

qPCR primer quality testing was conducted according to previously published protocols (e.g. Caballero-Solares et al., 2017). In the current study, quality and amplification efficiencies for each primer pair were assessed in three cDNA pools: control fish [week 7 (8°C)] and treatment fish [week 5 (3°C) and week 7 (1°C)]. Briefly, cDNAs were synthesized for each individual liver RNA sample (n=8 per group) and then pooled. Due to low expression levels, primers for *cirbp* and *serpinh1* were assessed using a 5-point 1:3 dilution series (in duplicate) starting with cDNA representing 50 ng of input RNA; while the rest of the genes were assessed using a 5-point 1:3 dilution series (in duplicate) starting with cDNA representing 10 ng of input RNA. As the primer pair for *cirbp* exhibited compression at the highest cDNA input amount, it was dropped and the standard curve generated using a 4-point dilution series. No-template controls (NTC) were included to test for contamination and primer dimers. The reported efficiencies (**Table S4**) are an average of the three values, with the exception of *hsp90aa1b* in which the values are based on the 3 and 1°C pools only. This was due to low expression levels in the 8°C pool. The efficiency and R^2 for each of the three standard curves for each primer pair were calculated using QuantStudio Real Time PCR Software (version 1.3) (Applied Biosystems/Thermo Fisher Scientific). All primer pairs used in the qPCR analyses had acceptable efficiencies (between 91 and 101%, **Table S4**), generated an amplicon with a single peak in the melt curve analysis, and had no primer dimers.

2.2.6.5. Selection of normalizer genes

Six candidate normalizer genes [60S ribosomal protein L32 (*rpl32*, BT043656), eukaryotic translation initiation factor 3 subunit D (*eif3d*, GE777139), elongation factor 1-alpha 1 (*ef1a1*, BT058669), elongation factor 1-alpha 2 (*ef1a2*, BT058669), polyadenylate-binding protein 1 (*pabpc1*, EG908498), and β-actin (*actb*; BG933897)] were assessed. The fluorescence threshold cycle (C_T) values of three samples from each group at 8°C and four samples from each group at all of the other sampling temperatures (n=48 in total) were measured (in duplicate) for these six normalizer genes using cDNA representing 5 ng of input total RNA. To identify the most stably expressed normalizers, we conducted a geNorm (Vandesompele et al., 2002) analysis within the qBase+ software package (Hellemans et al., 2007) (Biogazelle, Zwijnaarde, East Flanders, Belgium) on the C_T values obtained from the 48 samples. Based on this analysis, *eif3d* (geNorm M = 0.173) and *rpl32* (geNorm M = 0.183) were selected as the normalizer genes in the experimental qPCR analyses. For each candidate normalizer, the geNorm M value and the mean C_T values for each group in the study are presented in **Table S5**. In the experimental qPCR analysis, the selected normalizers were indeed stably expressed considering all samples and groups in the study (**Table S6**).

2.2.6.6 Experimental qPCR analyses

Expression analyses for the ten transcripts of interest (TOIs) in the 112 samples were performed as 5 separate studies; in each study, two of the TOIs and the two endogenous controls were assessed (in triplicate) across four plates. On each plate, a no-template control and a plate linker sample (to ensure that there was no plate-to-plate

variability) were included (both also tested in triplicate). cDNA representing 5 ng of input RNA (increased to 20 ng for study 5 due to low expression levels of *cirbp and serpinh1*) was used as template in the PCR reactions. The relative quantity (RQ) of each transcript was determined using the QuantStudio Real Time PCR Software (version 1.3) (Applied Biosystems/Thermo Fisher Scientific) relative quantification study application, with normalization to both *eif3d* and *rpl32* transcript levels, and with amplification efficiencies incorporated. For each TOI, the sample with the lowest normalized expression (mRNA) level was set as the calibrator sample (i.e., assigned an RQ value = 1.0). For each experimental qPCR study, the C_T values for the TOIs and endogenous controls that were used to calculate the RQ values are presented as means ± s.e.m. C_T values for each treatment group can be found in **Table S6**.

2.2.7 Ion measurements

Plasma sodium (Na^+) and potassium (K^+) plasma levels were analyzed using a PFP7 Flame Photometer (Jenway, Stone, Staffordshire, UK) following the manufacturer's instructions. Chloride (Cl^-) and osmolality were measured using a ChloroChek Chloridometer (model 3400, ELITech Group, Logan, UT) and a VAPRO vapor pressure osmometer (model 5600, ELITech Group, Logan, UT), respectively.

2.2.8 Statistical analyses

All data are reported as means ± standard error of the mean (s.e.m.). The data were analyzed using Rstudio Version 1.4.1106 R 4.0.5 (R Core Team, 2020, https://www.r-project.org/). A Bonferroni outlier test was used to identify outliers before

statistical analysis. Parameters measured were analysed by fitting linear mixed-effect models using the *lmer* function implemented in the *lme4* package in R (Bates et al., 2021). Statistical models were computed with the fixed interaction terms 'treatment' and 'sampling'. The random term 'tank' was used to account for between-tank variation (i.e., tank effects). Each model fit was graphically examined (i.e., using histograms and qqplots), and residuals were tested for normality (Shapiro–Wilk, $p<0.05$). Non-normal distributed data were power-transformed with a Box-Cox transformation (Atkinson et al., 2021) using the *boxcox* function implemented in the *EnvStats* (version 2.7.0) package in R (Millard, 2017) so that the data met the assumption of normality. Finally, when the model identified significant effects, least-squares means post-hoc tests with Bonferroni's *p*-value correction for multiple comparisons [using the *lsmeans* function in R (Length, 2016)] were used to identify significant differences between treatments at a particular temperature, and between sampling points (temperatures) within a treatment.

2.3 Results

2.3.1 Feed consumption, growth and survival

Feed consumption was approximately 0.7% body weight day^{-1} for all tanks for the first 2 weeks at 8°C. It remained at this level in the control tanks over the entire experiment. At 6°C, feed consumption was significantly lower (by 0.23 % body weight day^{-1}) in the cold-exposure fish as compared to the control fish, and continued to decrease until 1°C (**Figure 2-1**). At 1°C feed consumption is reported as ~0.1% body weight day^{-1}, but this value was actually lower as some pellets needed to accumulate in the tank before satiety was determined.

Fish from the control treatment grew (in weight and length) over the experiment. Nearly significant differences in weight were measured between the two treatments after the first ($p < 0.08$) and second ($p < 0.07$) weeks at 1°C (**Figure 2-2**) (n=8), and a significant difference in weight (~18% lower in the cold-exposure fish) was found at the end of the experiment when all remaining fish were measured; SGR = 0.45 ± 0.3 and 0.19 ± 0.03, respectively (**Table 2-1**). A similar result was noted for length, but there was no difference in CF at the end of the experiment (**Table 2-1, Figure 2-2**). Interestingly, while HSI was not different before the fish reached ~1°C, it was 35% higher in the cold-exposure fish after 2 weeks at this temperature (1.57 vs. 1.15%) (**Table 2-2, Figure 2-2**). Survival was also similar for both treatments until 1°C. However, during the last 2 weeks at 1°C, ~5% of the

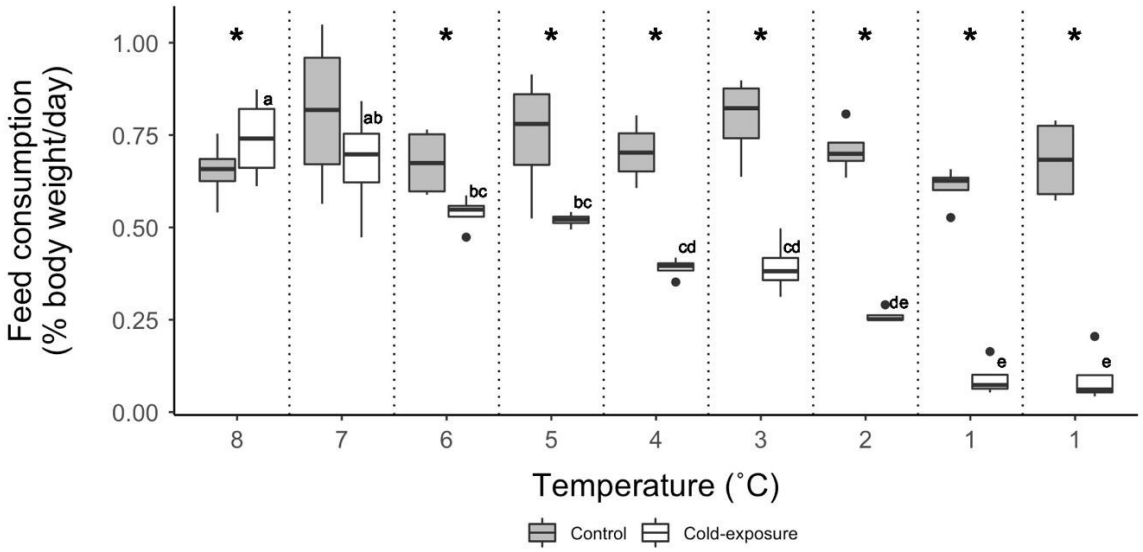

Figure 2-1. Weekly feed consumption during the experiment. Fish were fed to satiation twice daily. The lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. * Indicates a significant ($p < 0.05$) difference between control and cold-exposure fish at a given temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

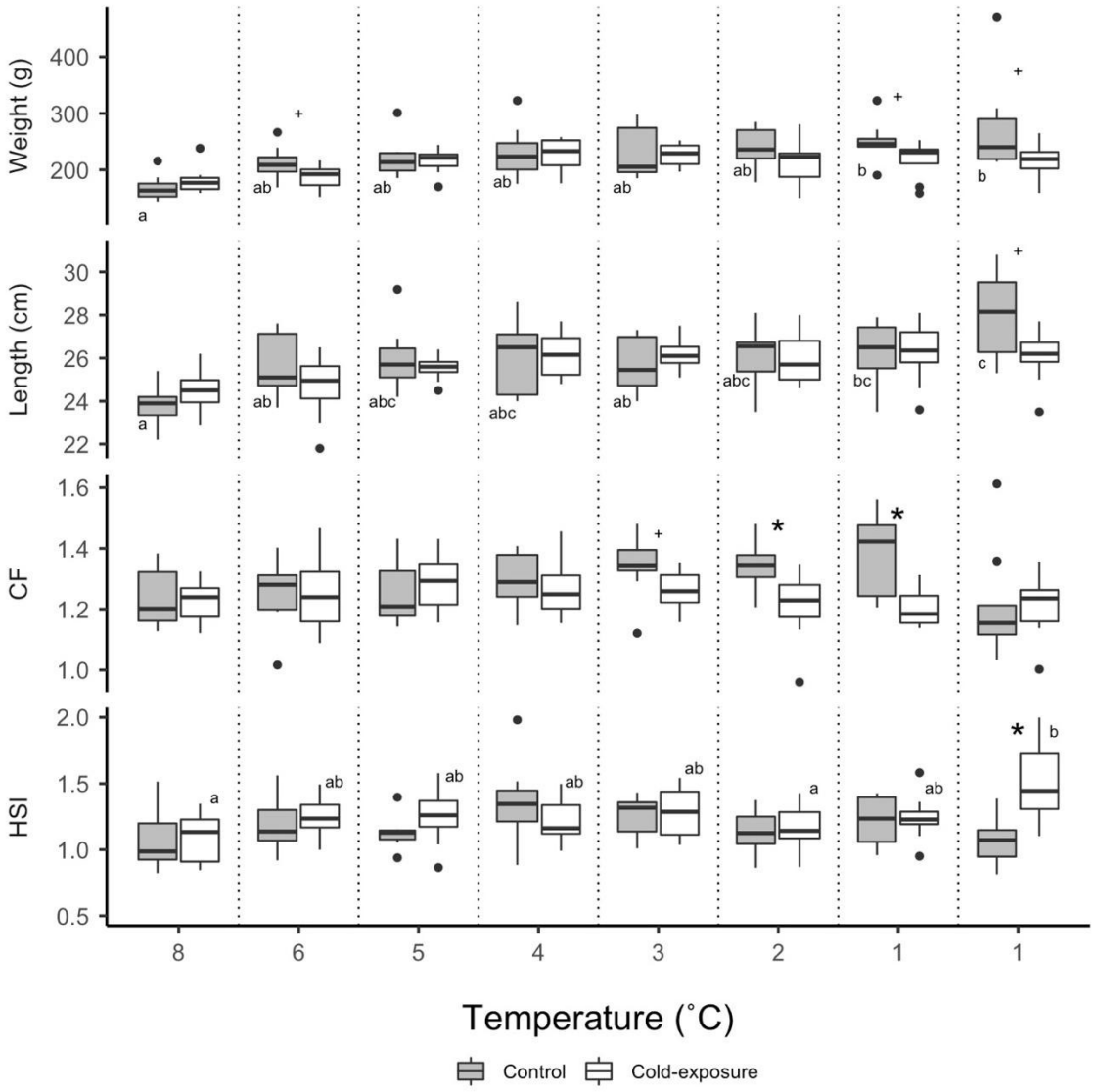

Figure 2-2. Changes in weight (g), length (cm), hepatosomatic index (HSI) and condition factor (CF) as temperature was lowered from 8 to 1°C in the cold-exposure group compared with the control group. Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. $^{+}$ Indicates a near significant difference ($p < 0.1$), whereas * indicates a significant ($p < 0.05$) difference, between control and cold-exposure fish at a particular temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

Table 2-1. Morphometric measurements for fish exposed to a temperature decrease from 8 to 1°C at a rate of 1°C/week and subsequently maintained for two weeks at 1°C ('cold-exposure), as compared to control fish (held at a constant temperature of 8°C). P values in bold type are significant ($p < 0.05$). HSI: hepatosomatic index, CF: condition factor.

	Control	Cold-exposure	**P-value**
n	54	59	
Mass (g)	248.6 ± 6.1	203.7 ± 4.3	**< 0.001**
Length (cm)	26.9 ± 0.2	25.4 ± 0.2	**< 0.001**
HSI	1.16 ± 0.04	1.57 ± 0.05	**< 0.001**
CF	1.27 ± 0.02	1.24 ± 0.02	0.3267
Score	3.8 ± 0.3	7.2 ± 0.5	**< 0.001**
Specific growth rate (% body weight day^{-1})	0.45 ± 0.3	0.19 ± 0.03	**< 0.05**

cold-exposure fish died (**Figure 2-3**). Fish that died/were culled all had similar traits. They were lethargic, swimming at the surface, and the affected animals had considerable scale loss, fin rot (pectoral and caudal) and severe ulcerative lesions/erosion on the snout (**Figure S8**). A visual inspection of the remaining animals in the experimental tanks also revealed that these animals were generally in poorer condition than the control fish; this conclusion is based on the considerably lower condition scores for the cold-exposure fish (**Table 2-1**).

2.3.2 Plasma enzyme levels, and indicators of stress and metabolism

In this experiment we measured a number of enzymes in the plasma as indicators of tissue damage. There were no differences in plasma enzyme levels for the control fish over the experiment. For the cold-exposure fish, plasma enzyme levels were significantly different between 8 and 5-to-1°C for aspartate aminotransferase (AST); 8, and 4 and 1°C for creatine kinase (CK); and 8 and 4°C for lactate dehydrogenase (LDH) (**Figure 2-4**). The only differences between control and cold-exposure fish were at 1°C where plasma AST levels were lower for cold-exposure fish after the first ($p<0.1$) and second weeks ($p<0.05$) (**Figure 2-4**).

Circulating cortisol levels were ~5-10 ng/ml in the control group (values indicative of unstressed fish) over the duration of the experiment. However, cortisol levels in cold-exposure fish began to increase noticeably compared to control fish by 2°C and were significantly higher (by 2-3 fold) once the fish reached 1°C (**Figure 2-5**). Plasma glucose increased in both groups between 8 and 6°C (from ~3.4 to 4.6 mM).

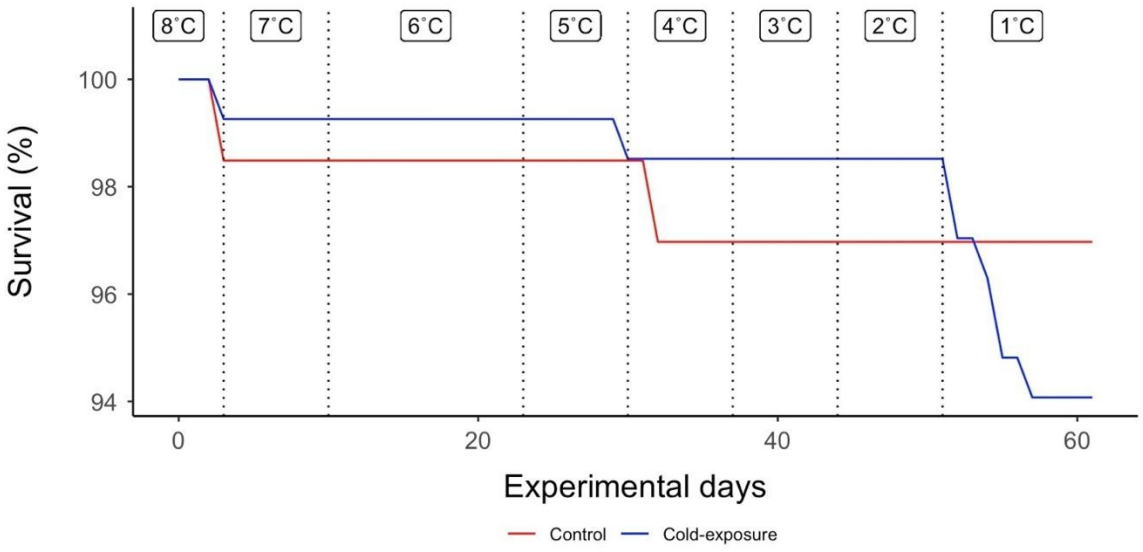

Figure 2-3. Survival (%) for control fish (red) and cold-exposure fish (blue). n=4 tanks per treatment. Salmon that were culled were included as mortalities.

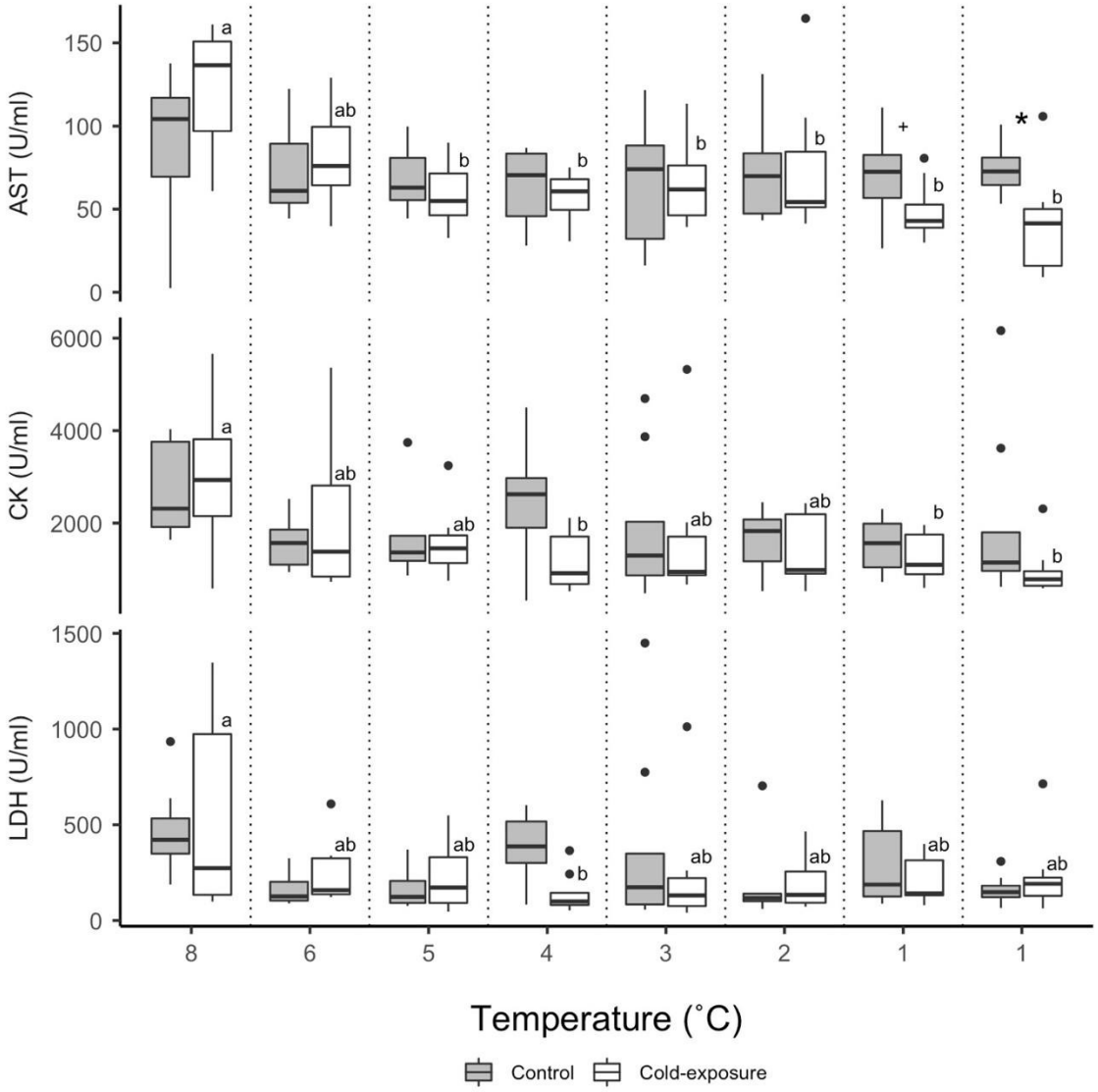

Figure 2-4. Plasma activity of aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) as temperature was lowered from 8 to 1°C in the cold-exposure group as compared with the control group. Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. $^{+}$ Indicates a near significant difference ($p < 0.1$), whereas * indicates a significant ($p < 0.05$) difference between control and cold-exposure fish at a particular temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

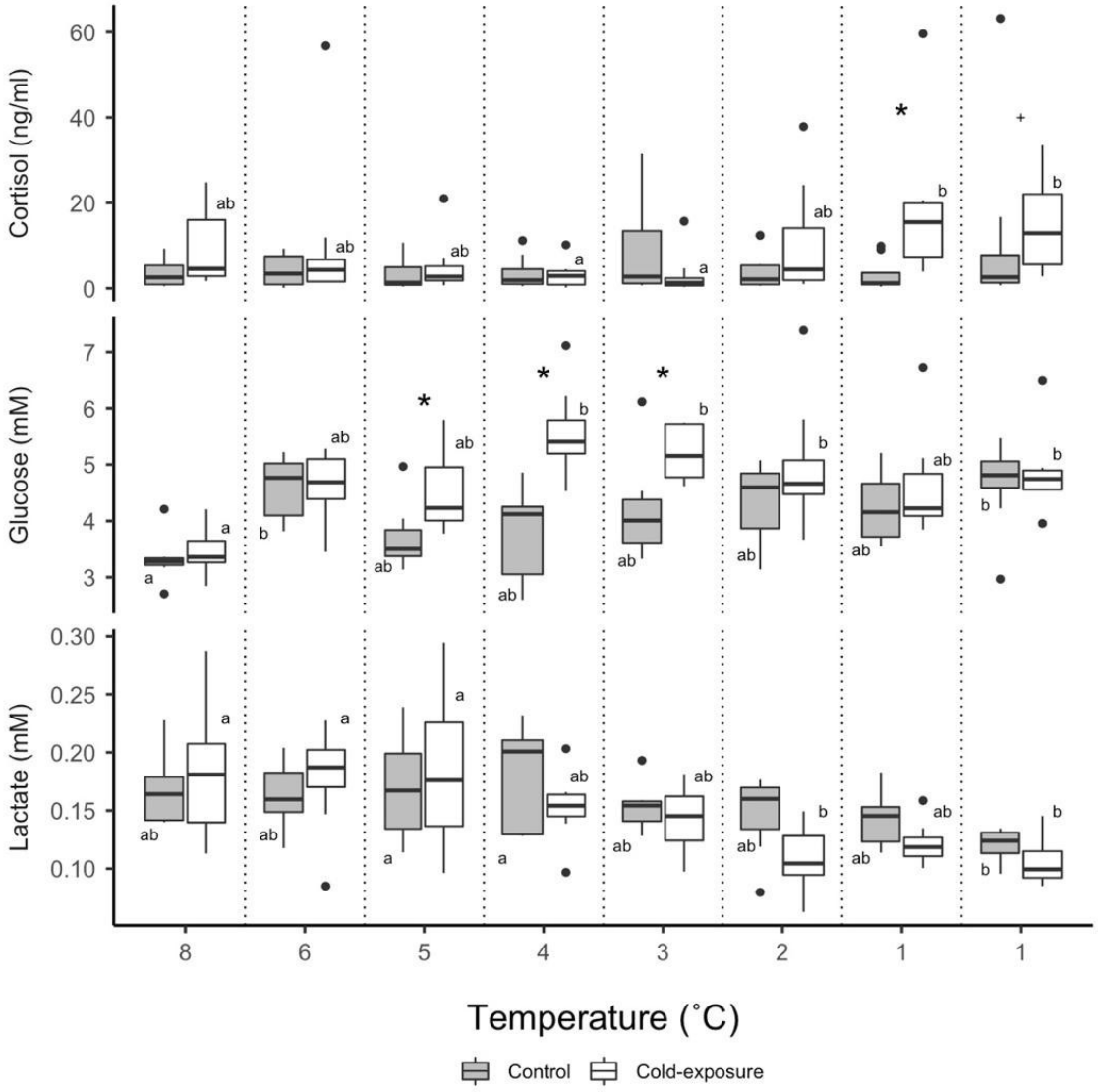

Figure 2-5. Plasma cortisol, glucose and lactate concentrations as temperature was lowered from 8 to 1°C in the cold-exposure group as compared with the control group. Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. $^{+}$ Indicates a near significant difference ($p < 0.1$), whereas * indicates a significant ($p < 0.05$) difference between control and cold-exposure fish at a particular temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

However, the temperature-dependent glucose response differed between the groups after this point in the experiment. Glucose levels in the cold-exposure fish increased transiently to ~5.6 mM, and were significantly higher than control fish between 5 and 3°C (**Figure 2-5**). Plasma lactate levels did not differ between the groups, but fell from ~0.17 mg/ml to ~0.12 mM over the course of the experiment.

2.3.3 Changes in stress-related gene expression in liver

Transcript expression levels of four *hsp90a* paralogues were measured: two *hsp90a* alpha 1 paralogues (*hsp90aa1a, hsp90aa1b*) and two *hsp90a* beta paralogues (*hsp90ab1a* and *hsp90ab1b*). In the control group, there were no significant differences in the expression level of any of the four *hsp90* transcripts throughout the experiment. In contrast, in the cold-exposure fish, expression levels of *hsp90aa1a, hsp90aa1b and hsp90ab1b* started to increase ($P < 0.05$) at approximately 5°C and continued to increase as temperature fell. At 1°C, expression levels of these genes were ~25-, 10- and 1.4-fold higher, respectively, as compared to time-matched controls. In contrast, the first significant temperature-dependent effect for *hsp90ab1a* was not observed until 1°C, where expression levels were 1.2-fold higher as compared with control fish (**Figure 2-6**).

Transcript expression levels of the four *hsp70* genes were stable in the control group until the last time point (sampling). At that point, expression was significantly higher than that recorded at the first sampling, with the expression for *hsp70a, hsp70b, hsp70c and hsp70d* elevated by 1.1-, 1.8-, 2.1- and 1.3-fold, respectively. In cold-exposure fish, there were no significant differences in *hsp70a* transcript levels as temperature was lowered. This is in contrast to all of the other *hsp70* paralogues, whose

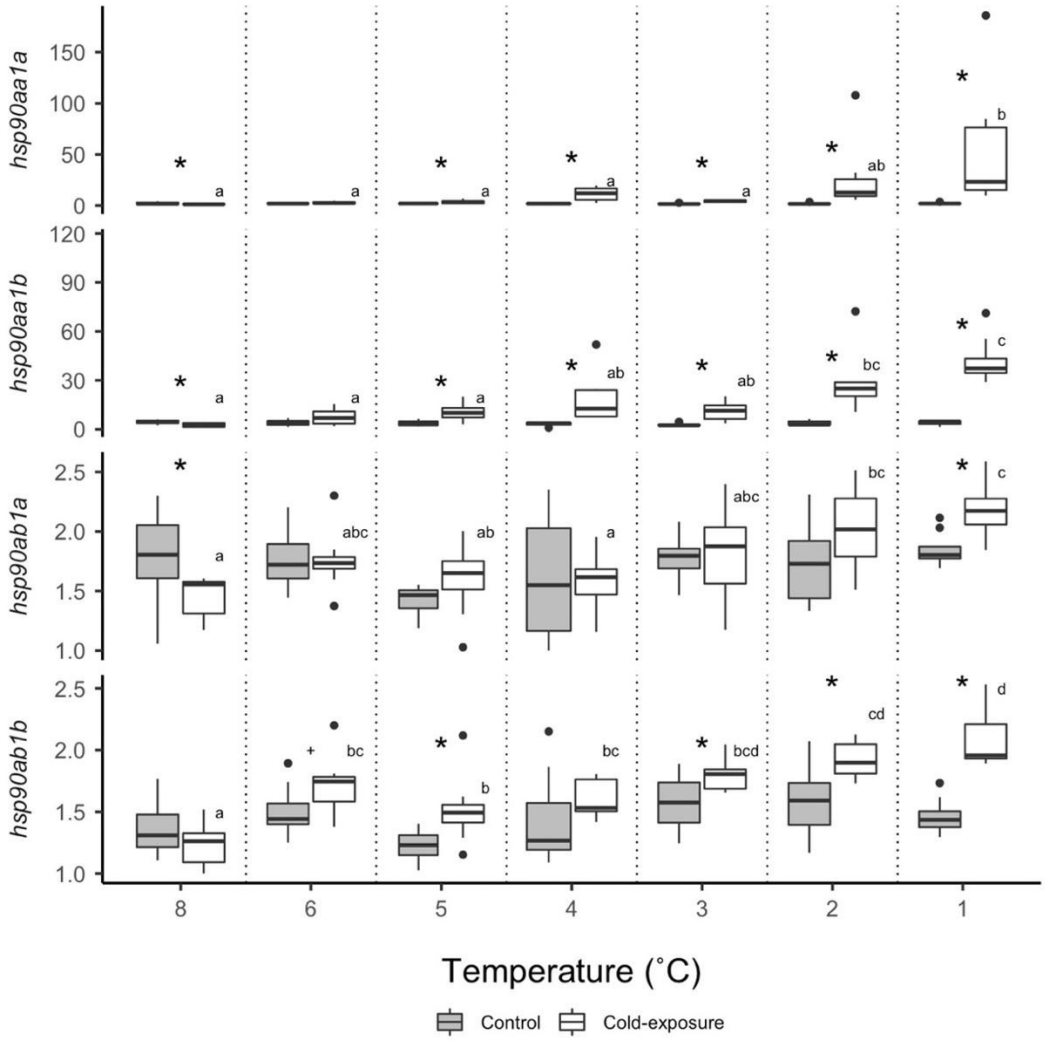

Figure 2-6. Transcript expression levels of four cytosolic heat shock protein 90 paralogues as temperature was lowered from 8 to 1°C in the cold-exposure group as compared with the control group. Transcript levels are presented as relative quantity (RQ) values (i.e., values for the transcript of interest were normalized to both eif3d and rpl32 transcript levels, and were calibrated to the individual with the lowest normalized expression level for that given transcript. Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. $^{+}$ Indicates a near significant difference ($p < 0.1$), whereas * indicates a significant ($p < 0.05$) difference between control and cold-exposure fish at a particular temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

expression levels increased significantly as water temperature was lowered. A significant increase in hsp70b and hsp70c expression was evident ($p<0.05$ as compared to the control group) by 6°C and 5°C, respectively, and values were 2.8- and 2.9-fold higher as compared to control fish by 1°C. The expression of *hsp70d* also increased as temperature fell. However, the first significant difference, as compared to the control group, was not recorded until 2°C, and the fold-difference at this temperature and at 1°C ($p<0.1$) (1.3- and 1.2-fold as compared to controls) were not as high as for *hsp70b* and *hsp70c* (**Figure 2-7**).

Transcript expression levels of *cirbp* were variable in the control fish over the course of the experiment, with a 1.6-fold increase ($p < 0.05$) seen between the samplings of week 3 (lowest) and week 5 (highest). Cold-exposure fish had an interesting *cirbp* expression pattern. Expression levels increased by 2.2-fold ($p<0.05$) at 3°C compared to the start of the experiment, but then decreased significantly and returned to initial levels. That said, the expression of *cirbp* was significantly higher in cold-exposure fish as compared to control fish from 6 to 1°C. The expression of *serpinh1* varied between the samplings, but there were no significant differences between the treatments at any temperature / sampling point (**Figure 2-8**).

2.3.4 Impact on plasma ion levels and osmolality

At 8°C plasma levels of Cl^-, K^+ and Na^+ were 108.4 ± 2.5, 3.5 ± 0.1 and 146.7 ± 0.8 mM respectively. Between 8 and 6°C, there was a significant increase in plasma Na^+ levels (to 163.3 ± 1.7 mM), and this was associated with an approximately 6.5% increase

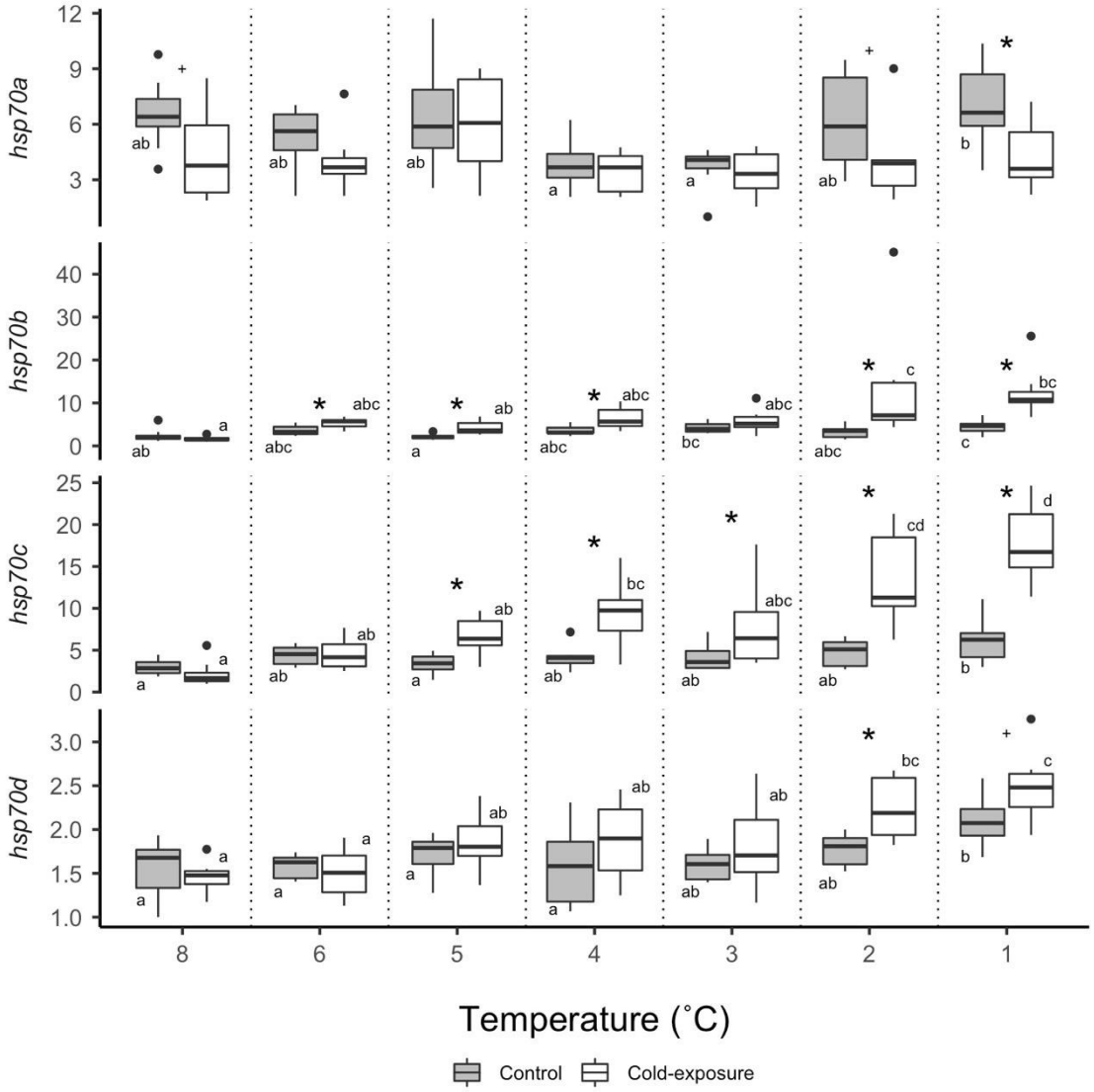

Figure 2-7. Transcript expression levels of four heat shock protein 70 paralogues as temperature was lowered from 8 to 1°C in the cold-exposure group as compared with the control group. Transcript levels are presented as relative quantity (RQ) values (i.e., values for the transcript of interest were normalized to both eif3d and rpl32 transcript levels and were calibrated to the individual with the lowest normalized expression level of that given transcript). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. $^{+}$ Indicates a near significant difference ($p < 0.1$), whereas * indicates a significant ($p < 0.05$) difference between control and cold-exposure fish at a particular temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

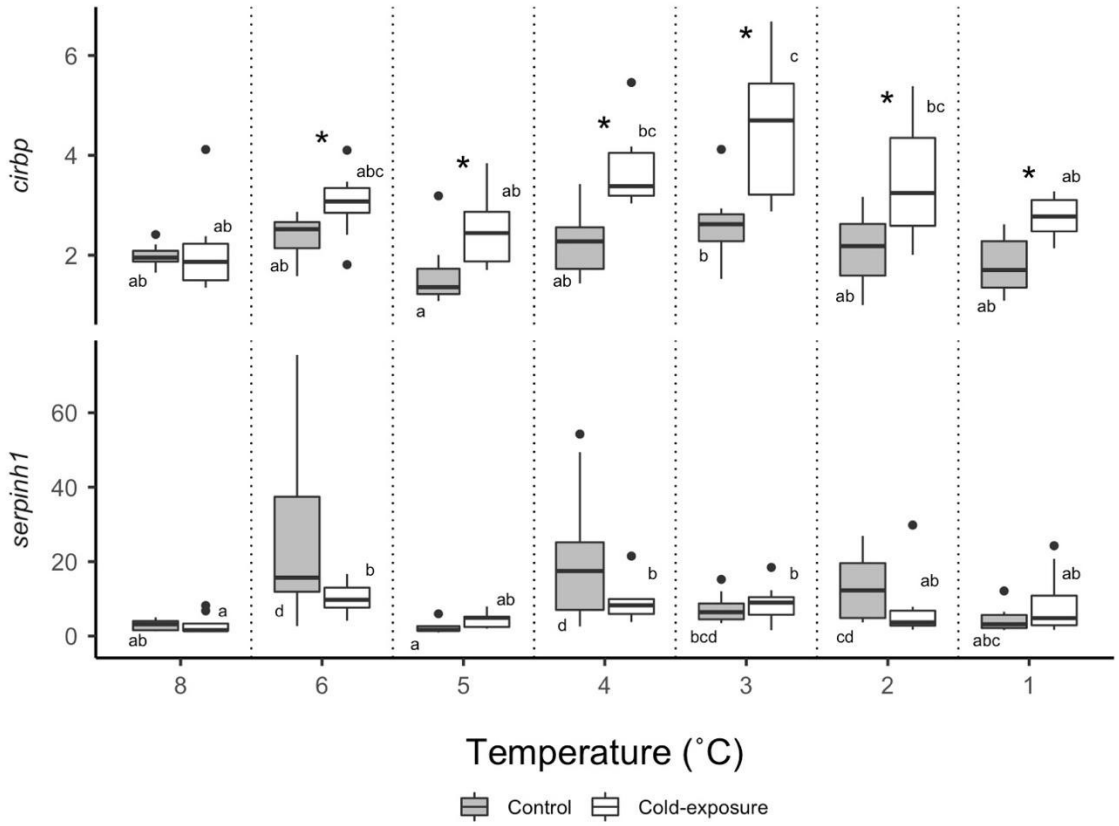

Figure 2-8. Transcript expression levels of two stress-response related proteins as temperature was lowered from 8 to 1°C in the cold-exposure group as compared with the control group. Transcript levels are presented as relative quantity (RQ) values (i.e., values for the transcript of interest were normalized to both eif3d and rpl32 transcript levels and were calibrated to the individual with the lowest normalized expression level of that given transcript. Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. $^{+}$ Indicates a near significant difference ($p < 0.1$), whereas * indicates a significant ($p < 0.05$) difference between control and cold-exposure fish at a particular temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

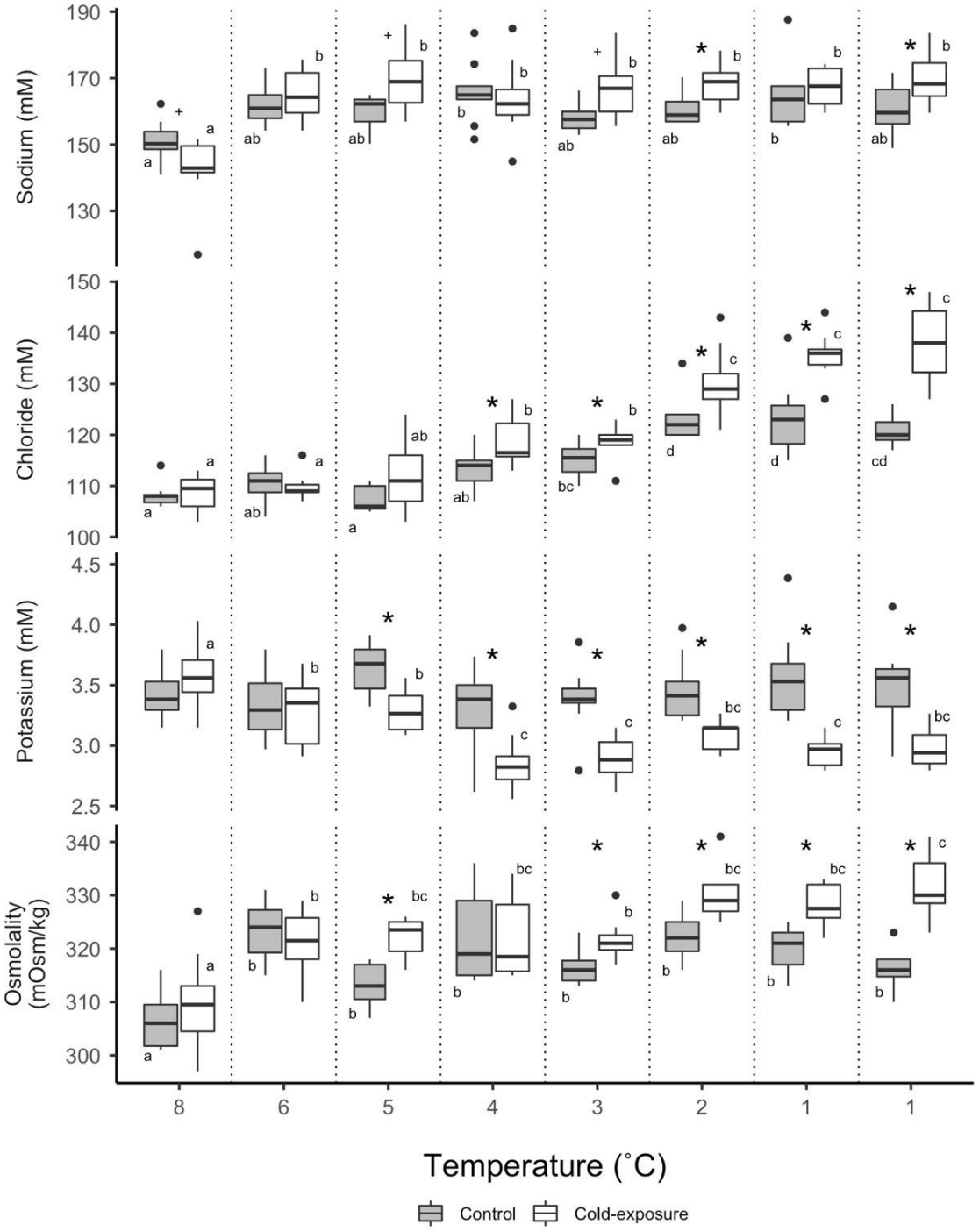

Figure 2-9. Plasma chloride (Cl^-), potassium (K^+) and sodium (Na^+) levels, and plasma osmolality as temperature was lowered from 8 to 1°C in the cold-exposure group as compared with the control group. Lower and upper box boundaries and the 25th and 75th percentiles, respectively, the line inside the box is the median value, and the lower and upper error lines are the 10th and 90th percentiles, respectively. Filled circles indicate data points falling outside 10th and 90th percentiles. $^+$ Indicates a near significant difference ($p < 0.1$), whereas a * indicates a significant ($p < 0.05$) difference between control and cold-exposure fish at a particular temperature. Dissimilar letters indicate a significant difference between samplings within a treatment (control and cold-exposure; n=8).

in plasma osmolality (308 ± 2 mOsmol kg^{-1} to 322 ± 1 mOsmol kg^{-1}) (**Figure 2-9**). Cooling (cold-exposure) began to affect plasma ion concentrations beginning at 4–5°C, with significant increases and decreases in $[Cl^-]$ and $[K^+]$, respectively. $[Na^+]$ was significantly increased in cold-exposure fish at 2 and 1°C compared to control fish, but this change was not as evident as that for $[Cl^-]$. The increase in $[Cl^-]$ was more pronounced as temperature decreased, and after 2 weeks of exposure to 1°C, plasma $[Cl^-]$ was approximately 20 mM higher relative to the control group (137.6 ± 2.8 vs. 120.9 ± 1.0 mM, respectively). Given the large increase in $[Cl^-]$ (and moderate increase in $[Na^+]$) as compared to the decrease in $[K^+]$ (to ~3.0 mM), plasma osmolality was consistently higher in the cold-exposure animals relative to the control group by 3°C, and was ~5% (15 mM) higher by the end of the experiment.

2.3.5 Does time post-feeding affect plasma biochemical measurements?

To examine whether the change in sampling protocol required to abide by Covid-19 policies in the JBARB might have affected the results, we performed a small additional experiment at 6°C, where the same plasma parameters were measured after salmon had been fasted for 6 and 24 hours (**Figures S9-11**). The only value that changed significantly was that of plasma glucose. This parameter was higher (4.2 $\pm$ 0.2 mM) at 6 h post-feeding relative to 24 h post-feeding (3.4 $\pm$ 0.2 mM), which is consistent with the findings of the main experiment (**Figure 2-5 and S10**). Thus, it appears that the change in sampling time with respect to when the fish were fed did not greatly influence the changes in parameters reported for the cold-exposure fish between 8 and 6°C in the main experiment.

2.4 Discussion

To improve the sustainability of aquaculture in regions that experience cold winters, it is essential that we understand at what temperatures Atlantic salmon experience physiological disturbances, stress and mortality. In this study, we exposed Atlantic salmon to decreasing temperatures that mimicked seasonal declines in Atlantic Canada from 8 to 1°C to assess temperature-dependent changes in feeding and growth, and in physiological parameters related to stress, osmoregulation, metabolism and tissue damage.

2.4.1 Growth and appetite

The first measurable impact of falling temperatures in our study was a decrease in feeding, which started at 6°C and resulted in a complete loss of appetite by 1°C (**Figure 2-1**). Liu et al. (2020) reported that Atlantic salmon parr subjected to an incremental decrease in temperature stopped feeding at 2°C, and a decrease in feed intake at low temperatures is well documented for many fish species (Elliott, 1991; Islam et al., 2020; Kehoe and Volkoff, 2008; Tort et al., 2004). Feed intake at low temperatures may be primarily reduced because of lower metabolic requirements (Ibarz et al., 2007; Porter et al., 2022), and generally leads to reduced growth in fish (Power et al., 2000; Weber and Bosworth, 2005). Indeed, salmon growth was reduced by 58% during the seasonally-relevant temperature decrease employed in this study. Surprisingly, few other long-term experiments that mimic fall/winter decreases in coastal seawater temperatures have been conducted. However, there are some studies that reported the relationship between

temperature, and fish feed intake and growth (Islam et al., 2021, 2020; Sánchez-Nuño et al., 2018), including those on Atlantic salmon. For example, Handeland et al. (2008) reared Atlantic salmon at 6°C, and after 12 weeks, body weight was one-third and feed intake was one-half of that measured in conspecifics reared at 14°C. Of note, our experiment was conducted using a constant 12h light :12 h dark photoperiod, given facility requirements. However, further experiments should be performed using decreasing temperatures and a natural photoperiod regime if we are to completely and accurately characterize the relationship between water temperature, photoperiod and fish appetite and growth during this challenging period of the Atlantic salmon production cycle (Døskeland et al., 2016; Imsland et al., 2014).

2.4.2 Physiological changes between 8 and 6°C

Plasma glucose increased between the 8 and 6°C samplings (**Figure 2-5**), however this was most likely associated with the differences in the time of last feeding and time of day. **Figure S10** shows that plasma glucose levels were significantly higher in salmon sampled 6 h vs. 24 h post-feeding, and Jia et al. (2018) reported higher plasma glucose in turbot (*Scophthalmus maximus*) at 6 h compared to 24 h post-feeding. The only other changes in fish exposed to a decrease in temperature from 8-6°C were increases in both plasma Na^+ and osmolality (**Figure 2-9**). Given that these changes were also found in the control fish (those held at a constant temperature of 8°C), we expected that they were also related to the change in the time of feeding. However, this hypothesis was not borne out by the results presented in **Figure S11**, and thus, the potential reasons for the increase in plasma [Na^+] and osmolality are unknown.

2.4.3 Plasma enzymes

Animal blood contains low levels of cellular enzymes from the normal physiological processes of cellular leakage and apoptosis (Boyd, 1983). However, when tissues and organs are damaged, cells lyse and release their contents into the bloodstream where elevated values can be measured (Neff, 1985), and the presence of certain elevated enzyme levels is used as a diagnostic tool in veterinary and human medicine (Boyd, 1983; Brewster, 2018; Klein et al., 2020). However, our results provide no indication that the salmon experienced tissue damage at temperatures down to 1°C, with values either being unchanged or decreasing as the salmon were lowered to 1°C (**Figure 2-4**). Lower plasma levels for AST are in line with other research showing that plasma enzyme levels decrease with temperature (Sandnes et al., 1988; Sauer and Haider, 1977). In contrast, Liu et al. (2020) exposed Atlantic salmon parr to a decline in temperature from 16 to 1°C at a rate of ~2°C per week, and reported 5-10 fold increases in plasma AST, alkaline phosphatase (ALP), LDH and alanine aminotransferase (ALT) starting at 4 and 6°C without mortalities. The data from Liu et al. (2020) strongly suggest that there was tissue damage in important organs (including the liver) at these temperatures. It is possible/probable that their results differ from those of our study, and most of the literature, due to the rapid decline in temperature used (from 16 to 1°C in 6 weeks). This may not have allowed the fish the time to acclimate appropriately, as temperatures were reduced to the fish's lower lethal limit. However, there is an alternate explanation. Liu et al. (2020) do not report the proximate composition of the diet they used, and it is possible that their diet had a higher lipid content than used in the current study or that of Sandnes et al., (1988), and that this in combination with low temperature resulted in liver damage

and elevated plasma enzyme levels. Dessen et al. (2021) showed that at 5°C an increase in lipid content in the diet led to high plasma levels of ALT and ALP, and significant mortalities in Atlantic salmon. That said, fish species with warmer thermal niches than Atlantic salmon can show signs of tissue/liver damage (i.e., higher plasma enzyme levels) when held at low temperatures; e.g., Nile tilapia (*Oreochromis niloticus*; Panase et al., 2018), European seabass (*Dicentrarchus labrax*; Islam et al., 2021) and gilthead sea bream (*Sparus aurata*; Gallardo et al., 2003).

2.4.4 Plasma metabolites

At the high end of a fish's thermal range, oxygen delivery to the tissues falls below the animal's needs. Fish then rely on anaerobic metabolism to fulfil/partially fulfil their energetic requirements (Eliason et al., 2013; Jeffries et al., 2012) and this results in elevated plasma lactate levels (Clark et al., 2008; Eliason et al., 2013; Gallant et al., 2017). Similarly, fish exposed to a 'cold-shock' may need to rely on anaerobic metabolism to meet energy demands (Foss et al., 2012; Hyvärinen et al., 2004; Kuo and Hsieh, 2006), and long-term acclimation to cold temperatures (30 days) increases plasma lactate levels (from 3.5 to 4.5 mM) in European sea bass (Islam et al., 2021). The salmon in the current experiment were exposed to a realistic seasonal decline in temperature (from 8°C to 1°C), and there was no indication that they had to rely on anaerobic metabolism to meet their metabolic requirements (**Figure 2-5**). This is consistent with a recent study on Atlantic salmon published by Porter et al. (2022), and with unpublished data (Porter and Gamperl, in prep.) on this species. Both studies showed that this species can elevate its metabolic rate by ~4-fold at 1°C during a critical swimming speed (U_{crit})

test (i.e., their capacity for oxygen delivery and oxidative metabolism is not compromised at this temperature).

In this study, plasma glucose was elevated as compared to control fish when the cold-exposure fish began to experience ionic dysregulation at 4-5°C (**Figures 2-5 and 2-9**). This is in accordance with other studies where plasma osmolality and/or plasma [Na^+] and [Cl^-], and glucose levels, varied together (Fiess et al., 2007; Islam et al., 2021). This increase in plasma glucose may be required to meet the increased energy requirements for osmoregulation as glucose is the main source of energy for gill ionoregulation (Tseng and Hwang, 2008). However, the hyperglycemia measured between 3 and 5°C also coincided with reduced feeding. Sheridan and Mommsen (1991) fasted adult coho salmon (*Oncorhynchus kisutch*) for 3 weeks. In their study, hyperglycemia was recorded in the first week of fasting. However, after 3 weeks of fasting, glucose was back to 'normal' levels, and energy production was maintained by lipids as evidenced by plasma hyperlipidemia. Liu et al. (2020) also found higher plasma glucose levels in Atlantic salmon at 4°C during a decrease from 16 to 1°C, and reported that this was followed by a peak in plasma triacylglycerol levels and the cessation of feeding at 2°C. Glycogen reserves are a potential source for the reported elevated levels of plasma glucose. However, the *in vitro* production of glucose at low temperature by rainbow trout hepatocytes could not be accounted for by the rate of glycogenolysis, and thus, gluconeogenesis from other precursors was clearly involved (Seibert, 1985). In a metabolomic study on gilthead seabream, Melis et al. (2017) reported that both glycogenolysis and gluconeogenesis were associated with decreasing temperatures. However, when the same fish were acclimated to a constant low temperature,

gluconeogenesis was the main form of glucose production. In our study, hyperglycemia between 3 and 5°C could signify a change in the source of energy used in the weeks leading up to the coldest parts of winter; from feed to stored lipids, with a transition period utilizing glycogen reserves. However, when temperature stabilizes during winter, and feeding is minimal or stops, glucose levels are maintained through gluconeogenesis from stored lipids.

Plasma [cortisol] is an established and well accepted measurement of stress in fish (Carey and McCormick, 1998; Fiess et al., 2007; Hjelmstedt et al., 2021) including that due to cold exposure (Chen et al., 2002; Islam et al., 2021; Panase et al., 2018; Staurnes et al., 1994). After an acute stress, plasma cortisol in Atlantic salmon can increase to values between 100 and 500 ng ml^{-1} before it eventually returns to pre-stress levels (Barton et al., 1986; Barton et al., 1988; Carey and McCormick, 1998; Iversen et al., 1998). In our experiment, cortisol levels started to increase at 2°C, and were higher than those measured in the control fish at 1°C (**Figure 2-5**). This indicates that the fish were stressed by exposure to these temperatures. However, the values were low (~18 ng ml^{-1}) as compared to those recorded following an acute stressor. This low, but constant, level of plasma cortisol suggests that salmon are under chronic stress at 1°C. As a stress hormone, cortisol plays two main roles. Cortisol is released for mineralocorticoid functions and stimulates NKA activity and salt excretion in saltwater (McCormick et al., 2008). Thus, the increase in [cortisol] at ~2°C, measured ~1-2°C after the increase in plasma [Cl^-] and osmolality, is intuitive in this regard. Cortisol is also released for glucocorticoid functions, and stimulates the release of glucose through gluconeogenesis and glycogenolysis (Barton et al., 1986; Butler, 1968; Faught and Vijayan, 2016;

Wendelaar Bonga, 1997). Finally, there is considerable data which relates an increase in circulating cortisol to fasting in fish (Blom et al., 2000; Hvas et al., 2021; Jia et al., 2018; Kelley et al., 2001; Varnavsky et al., 1995), although decreases (Farbridge and Leatherland, 1992; Jørgensen et al., 1999) or inconclusive responses (Holloway et al., 1994; Pottinger et al., 2003) have also been reported. The relationship between the natural cessation of feeding at low temperatures in salmon and plasma cortisol has not been investigated but our results suggest that cortisol may play a role in maintaining glucose production when Atlantic salmon cease feeding at the lower end of their thermal range.

2.4.5 Expression of stress-related genes

The liver was chosen as the tissue to examine changes in the transcript expression level of genes with functional annotations related to the stress response due to its central role in metabolism and previously observed changes in its transcriptomic response to thermal stress. Indeed, stress-related protein transcript expression in the liver has been shown to be upregulated by high temperatures, and rapid increases and decreases in temperature (Beemelmanns et al., 2021c; L. Liu et al., 2020; Vergauwen et al., 2010; Xu et al., 2018).

Heat shock proteins are highly conserved molecular chaperones that are constitutively expressed in most cells, and have essential roles in the proper folding of newly synthesized proteins and in the re-folding of damaged proteins (Iwama et al., 1998; Mohanty et al., 2018). They are also induced when faced with a variety of stressors and specific conditions (Roberts et al., 2010). This is the first study to measure the transcript

expression levels of four paralogues of *hsp70* and of cytosolic *hsp90* following a temperature challenge in salmonids. Specific paralogues of these genes were highly upregulated (> 5-fold: *hsp90aa1a, hsp90aa1b, hsp70b* and *hsp70c*) during the gradual temperature decrease (1°C $week^{-1}$) whereas others showed only modest increases (< 1-fold: *hsp70d, hsp90ab1a* and *hsp90ab1b*) or no change (*hsp70a*). The paralogues of *hsp90* have been categorized in previous studies as constitutive (*hsp90ab1a* and *hsp90ab1b)* or inducible (*hsp90aa1a* and *hsp90aa1b*) (Garcia de la serrana and Johnston, 2013; Millson et al., 2007), and the expression pattern found in the present study is consistent with these previous studies where the expression of constitutive forms was mostly stable, whereas that of the inducible forms was up-regulated at low/declining temperatures (**Figure 2-6**). The characterization of *hsp70* paralogues as inducible vs. constitutive isoforms is not as clear as for *hsp90* since the multiple paralogs of *hsp70* have not been analyzed in a single study on Atlantic salmon (Beemelmanns et al., 2021c; Garcia de la serrana and Johnston, 2013; Lewis et al., 2016). The current study shows that slowly reducing seawater temperature to 1°C induced the expression of *hsp70b* and *hsp70c* (**Figure 2-7**). Thus, there appears to be a similar response in *hsp70* and *hsp90* expression to incremental increases and decreases in temperature that are close to the limits of the Atlantic salmon's thermal range (Beemelmanns et al., 2021b). In contrast to our findings, Liu et al., (2020), measured stable *hsp70* expression in tiger barb (*Puntius tetrazona*) liver under cold-stress, while expression in brain, gill and muscle was up-regulated. It is difficult to compare our data with those in previous studies as these fish were exposed to 'cold stress' or 'cold-shock' (i.e., much faster rates of temperature decrease; generally hours), and it is well known that the rate of temperature change has

considerable impacts on the magnitude and temporal nature of heat shock protein responses (Donaldson et al., 2008; Reid et al., 2022). It is important to note that *hsp70* and *hsp90* have been shown to be up-regulated in different fish under hyperosmotic stress (Choi, 2010; Deane et al., 2002; Pan et al., 2000; Peng et al., 2016). Given that the expression of both of these gene families was first increased at 5°C (**Figures 2-6, 2-7 and 2-9**), when osmoregulatory issues were first detected, it is possible that the increase in *hsp* expression at this temperature may be at least partially related to an increase in plasma osmolality. However, plasma osmolality did not change as temperature was lowered further, and thus, the large increases in the expression of most of the *hsp* genes at 2 and 1°C must specifically reflect the effects of cold temperatures. Finally, changes in *hsp* expression as temperature was lowered preceded changes in plasma cortisol by 2-3°C (~4°C vs. 1-2°C), indicating that these cellular measures are a more sensitive indicator of stress related to long-term decreases in temperature than cortisol. This is consistent with recent data collected on Atlantic salmon warmed to their incremental thermal maximum (IT_{Max}) at 0.2°C day^{-1}. Beemelmanns et al., (2021b) showed that liver *hsp90aa1* expression increased by 18°C, whereas plasma cortisol did not increase in these same fish until mortalities began at 22°C (Zanuzzo et al., in prep).

CIRBP stabilizes mRNA, is implicated in many cellular processes (Zhong and Huang, 2017), and it has been suggested that it represents a universal marker of cold exposure (Gracey et al., 2004). Further, the transcript expression of this gene is downregulated with heat-stress in several studies on salmonids (Akbarzadeh et al., 2018; Beemelmanns et al., 2021c; Jeffries et al., 2014, 2012). *In vitro* studies using rainbow trout tissues exposed to acute cooling from 5 to 0°C do not report a change in the

expression of *cirbp* (Borchel et al., 2017). However, the exposure to 0°C was only for 80 minutes, and may have been too short to elicit a change in *cirbp* expression. Rapid exposure to low temperatures has led to increases in *cirbp* in zebrafish (*Danio rerio*) after 4 (Hung et al., 2016) and 24 h (Chou et al., 2008), that subsequently to 'control' levels after their respective peak. This transient increase in *cirbp* has also been measured during longer periods of cold-exposure. For example, in common carp (*Cyprinus carpio* L.) exposed to a 13°C temperature decrease, *cirbp* levels peaked at 12 days and approached baseline level when the experiment ended after 22 days (Gracey et al., 2004). Similarly, we report a transient increase in the expression of *cirbp* in salmon exposed to a seasonally-relevant temperature decrease (**Figure 2-8**). The studies mentioned above all involved an acute cold-exposure, with the lower temperature maintained during the sampling period, whereas the temperature was incrementally decreased in the current study. It may be that, below a certain temperature, induction of *cirbp* expression is reduced or that there is a global acclimation response to decreasing temperatures that only elicits a transient increase in *cirbp*.

SERPINH1 plays an essential role in the stability and formation of collagen (Nagata, 2003), and has protective cellular functions such as the detoxification of reactive oxygen species (ROS) (Wang et al., 2016). Interestingly, while *serpinh1* has been reported to be a reliable marker of thermal stress in multiple studies (Akbarzadeh et al., 2018; Beemelmanns et al., 2021b; Houde et al., 2019; Swirplies et al., 2019), there are no/very limited data on the effects of exposure to cold temperatures on its expression. In this study, *serpinh1* expression did not change as temperature was slowly reduced from 8

to 1°C (**Figure 2-8**). It would be interesting to analyze all four paralogs of *serpinh1* to see if the paralogs have different expression profiles.

2.4.6 Osmoregulatory disturbance

Changes in acclimation temperature lead to ion dysregulation in European sea bass (Islam et al., 2021), gilthead seabream (Vargas-Chacoff et al., 2020), Atlantic cod (*Gadus morhua*; Staurnes et al., 1994), Mozambique tilapia hybrids (*Oreochromis mossambicus* x *O. urolepis hornorum*; Sardella et al., 2004), Atlantic salmon (Staurnes et al., 2001) and many other fish species (Burton, 1986). Low temperature exposure often results in an increase in plasma osmolality and/or in $[Na^+]$ and $[Cl^-]$ that trend towards the environment's salinity (O'Grady and Devries, 1982; Prosser et al., 1970). This implies changes in the influx and efflux of ions that result in a net increase in plasma osmolality for fish in saltwater, and a decrease in this parameter for fish in freshwater. There is considerable data in support of this hypothesis. For example, Arctic and Antarctic fish are known to have higher osmolality than temperate/tropical fish (O'Grady and Devries, 1982). In addition, Arctic, Antarctic and cold-acclimated fish have a higher concentration of $[Na^+]$ and $[K^+]$ in the muscle as compared to temperate warm acclimated fish (O'Grady and Devries, 1982; Prosser et al., 1970). We show a gradual increase in salmon plasma $[Cl^-]$ with falling temperature, and smaller and less consistent increases in $[Na^+]$ (**Figure 2-9**). If a reduction in osmoregulatory capacity/function increases the influx of all ions, it is curious that increases in plasma Na^+ did not match the increase in Cl^-. The increase in plasma osmolality would leave the fish's cells bathed in a hyperosmotic medium that would then lead to a reduction in their volume due to an osmotically driven water loss.

However, fish can regulate cellular volume by modulating intracellular solutes, and this can result in a regulatory volume increase (RVI) when exposed to hyperosmotic stress (Chamberlin and Strange, 1989). RVI mainly involves the cellular uptake of Na^+, Cl^- and K^+ (Jentsch, 2016), with Na^+ being the first ion to enter the cell to maintain cellular volume (Jentsch, 2016). For example, winter flounder (*Pseudopleuronectes americanus*) red blood cells increase their relative cell membrane permeability for Na^+ to regulate cellular volume, and this leads to increases in cellular [Na^+] and thus a reduction in extracellular [Na^+] (Cala, 1977). Perhaps the osmotically driven RVI response is the basis for the reduced plasma [Na^+] relative to [Cl^-] observed in this study. The increase in [Cl^-] relative to [Na^+] in the plasma may result in a reduction in strong ion difference (not all anions and cations were measured) and would need to be electro-chemically balanced in some way, possibly through a reduction in plasma [HCO_3^-] or an increase in plasma [H^+]. It is unlikely, however, that low temperature exposure would be associated with a reduction in pH, as there is a well known increase in blood pH with lower temperature in fish (Cameron, 1978; Rahn and Baumgardner, 1972) and there was no significant increase in plasma lactate at low temperature to indicate a metabolic acidosis. In addition to HCO_3, other anions may follow Na^+ and K^+ into the cells to maintain electro-chemical balance, such as negatively charged amino acids (e.g., aspartic acid and glutamic acid) that have been reported to be important in maintaining the electro-chemical balance of cells (Chamberlin and Strange, 1989). Thus, the implications of the large increase in plasma [Cl-] at low pH on acid-base status, and the possible role played by other anions, are clearly areas worthy of further investigation.

Byrne et al. (1972) and Islam et al. (2021) reported a decrease in Atlantic salmon and sea bass plasma [K^+] over a range of salinities at low temperatures, respectively, and three year-old Atlantic salmon acclimated to 1.5°C in freshwater had decreased plasma [K^+] compared to fish acclimated to 10°C (Virtanen and Oikari, 1984). K^+ ions are mainly found inside cells, are used to maintain cellular volume and membrane potential, and play a major role in osmoregulation (Epstein et al., 1980; Evans et al., 2005; Partridge and Lymbery, 2008). Whereas, [Na^+] is much lower in the intracellular vs. extracellular compartment (Pörtner et al., 1998). This gradient is maintained by pumps, mainly Na^+-K^+-ATPase (NKA). This is an active type of transport (i.e., requiring ATP), where 2 K^+ ions enter the cell in exchange for 3 Na^+ ions (Hiroi and McCormick, 2012). Pörtner et al. (1998) presented a model of cellular adjustments in NKA activity during seasonal cold adaptation where, to keep the increase in plasma [Na^+] out of the cells, K^+ needs to be pumped into cells, and this results in a decrease in its plasma concentration. The same thing would happen at the gills to eliminate Na^+ and Cl^- from the plasma. In addition, low temperatures decrease the permeability of membranes to K^+ (Raynard and Cossins, 1991), and thus passive K^+ influx. It has been reported that Atlantic cod at 1°C had increased gill NKA activity compared to fish acclimated to 8°C (Staurnes et al., 1994) and that gilthead sea bream experienced a similar increase in activity of gill NKA when held at 12°C vs. 19°C (Vargas-Chacoff et al., 2020). Thus, collectively, low temperatures may lead to increased osmolality, plasma [Cl^-] and NKA activity, but a decrease in plasma [K^+].

2.4.7 Low temperature exposure

It is difficult to discern if the chronic stress observed in our experiment at 1°C is due to a lack of food/feeding, high plasma osmolality, or cold-stress. However, the 5% mortality recorded in the last two weeks of the study is a clear indication of a developing condition at low temperature. Cortisol is known to suppress the immune system, and limits the fish's ability to signal that an infection has occurred and to mount an inflammatory response (Coutinho and Chapman, 2011). Fish in the cold-exposure group had clear signs of an opportunistic infection (although specific pathogens were not identified). Furthermore, the two indices of liver size / function were impacted at 1°C. HSI was higher, while surprisingly, the circulating level of AST was surprisingly lower. At this point in the experiment, the fish had essentially ceased feeding, and it is well known that fasted Atlantic salmon use their lipid reserves to provide the necessary energy to maintain activities (Cook et al., 2000; Rørvik et al., 2018). It was previously mentioned (in Section 2.4.4) that fasting promotes the use of lipids for gluconeogenesis, and cortisol promotes gluconeogenesis from lipid precursors and increases global lipolytic activity in tissues including the liver (Sheridan, 1986; Van Der Boon et al., 1991). This latter result contradicts our findings of an increase in liver size in Atlantic salmon at 1°C. However, low temperatures have been shown to reduce lipid oxidation in the Atlantic salmon liver (Fonseca-Madrigal et al., 2006), and Hochachka and Hayes (1962) showed that low acclimation temperature (4 vs 15°C) tends to promote lipid synthesis instead of glycogen synthesis in liver and muscle homogenates of brook trout (*Salvelinus fontinalis*). Given the Atlantic salmon's low metabolic rate when acclimated to 1°C (~25 mg kg^{-1} hr^{-1}; Porter et al., 2022) and reduced hepatic lipid oxidation at low temperatures (Fonseca-Madrigal et al., 2006), it is possible that the lipids released from

other tissues for gluconeogenesis due to the cessation of feeding, and the rise in cortisol, create a mismatch between fatty acid synthesis and oxidation in the liver. This would result in an increase in lipid deposition in the liver, a hypothesis that is supported by the higher HSI for the salmon at 1°C in this study (**Table 2-1 and Figure 2-2**) and studies on sea bass and sea bream when exposed to cold temperatures for prolonged periods (Ibarz et al., 2010; Islam et al., 2020). At such temperatures, these species develop 'Winter Syndrome', also called 'Winter Disease', which is associated with increased lipolytic activity in the muscle and perivisceral fat, while lipogenesis is promoted in the liver leading to an increased HSI (Ibarz et al., 2007, 2005). Indeed, when Atlantic salmon are held at 3°C for 5 weeks, ~30% become moribund / develop symptoms of 'Winter Syndrome' (Ibarz et al., 2010), and these fish have an HSI of 2.5 vs. 1.3 in asymptomatic fish, with similar observations in the present study. This condition in Atlantic salmon is covered in more detail in Chapter 4.

2.5 Summary and Conclusions

This study reveals a number of important physiological changes in Atlantic salmon when exposed to an incremental decline in seawater temperatures. When cooled from a starting temperature of 8°C: appetite decreased starting at 6°C; at 4-5°C, the fish experienced ion dysregulation accompanied by changes in metabolite levels and increases in liver stress-related gene expression; and at 2°C, an increase in cortisol was observed that became more evident at 1°C, and the liver became enlarged greatly and plasma AST levels decreased (a clear indication of alterations in liver function).

The current study identifies plasma Cl^-, and liver *hsp70b, hsp70c, hsp90aa1a* and *hsp90aa1b*, as the best candidate biomarkers of cold-stress in Atlantic salmon, and

further reinforces that cortisol is not a good indicator of long-term temperature changes (stress) as previously thought. During the two last weeks of the experiment at 1°C, significant mortalities were recorded indicating that there are important welfare issues at low temperatures. Since changes to the physiology of Atlantic salmon were measured throughout the temperature decrease, and we only monitored the salmon's physiology and mortality for two weeks at 1°C, it is possible that the issues identified would become more problematic/severe if the fish are held at cold temperatures for prolonged periods. Indeed Chapter 4 of this thesis (Vadboncoeur et al., in prep) found significant welfare issues (erratic swimming, fin rot, and facial/jaw erosion and ulceration) and reported 30% mortality, when these same populations of salmon were acclimated for 5 weeks at 3°C. Thus, it is likely that bacterial infections are concomitant with the physiological disturbances experienced by the fish at cold temperatures, and clear that management strategies should be developed and implemented to ensure salmon health and welfare during the coldest months of the year.

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CHAPTER 3: 'Cold-Shock' Has Few Physiological Effects on Cultured Atlantic Salmon (*Salmo salar*) Acclimated to Low Temperatures

Abstract

With climate change, winter storms are predicted to increase in frequency and intensity, and result in rapid declines in water temperatures in sea-cages due to the mixing of deeper waters with surface waters in contact with cold air. Given that winter storms have been identified as a cause of mortality events in Atlantic salmon held in sea-cages, we acclimated fish for 5 weeks to 3°C and: 1) maintained one group of fish at 3°C (control group); and 2) exposed two other groups to a rapid decline in temperature from 3 to 0°C over 3h, and then held them at this temperature for 4 or 24 h (i.e., a 'cold-shock'). At the end of the experiment, the temperature for these latter groups was returned to 3°C, and the fish were recovered for 5 days. Plasma samples were taken before and at the end of the 'cold-shock' (i.e., while still at 0°C), and at 6, 24, 72 and 120 hours after the fish were returned to 3°C. A moderate stress response was measured after the 'cold-shock' with: cortisol levels transiently increasing to ~30 and ~25 ng ml^{-1} in fish exposed to 0°C for 4 and 24h, respectively, within the first 24 hours after the 'cold-shock'; and glucose increasing from ~3.8 mM to 5.5-6.0 mM. There were no changes in plasma lactate, indices of tissue damage, or in plasma ion concentrations. However, gill Na^+-K^+-ATPase activity was ~5-fold higher in fish given a 'cold-shock' as compared to those maintained at 3°C. Finally, there were no mortalities within 120h of the end of the 'cold-shock'. Collectively, this information indicates that Atlantic salmon have the capacity to withstand rapid declines in seawater temperature close to 0°C, and suggests that such rapid drops in temperature are unlikely to be responsible for winter-related mortalities at salmon cage-sites in Iceland and Canada.

3.1 Introduction

Temperature is the dominant abiotic factor affecting ectotherms like Atlantic salmon (Fry, 1958), and this species' performance in culture (i.e., growth, food conversion ratio etc.) is maximized between 12 and 19°C depending on life stage (Elliott and Elliott, 2010). The annual temperature range at sea-cages in Norway generally varies between 3 and 17°C (Oliveira et al., 2021), whereas these temperature ranges are 9 to 19°C in Chile (Narváez et al., 2019; Soto and Norambuena, 2004) and 6 to 23°C in Australia (Dempster et al., 2016; Wade et al., 2019). In contrast, winter temperatures in Iceland and Canada can decrease below 3°C for more than two months of the year, and approach 0°C [(Björnsson et al., 2007; Sandrelli et al., In Prep (**Figure S1**)]. Further, winter mortality events associated with harsh winter conditions have been reported in Atlantic Canada and Iceland in recent years (Ćirić, 2020; Huffman, 2019; Pennell, 2014; The Fish Site, 2022; Undercurrent News, 2020; Willick, 2019). Some of these events were associated with seawater temperatures where Atlantic salmon plasma could freeze (-0.5 to -0.8°C) (Fletcher et al., 1988; Huffman, 2019; Hurst, 2007; Pennell, 2014), however, this was not for all cases.

Surprisingly, there has been little effort to understand the impacts of cold temperatures (< 5°C) on salmon physiology, especially given that winter storms are predicted to increase in frequency and intensity (IPCC, 2022), and this will lead to episodes where deeper waters mix with surface waters in contact with cold air resulting in rapid declines in temperature (Szekeres et al., 2016). Further, although it is well established that rapid declines in temperature result in a stress response and possibly mortalities in salmonids (Donaldson et al., 2008; Reid et al., 2022), and that low

acclimation temperatures do have an impact on the physiology and stress response of fish (Barnes et al., 2014; Madaro et al., 2018; Porter et al., 2022; Tang et al., 2022), no study has taken a comprehensive look at how acute exposure to temperatures close to 0°C (as would be associated with winter storms at cage-sites) impacts the physiology of Atlantic salmon.

Thus, we acclimated Atlantic salmon post-smolts to 3°C for 5 weeks to mimic temperature conditions in Atlantic salmon sea-cages in the middle of winter [**Figure S1**; Björnsson et al., (2007)]**;** and 1) maintained one group of fish at 3°C (control group); and 2) exposed two other groups to a rapid decline in temperature from 3 to 0°C over 3h, and maintained them at this temperature for 4 or 24 h (i.e., a 'cold-shock'). At the end of the experiment, the temperature for these latter groups was restored to 3°C, and the fish were subsequently recovered for 5 days. The fish were sampled for plasma before the 'cold-shock', at the end of the 'cold-shock', and at 6, 24, 72 and 120 h after their return to 3°C. The plasma was analyzed for biomarkers associated with stress (cortisol and glucose), ion levels and osmolality, and indices of tissue damage [i.e., lactate dehydrogenase (LDH); creatine kinase (CK); and aspartate aminotransferase (AST)]. In addition, gill Na^+-K^+-ATPase activity was measured prior to, and 72 h after exposure to 0°C for 24 h. The results from this study provide strong evidence that Atlantic salmon are not negatively impacted by such 'cold-shocks' (at least not directly), and suggest that such events are unlikely to be the primary reason for cage-site mortalities in winter in Iceland and Norway.

3.2 Materials and Methods

This study was approved by the Animal Care Committee of Memorial University of Newfoundland and Labrador (protocol #21-02-KG). All procedures conducted on the salmon were performed in accordance with the Canadian Council on Animal Care's Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (Canadian Council on Animal Care, 2005).

3.2.1 Experimental animals

The Atlantic salmon used in these experiments were raised in a commercial hatchery in New Brunswick (Canada), and transferred as pre-smolts to the Dr. Joe Brown Aquatic Research Building (JBARB) (Department of Ocean Sciences, Memorial University of Newfoundland and Labrador) in January of 2021. These fish were initially held for 6 months in a 3000 l tank. This tank was supplied with flow-through seawater (32 ppt salinity) at ~6-8°C and ~100% air saturation. The photoperiod was 12hlight : 12h dark. The fish were fed to apparent satiation daily with 3 mm commercial pellets (Signature Salmon, Northeast Nutrition, Truro, NS, Canada).

3.2.2 Experimental protocol

The Atlantic salmon (225 fish; 343 ± 15 g) were separated into three 1000 l tanks (75 fish $tank^{-1}$; **Figure S2**) supplied with ~6°C flow-through seawater at ~100% air saturation at 7.5 l min^{-1}, and with a 12h light : 12h dark photoperiod. Feed was offered by hand to all tanks every other day until apparent satiation was achieved. The fish were

considered satiated when pellets began accumulating at the bottom of the tank. The fish were fasted 24 h before any sampling.

The fish were allowed three weeks of acclimation at ~6°C. The temperature was then decreased from 6 to 3°C over 10 days, and the fish were acclimated to this latter temperature for 5 weeks. Finally, the tanks were separated into three treatments: 1) a constant temperature of 3°C (control group); 2) holding at 3°C, followed by a 4 h exposure to 0°C, and a return to 3°C; and 3) '2', but with 24 h of exposure to 0°C. A diagram detailing the timing and duration of changes in water temperature in the three treatments is included as **Figure 3-1**. The two different 'cold-shock' durations (4 and 24 h) were performed at different times because of the limited cooling capacity of our glycol chiller (Technical Services, Memorial University). It could only maintain a temperature of 0°C in one tank at a time while keeping the others at a constant temperature of 3°C. When exposing the fish in one of the tanks to a 'cold-shock', the water temperature was decreased over 3 hours from 3 to 0°C, and then maintained at this temperature for 4 or 24 hours depending on the treatment. At the end of the 'cold-shock', the temperature in the tanks was returned to 3°C over 1 hour.

3.2.3 Sampling regime

Apparently healthy fish (behaving normally and with no external signs of any health or welfare related issue) [see Vadboncoeur et al. (Chapter 4; in Final Prep)] were haphazardly netted, and anesthetized in seawater containing 0.6 g l^{-1} MS-222 (Syndel Laboratories Ltd, Vancouver, BC, Canada). The first samples were taken at the end of the three weeks at 6°C, where 3 fish per tank had their first gill arch sampled (see details

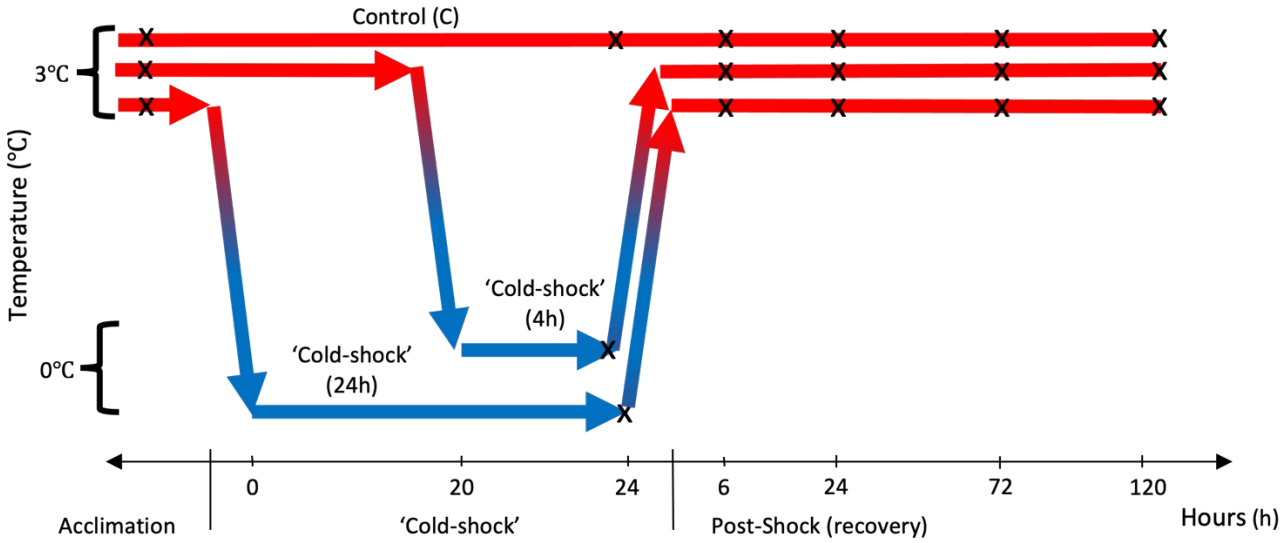

Figure 3-1. Protocol used for the 'cold-shock' experiment. Fish in the three treatments were held in separate tanks. The 'X' represents time points at which fish (n=8) from all three treatments were sampled. The temperature was decreased from 3 to 0°C over 3 hours, and returned to 3°C after the 'cod-shock' in 1 h. The color shift from red to blue represent the change in temperature from 3°C (red) to ~0°C (blue).

below) for Na^+-K^+-ATPase (NKA) activity. The first 3°C blood sample was taken (see details below) 2 days before the 'cold-shock' (these values referred to as 'initial'), with 3 fish sampled per tank (n=9). Blood samples were collected from eight fish per tank at the end of the 'cold-shock' while they were still at 0°C (either 4 or 24 hours after the start of the 'cold-shock'), and at 6, 24, 72 and 120 hours after being returned at 3°C. To obtain the eight control (time-matched) samples, four fish from the control tank (maintained at 3°C) were collected during each sampling of the 'cold-shock' treatments. All fish were measured for weight and fork length. Their blood was then sampled by caudal puncture prior to removal of their liver for the determination of hepatosomatic index (HSI).

Hepatosomatic index was calculated on every sampled fish according to the following formula:

$$HSI = 100 [W_L/W_t]$$

where W_t (g) was total body weight and W_L was liver weight.

3.2.4 Plasma and gill collection

The anesthetized fish were bled by caudal puncture within 3 min of netting. Blood samples were transferred to 2 ml Eppendorf® tubes containing 75 µl of saline containing lithium heparin (Sigma Chemical Co., Oakville, On, Ca; 1000 units per ml), shaken by inversion, and then centrifuged at 1,100 *xg* for 1 minute. The plasma was then distributed into a number of Eppendorf® tubes for the various analyses, and immediately frozen in liquid nitrogen prior to beieng stored at -80°C for later analysis.

Additionally, gill tissue was collected from the fish sampled at 6°C, 3°C ('Initial') and at 3°C 72 h after the 24 h 'cold-shock'. The first gill arch on the left side of the fish

was taken out, placed whole in a 2 ml Eppendorf® tube, flash frozen in liquid nitrogen, and then stored at -80°C for later analysis.

3.2.5 Plasma analyses

3.2.5.1 Spectrophotometry

Samples were thawed and diluted to the desired concentration to allow for accurate readings. All enzyme activities were measured at 25°C using a SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA, USA). These assays were developed for this experiment using established protocols on human plasma (Bergmeyer et al., 1978), fish plasma (Casillas et al., 1982), fish liver (Treberg et al., 2002) and fish muscle (Treberg et al., 2003). All chemicals were purchased from Sigma Aldrich (Oakville, OB, Canada) unless otherwise stated.

Assay conditions were as follows:

Aspartate aminotransferase (AST; E.C. 2.6.1.1). 50 mM imidazole (pH 7.4), 200 mM aspartate, 0.2 mM NADH, 0.05 mM pyridoxal–5–phosphate and 14.5 U/ml malate dehydrogenase. The reaction was initiated with 7.0 mM α-ketoglutarate.

Lactate dehydrogenase (LDH; E.C. 1.1.1.27). 50 mM imidazole (pH 7.4) and 0.2 mM NADH. The reaction was initiated by the addition of 1 mM of pyruvate.

Creatine kinase (CK; E.C. 2.7.3.2). 50 mM Imidazole (pH 7.4), 5.0 mM $MgCl_2$, 2 mM ADP, 0.8 mM NADP, 5 mM glucose, 10 mM AMP, 2 U/ml hexokinase, 2 U/ml glucose–6–phosphate dehydrogenase, and 10 μM P1,P5-Di(adenosine-5'-pentaphosphate). The reaction was initiated by the addition of 50 mM of phosphocreatine.

Glucose. The sample was deproteinized in 1:4 0.6% Protocatechuic acid. 250 mM Imidazole (pH 7.8), 5 mM $MgSO_4$, 10 mM ATP, 0.8 mM $NADP^+$ and 10 U/ml glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of 10 U/ml hexokinase.

Lactate. The sample was deproteinized in 1:4 0.6% Protocatechuic acid. 200 mM glycine buffer solution and 25 mM NAD. The reaction was initiated by the addition of 400 U/ml of lactate dehydrogenase.

Cortisol levels in the plasma were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (NEOGEN Corp. Lexington, KY, USA).

3.2.5.2 Ion measurements

Plasma sodium (Na^+) and potassium (K^+) plasma levels were analyzed using a PFP7 Flame Photometer (Jenway, Stone, Staffordshire, UK) following the manufacturer's methods for plasma analysis. Chloride (Cl^-) and osmolality were measured according to manufacturer instructions using a ChloroChek Chloridometer (model 3400) and a VAPRO Vapor Pressure Osmometer (model 5600; ELITech Group, Logan, UT), respectively.

3.2.6 Na^+-K^+-ATPase activity

Gill Na^+-K^+-ATPase (NKA) activity was measured as described in McCormick (1993). SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% sodium deoxycholate, pH 7.3), Salt mix (105 mM NaCl, 21 mM KCl, 5.2 mM $MgCl_2$(hex), 50 mM imidazole, 5 mM sodium azide, pH 7.5) and Imidazole buffer (50 mM imidazole, pH 7.5) were prepared in advance and stored at 4°C for up to 1 month. pH was adjusted using 0.1 N HCl. On the day of assay, assay solutions were prepared as follows: ATPase mix

[3.1 mM ATP, 2.4 mM phosphoenolpyruvate (PEP), 0.21 mM NADH, excess (10-17 units ml^{-1}) pyruvate kinase (PK), excess (~61 units ml^{-1}) lactate dehydrogenase (LK), dissolved in Salt mix], and Ouabain (OB) solution (26.7 mM ouabain, dissolved in imidazole buffer at 85°C then kept in the dark on ice).

Gill lamellae (mean: 0.054 ± 0.0003 g) were homogenised in 1 ml of SEID buffer at 4°C using a Bullet Blender Storm 24 (Next Advance, Troy, NY, USA) and Precellys ceramic (zirconium oxide) beads (Bertin Corp., Rockville, MD, USA). Homogenates were spun for 30 seconds (at 5000 *xg* at 4°C), and the supernatant retained for use in the assay. On ice, 10 µl of sample was added to each of 6 wells of a microplate (Corning, costar 9017). Two hundred µl of control mix (comprised of: 190.2 µl of ATPase mix and 9.8 µl of imidazole buffer) was added to each of the first three wells, and 200 µl of NKA mix (190.2 µl of ATPase mix, 9.8 µl of OB solution) was added to wells 4-6. A kinetic assay was then run on a SpectraMAX 190 microplate spectrophotometer with SoftMax Pro software (Molecular Devices, San Jose, CA, USA) at 340 nm at 25°C for 45-60 minutes until the reaction was finished.

An ADP standard curve was used to convert OD to rate of ADP production, and the difference between the 'Control' and NKA mix OD values was taken as NKA activity. NKA activity was standardized per mg of protein. Protein concentrations were determined separately using a Bradford Assay (Bradford, 1976) and bovine serum albumin standards. All biochemical reagents for the measurement of NKA activity were obtained from Sigma Aldrich.

3.2.7 Statistical analyses

All data are reported as means ± standard error of the mean (s.e.m.). The data were analyzed using Rstudio Version 1.4.1106 R 4.0.5 (R Core Team, 2020, https://www.r-project.org/). A Bonferroni outlier test was used to identify outliers before statistical analysis. Parameters were analysed by fitting linear models using the *lm* function. Statistical models were computed with 'treatment' and 'sampling' as main effects. Each model fit was graphically examined (i.e., using histograms and qqplots) and residuals tested for normality (Shapiro–Wilk, $p<0.05$). Non-normally distributed data were power-transformed using a Box-Cox transformation (Atkinson et al., 2021) using the *boxcox* function implemented in the *EnvStats* (version 2.7.0) package in R (Millard, 2017) so that the data met the assumption of normality. Finally, when the model identified significant effects, least-squares means post-hoc tests with Bonferroni's p-value correction for multiple comparisons [using the *lsmeans* function in R (Lenth, 2016)] were used to identify significant differences between treatments at a particular sampling point, and between sampling points within a treatment.

3.3 Results

3.3.1 Indices of stress and tissue damage

Plasma cortisol levels were 5 ± 2 ng ml^{-1} in fish at 3°C before the 'cold-shock' (**Figure 3-2**). Control fish had stable cortisol levels over the course of the experiment. The fish exposed to a 4 h 'cold-shock' had significantly higher levels of plasma cortisol at 24h post-exposure (31 ± 11 ng ml^{-1}) as compared to initial values. In contrast, the 24h 'cold-shock' resulted in a ~4-fold increase in plasma cortisol levels by the end of the period of exposure to 0°C (23 ± 7 ng ml^{-1}, **Figure 3-2**). Nevertheless, these increases in plasma

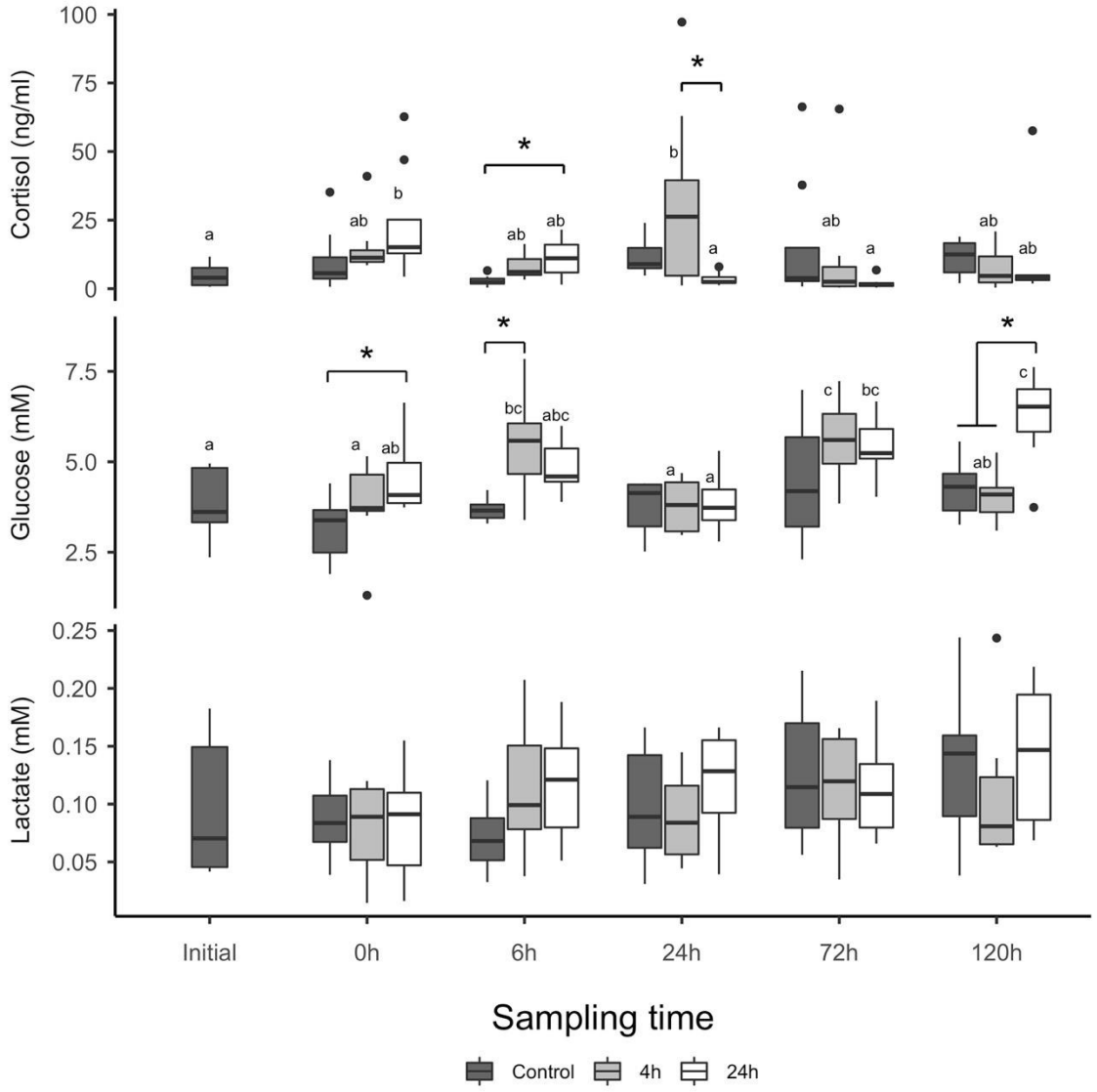

Figure 3-2. Plasma cortisol, glucose and lactate concentrations prior to the 'cold-shock' ('initial'), at the end of the period of exposure to 0°C (0h), and at 6, 24, 72 and 120 hours after water temperature was increased back to 3°C. Control fish were held at a constant temperature of 3°C, whereas the other groups were exposed to 0°C for 4 or 24 h). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. * indicates a significant difference ($p < 0.05$) between treatments at a specific sampling point. Dissimilar letters indicate a significant difference between samplings within a treatment (Control, 4h 'cold-shock', 24h 'cold-shock'; n=8).

cortisol were short-lived, and levels in both experimental groups were similar to those in control fish by 72h post-exposure. Glucose levels in the control group were also stable for the duration of the experiment (~3.2 – 4.5 mM). The fish exposed to 'cold-shock' for 4 hours had significantly higher glucose levels (5.5 ± 0.4 mM) as compared to control fish and pre-stress levels (3.8 ± 0.3 mM) at 6h and 72h post-stress, whereas those exposed to the 24h 'cold-shock' had significantly higher glucose levels at 72h (5.4 ± 0.3 mM) and 120h (6.2 ± 0.4 mM) as compared to pre-stress levels. Their glucose levels were also significantly higher than control values at the end of the 'cold-shock' (0h) and higher than the controls and salmon exposed to 4h of 'cold-shock' at 120h post-stress (**Figure 3-2**). There were no differences in plasma lactate levels between the treatments or sampling points, with levels ranging between 0.08 ± 0.01 mM to 0.13 ± 0.2 mM (**Figure 3-2**). There were also no significant differences in the plasma levels of enzymes indicative of tissue damage (AST, CK and LDH) between the treatments at any sampling point. Values ranged from ~40 to 65 U ml^{-1} for AST, ~420 to 600 U ml^{-1} for CK and ~90 to 190 U ml^{-1} for LDH (**Figure 3-3**).

3.3.2 Plasma ion levels, osmolality and Na^+-K^+-ATPase activity

No changes in plasma [Cl^-], [K^+] or [Na^+] were recorded between the groups or sampling points, with mean plasma levels for these ions of 139 ± 9 mM, 3.3 ± 0.3 mM and 196 ± 12 mM, respectively (**Figure 3-4**). Osmolality prior to the 'cold-shock' was 337 ± 6 mOsm kg^{-1}, and did increase when fish were exposed to 0°C. In fish exposed to the 4h 'cold-shock', osmolality was higher at 24h post-exposure (368 ± 13 mOsm kg^{-1}) as compared to initial values, and remained elevated until the experiment was completed (values 378 ± 13 and 370 ± 4.7 mOsm kg^{-1} at 72 and 120h after temperature was returned

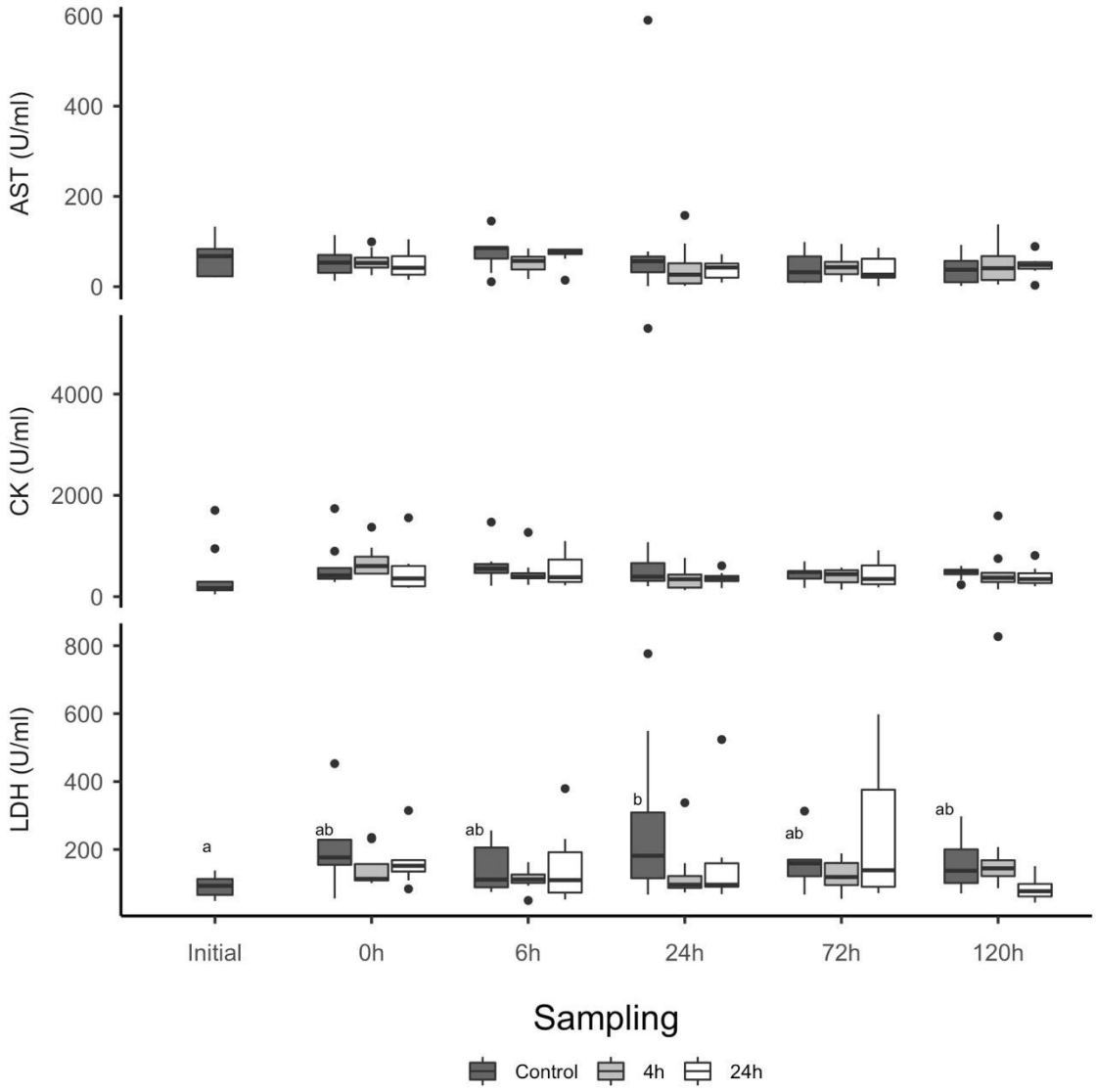

Figure 3-3. Plasma aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) activity prior to the 'cold-shock' ('initial)', at the end of the period of exposure to 0°C (0h), and at 6, 24, 72 and 120 hours after water temperature was increased back to 3°C. Control fish were held at a constant temperature of 3°C, whereas the other groups were exposed to 0°C for 4 or 24h). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. Dissimilar letters indicate a significant difference ($p < 0.05$) between samplings within a treatment (Control, 4h 'cold-shock', 24h 'cold-shock'; n=8).

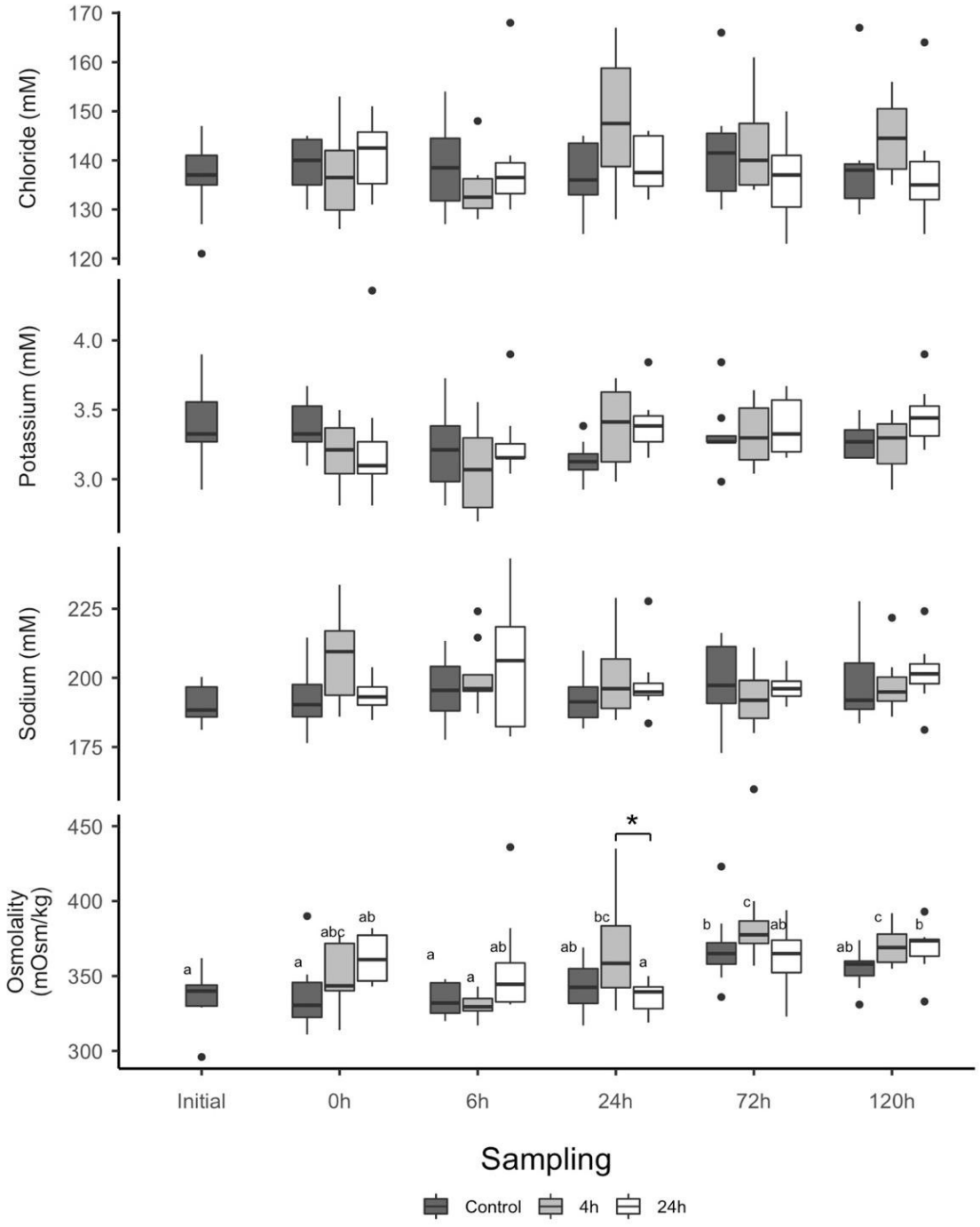

Figure 3-4. Plasma chloride (Cl^-), potassium (K^+) and sodium (Na^+) levels, and plasma osmolality, prior to the 'cold-shock' ('initial'), at the end of the period of exposure to 0°C (0h), and at 6, 24, 72 and 120 hours after water temperature was increased back to 3°C. Control fish were held at a constant temperature of 3°C, whereas the other groups were exposed to 0°C for4 or 24 h). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. * indicates a significant difference ($p < 0.05$) between treatments in a sampling point. Dissimilar letters indicate a significant difference between samplings within a treatment (Control, 4h 'cold-shock', 24h 'cold-shock'; n=8).

to 3°C). For fish exposed to 0°C for 24h, osmolality was not elevated until 120h after the 'cold-shock' as compared to initial levels (the value at this time point 368 ± 6.2 mOsm kg^{-1}). Nevertheless, none of these values were increased as compared to their time-matched controls; the levels in these fish were also higher at the end of the experiment. (**Figure 3-4**). There was no difference in NKA activity in the gill homogenates of salmon acclimated to 6°C and 3°C. However, the gill NKA activity of salmon in the 24h 'cold-shock' treatment was significantly higher (by ~5-fold) at the 72h post-exposure sampling point than measured in fish prior to being lowered to 0°C (**Figure 3-5**).

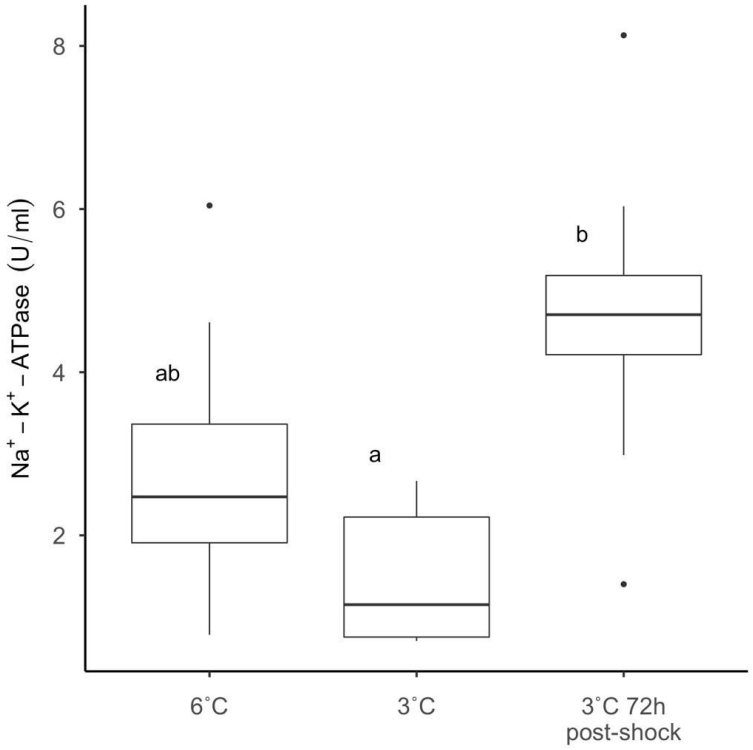

Figure 3-5. Box-plots of Na^+-K^+-ATPase activity in gill homogenates from salmon acclimated to 6°C (n=9), held at 3°C for 5 weeks (n=9), and exposed to a 24 h 'cold-shock' followed by 72 h of recovery at 3°C (n=7). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. Dissimilar letters indicate a significant difference ($p< 0.05$) between samplings.

3.4 Discussion

Winter storms are expected to increase in frequency and intensity with climate change (IPCC, 2022), and this could exacerbate the fish welfare/health and mortality issues that have been observed over the past decade in Atlantic Canada and Iceland during the winter months (CBC News, 2020; Ćirić, 2020; Pennell, 2014; The Fish Site, 2022; Undercurrent News, 2020; Willick, 2019). The current study shows that while Atlantic salmon experience a moderate stress response during a 24 h 'cold-shock', and after being returned to 3°C following both 4 and 24 h 'cold-shocks', there was no evidence of tissue damage or an ionoregulatory disturbance, and there we no mortalities. Thus, rapid declines in temperature to 0°C are unlikely to be associated with the loss of salmon during winter storms in these regions.

3.4.1 Indices of stress and tissue damage

The two exposures to 0°C resulted in a moderate stress response (cortisol ~25-30 ng ml^{-1}) in the Atlantic salmon used in this study that lasted up to 24 hours after the fish were moved back to 3°C (**Figure 3-2**). When Atlantic salmon are acutely stressed, cortisol levels reach 100-200 ng ml^{-1} (Barton et al., 1986, 1988; Carey and McCormick, 1998; Iversen et al., 1998). Interestingly, a rapid transfer from 13 to 10°C (the same temperature difference as in the current study) elicited a stronger cortisol response (to ~100 ng ml^{-1}) in post-smolt Atlantic salmon 1 hour post-transfer (Tang et al., 2022). Long acclimation periods at low temperature can lead to a decrease in the amplitude of the stress response through differential expression of corticoid receptors and decreases in plasma cortisol release in Atlantic salmon (Madaro et al., 2018; Tang et al., 2022). It is possible that a 3°C

drop in temperature is not that stressful for Atlantic salmon that are already acclimated to cold temperatures. However, there are no similar studies with which to compare our results, and thus, confirmation of this hypothesis would require additional research.

Glucose is an important metabolite and is released into the plasma when fish are exposed to acute stress (Jentoft et al., 2005; Rotllant and Tort, 1997). The initial increases in plasma glucose following the 'cold-shock' (i.e., at 0 and 24h ; **Figure 3-2**) were likely associated with the concomitant increase in plasma cortisol as this hormone is, in part, a glucocorticoid that stimulates the release of glucose through activation of gluconeogenesis and glycogenolysis (Barton et al., 1986; Butler, 1968; Faught and Vijayan, 2016; Wendelaar Bonga, 1997). A second increase in plasma glucose levels was observed in the fish exposed to 0°C for 4 and 24h at 72 h and 120 h following their return to 3°C, respectively. The increases in cortisol were similar to the magnitude of those measured in the first 24h after the 'cold-shock'. The reason(s) for this later increase in circulating glucose levels is not known as plasma cortisol was down to baseline levels during this period. (**Figure 3-2**). However, these results suggest that 'cold-shock' may be more than just an acute stressor, and that the duration of the fish's elevated glucose levels may depend on the length of exposure to 0°C.

Plasma lactate is used as a biomarker of anaerobic metabolism (Hvas and Oppedal, 2019; Jones, 1982). Increases in plasma lactate following a 'cold-shock' have been measured in salmonids originally held at preferred temperatures. Brown trout (*Salmo trutta*) exposed to 0.2°C for 10 minutes and recovered in 14°C water had maximum plasma lactate levels of 60 – 90 mM between 10 and 60 minutes and values that were elevated as compared to control fish for up to 10 hours post-exposure (Hyvärinen et al.,

2004). When exposed to a decrease in temperature from 16 to either 4 or 0°C for 5 or 1h, plasma lactate was increased (by 20-fold, to 11 mM) 1 hour post-shock only for Atlantic salmon exposed to a temperature to 0°C for 1h (Foss et al., 2012). In contrast, Porter et al. (2022) reported that plasma lactate was not elevated in Atlantic salmon 24 h after salmon were exposed to a temperature decrease from 8 to 1°C over 7h. The lack of an increase in plasma lactate in salmonids acclimated to cold temperatures (e.g., 3°C) after a 'cold-shock' to temperatures near 0°C may be due to the fish's low metabolic rate at this temperature (Porter et al., 2022).

Plasma enzymes are used as indices of cellular damage or apoptosis in general, or in specific tissues (Boyd, 1983). The low and steady plasma enzyme levels in this study (**Figure 3-3**) suggest that no cellular/tissue damage occurred during the 0°C exposure or during the salmon's subsequent recovery at 3°C. This data is hard to compare with the literature as 'cold-shock' experiments usually have protocols with larger temperature decreases than the one used here. However, there is some data. Orange-spotted grouper (*Epinephelus* coioid) had higher plasma AST levels when exposed to a 9°C (1.25-fold increase) and 12°C (2-fold increase) 'cold-shock' at 24h post-shock (Sun et al., 2019). Nile tilapia (*Oreochromis niloticus*) exposed to acute decreases in temperature of 2, 4, 8 and 12°C only had increased plasma levels of alanine aminotransferase (ALT; ~5-fold) at 48 h and AST (~4-fold) at 72 h after the largest temperature drop (12°C) (Panase et al., 2018). Collectively, this data suggests that tissue damage (as assessed by increases in plasma enzyme activity) only occurs when the drop in temperature is large. However, this would need to be confirmed by additional experiments.

3.4.2 Plasma ion levels, osmolality and Na^+-K^+-ATPase activity

'Cold-shocks' are known to challenge the osmoregulatory capacity of fishes, with plasma osmolality generally trending towards environmental values (Donaldson et al., 2008). The fish in the current experiment did not experience an osmoregulatory disturbance associated with the imposed 3°C 'cold-shock' (3 – 0°C; **Figure 3-4**). There were no changes in plasma [Cl^-], [Na^+] or [K^+], and plasma osmolality was never different from that of the control group (**Figure 3-4**). Similar to the data for lactate mentioned above, Foss et al. (2012) only reported increased plasma Na^+ at 6 h post-shock in Atlantic salmon exposed to the largest (and shortest) temperature decrease (16°C in 1h). The latter data suggest that the 'cold-shock' in our experiment was not large or long enough to compromise the salmon's osmoregulatory functions. This may be because cold-acclimation results in compensatory responses that allow the fish to osmoregulate effectively at cold temperatures. Indeed, killifish (*Fundulus heteroclitus*) acclimated for 24 days to 5°C are able to limit the passive influx of ions by remodelling their gill structure and increasing the level of polyunsaturated fatty acids in their gill cell membranes, all the while reducing energy demand (Barnes et al., 2014). The Atlantic salmon has also been shown to increase the level of polyunsaturated fatty acids in the gills with decreasing temperature (Liu et al., 2018). Similar adaptations after 5 weeks at 3°C could be the reason for the stable ion levels measured during and after the 'cold-shock' in the current experiment. Increasing the level of polyunsaturated fatty acids, and other 'remodelling' of the gills, would reduce the gill's permeability to certain ions as described for killifish above (Barnes et al., 2014). Furthermore, the fish were held at their previous acclimation temperature after the 'cold-shock', and this may not have required the fish to make any

physiological adjustments to maintain plasma ion levels at appropriate levels. However, the reported large increase in gill NKA activity following the 'cold-shock' (**Figure 3-5**) suggests that this protein might have played a role in limiting the increase in plasma ions as a consequence of 'cold-shock'. NKA in the gills of fish in seawater works in maintaining an electrochemical gradient that is responsible for the secretion of NaCl (Hwang and Lin, 2013). Gill NKA activity is known to increase with cold-acclimation. Gilthead sea bream had higher gill NKA activity at 12°C as compared to 19 and 26°C after 35 days of acclimation (Vargas-Chacoff et al., 2020). Staurnes et al. (1994) measured a similar response in the activity of Atlantic cod (*Gadus morhua*) gill NKA when this species was exposed to a rapid change in temperature from 8 to 1°C and after 17 days of acclimation to 1°C. Finally, low acclimation temperatures led to increased NKA activity in the gill tissue of fathead minnow (*Pimephales promelas;* Monroe et al., 2019) and increased NKA gene expression in the gills of European seabass (*Dicentrarchus labrax*; Islam et al., 2021).

A rapid response of NKA activity to a 'cold-shock' has not been described previously. However, cortisol is a known stimulator of NKA activity and transcription (McCormick et al., 2008), and thus, it could have played a role in the large increase in NKA measured in the current experiment at 72 h post-shock. Similarly, spottedtail goby (*Synechogobius ommaturus*) exposed to an acute increase in salinity had increased gill NKA activity that peaked after 12h and then settled to a new equilibrium at 96 h (Shui et al., 2018). Rapid changes in NKA activity have also been measured after 'heat-shock'. Blue green damselfish (*Chromis viridis*) experienced a 50% decrease in gill NKA activity 12 h after a 4°C 'heat-shock' (Tang et al., 2014). Rapid changes in the activity of NKA

have been linked to a number of metabolites and hormones (e.g., cortisol), and the recruitment of NKA to the cellular membrane through endosomic transport (Blanco and Mercer, 1998; Carranza et al., 1998; Chew et al., 2014; McCormick et al., 2008; Tipsmark and Madsen, 2001).

3.4.3 Comparisons between 'cold-shock' studies

It is difficult to compare the physiological response to 'cold-shock' between different studies. 'Cold-shock' is an investigational tool that is not standardized and it has been performed under a variety of protocols (Donaldson et al., 2008; Reid et al., 2022). Many parameters need to be controlled, that can have significant impacts on the measured parameters. The amplitude of the decrease in temperature used is quite variable. For example, Ji et al. (2016) exposed turbot (*Scophthalmus maximus*) to a decrease in temperature of 17°C when examining this species' stress response, while Tang et al. (2022) used a 3°C decrease in temperature to examine the stress response of Atlantic salmon. The rate of temperature change also varies widely between studies. Some investigators transfer fish directly from one temperature to another (Hyvärinen et al., 2004; Inoue et al., 2008), while others take multiple hours/days to achieve the same temperature change (Islam et al., 2021; Panase et al., 2018). Finally, the duration and temperature of acclimation are also important to consider as they can lead to specific physiological responses (Barnes et al., 2014; Madaro et al., 2018; Tang et al., 2022). To date 'cold-shock' protocols have generally been used to emulate real-world events, and while not standardized to a specific physiological outcome [e.g., as the loss of equilibrium in critical thermal maximum (CT_{Max}) and minimum (CT_{Min}) tests], they are clearly relevant

and important. In fact, keeping such studies relevant to culture or wild conditions should be the goal/aim of such studies, if we are to avoid the problems that have occurred in research aimed at understanding the physiology of fish, and their survival, at high temperatures (Lefevre et al., 2021; Ørsted et al., 2022). With climate change, there has been an explosion of researchers performing upper thermal tolerance tests. They often use protocols (e.g., Casselman et al., 2012) with questionable relevance to a fish in its natural environment or in aquaculture for ease of use (Desforges et al., 2021; Ørsted et al., 2022; Sandrelli and Gamperl, 2022). These studies have sometimes done more to 'muddy the waters' than to advance the field and provide valuable information of use to fisheries managers, aquaculture personnel and/or conservation biologists (Lefevre et al., 2021).

3.5 Conclusion

In our experiment, Atlantic salmon acclimated to 3°C (a 'cold' temperature for this species) and rapidly exposed to 0°C showed signs of moderate stress (increased plasma glucose levels and cortisol values of ~25 – 35 ng ml^{-1} post-exposure). However, there were no effects of this protocol on plasma lactate, ion concentrations (Cl^-, Na^+ and K) or osmolality, and the only other substantive change was an increase in NKA activity following the 0°C exposure. This latter physiological change can be seen as an adaptive response, and given that neither of the 'cold-shock' protocols resulted in any mortality, 'cold-shock' is unlikely to be a major contributor to winter-related mortalities at salmon cage-sites in Atlantic Canada, Iceland and northern regions of Norway where cold temperatures are experienced.

During the acclimation period to 3°C in this experiment, a number of moribund fish had to be removed. These fish had ulcers to the head and jaw, and when examined had large, pale and friable livers, higher plasma AST levels than 'asymtomatic fish', and signs of issues with ionoregulation (see Chapter 4). The latter symptoms are very similar to a condition called 'Winter Syndrome' or 'Winter Disease' in fish such as sea bream and yellow drum [*Sparus aurata*, and *Nibea albiflora*; respectively; Ibarz et al., 2010; Song et al., 2019)], whereas the ulcers of the jaw/head are characteristic of an opportunistic infection such as *Tenacibaculum* sp. (tenacibaculosis) which occurs at cold temperatures (Olsen et al., 2011; Spilsberg et al., 2022). It is very likely that these two conditions, in combination, are largely responsible for fish mortality events at cage-sites that are not associated with 'winter chill' [where temperatures fall below the freezing point of salmon blood, -0.5 to -0.8 (Fletcher et al., 1988; Hurst, 2007)]. Additional research is currently underway to further understand whether 'Winter Syndrome' does occur in Atlantic salmon at cold temperatures, to understand its aetiology, and its relationship to opportunistic pathogenic infections and salmon mortality.

3.6 References

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CHAPTER 4: Low Seawater Temperatures are Associated with Liver Lipid Accumulation and Dysfunction, Ionoregulatory Disturbance and Opportunistic Infections in Cultured Post-Smolt Atlantic Salmon (*Salmo salar*)

Abstract

To ensure the sustainability of fish aquaculture, all sources of stress that could lead to issues with health and welfare and/or a loss of production should be examined, and if possible, mitigated. High temperatures, nutrition, pathogens, and the interactions between them, have been the focus of many studies. In contrast, while low temperatures are also a challenge to salmon production in several countries (regions), there has been limited research on this topic. In recent lab-based experiments, some Atlantic salmon (*Salmo salar*) reared at 8°C and below became lethargic and swam at the water's surface, and later developed ulcers to the head and jaw (symptoms similar to tenacibaculosis in Norwegian salmon aquaculture), and this was associated with significant mortalities. However, when fish with 'early' and 'advanced' symptoms were further examined, their livers were found to be large, pale and friable. Further, analysis of plasma samples showed that fish with this aetiology had: aspartate aminotransferase levels that were increasing; difficulty osmoregulating and producing enough glucose to maintain homeostasis; and high circulating cortisol levels [~100 ng ml^{-1}] when symptoms became severe / advanced. This set of physiological symptoms is very similar to a condition referred to as 'Winter Syndrome' in cultured gilthead sea bream and other species. Thus, it appears that 'Winter Syndrome', described here for the first time in Atlantic salmon, alone or in combination with opportunistic infections, results in lipid deposition in the liver, reduced/compromised liver functions and metabolic collapse that ultimately results in significant losses.

4.1 Introduction

Skin ulcers in Atlantic salmon (*Salmo salar*) aquaculture are problematic and can lead to large losses of fish at cage-sites (Bruno et al., 1998; Godoy et al., 2010; Lunder et al., 1995; MacKinnon et al., 2020, 2019; Spilsberg et al., 2022). The difficulty in addressing issues with skin ulcers in Atlantic salmon is that multiple pathogens (e.g., *Moritella viscosa, Tenacibaculum spp., Aliivobrio wodanis* etc.), and different environmental conditions, have been associated with this aetiology. For example, salmon farms on Canada's east coast have reported skin ulcer outbreaks above 10°C (MacKinnon et al., 2020, 2019), whereas Norwegian farms typically experience outbreaks of ulcers at temperatures below 7°C (Spilsberg et al., 2022; Tunsjø et al., 2007). Further, although ulcers at low temperatures (7°C) at Canadian aquaculture cage-sites have not been reported in the literature, industry personnel in Atlantic Canada have indicated anecdotally that mortalities with ulcers are regularly removed during the colder months of the production cycle (Pers. Comm., cage site manager NL).

Due to improved monitoring, or greater impacts on production, ulcers associated with cold temperatures have been studied much more in Norway. *Moritella viscosa* was identified as the main causative agent in the development of 'winter-ulcer disease' (Karlsen et al., 2014), and vaccination was shown to be effective in preventing disease in salmon going to sea-cages (Karlsen et al., 2017). However, despite vaccination against *M. viscosa*, ulcers at low temperature continue to have significant impacts on production, and different pathogens have been associated with the disease (e.g., *Aliivibrio wodanis* and *Tenacibaculum spp*.) (Nowlan et al., 2021; Småge et al., 2018, 2017). In Norway, outbreaks of *Tenacibaculum spp*. are known to cause different clinical signs depending on

the period of the year. Flank lesions have been reported in late fall and in late winter/spring, whereas lesions to the head and jaws have mostly been reported in late winter/spring. Head lesions/ulcers are associated with acute mortality (Spilsberg et al., 2022), and *Tennacibaculum maritimum* is known to cause mouth rot on the west coast of North America (Frisch et al., 2018), but not skin lesions (ulcers) associated with low temperatures.

Low rearing temperatures have also been shown to be stressful for many fish species (Reid et al., 2022). A condition has been identified in cultured gilthead sea bream (*Sparus aurata*) and other species [e.g., sea bass, *Dicentrarchus labrax* (Younes et al., 2020); yellow drum, *Nibea albiflora* (Song et al., 2019)], where the fish start to swim at the surface in a lethargic/tail down and disoriented state, and eventually become moribund. It is referred to as 'Winter Syndrome' or 'Winter Disease' in sea bream and is associated with increases in liver size, metabolic dysfunction and opportunistic infections (Ibarz et al., 2010b). We recently conducted 3 experiments where post-smolt Atlantic salmon were held at 8°C and below, for prolonged periods: 1) we performed an incremental temperature decrease at 1°C $week^{-1}$ from 8 to 1°C, with fish maintained for an additional week at 1°C, and 5% mortalities were recorded during the last two weeks of the experiment; 2) when acclimated to 3°C for 5 weeks, an average of 23% mortalities were recorded over the last three weeks; and finally 3) tanks acclimated to 8, 4 or 1°C experienced 40, 33 and 30% mortalities over a 12 week period, respectively. The moribund fish all had similar symptoms to those described above for sea bream and yellow drum with 'Winter Syndrome / Disease'. Thus, it is possible that this metabolic syndrome experienced by other cultured species, which is known to create favorable

conditions for the development of opportunistic infections (Contessi et al., 2006; Mohammed and Peatman, 2018; Tort et al., 1998), may also occur in Atlantic salmon.

In this study, plasma from affected ('symptomatic') fish was analyzed for biomarkers of stress, osmoregulatory disturbance and tissue damage/liver failure to examine whether Atlantic salmon may also experience 'Winter Syndrome', and its possible association with skin/fin related infections. Ultimately, it is hoped that such research will lead to strategies (i.e., through changes in management / rearing protocols, modifications to diet formulations and/or the development of new vaccines) that would safeguard these fish during the winter months, reduce production losses, and improve fish health and welfare.

4.2 Materials and Methods

This study was approved by the Animal Care Committee of Memorial University of Newfoundland and Labrador (protocol #21-02-KG). All procedures conducted on the salmon were performed in accordance with the Canadian Council on Animal Care's Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (Canadian Council on Animal Care, 2005).

4.2.1 Experimental animals

The Atlantic salmon used in these experiments were raised in freshwater in a commercial hatchery in New Brunswick (Canada) and transferred to seawater at the Dr. Joe Brown Aquatic Research Building (JBARB) (Department of Ocean Sciences, Memorial University) in January of 2021. These fish were initially held in a 3000 l tank supplied with flow through seawater (32 ppt salinity), with temperature maintained at ~6-

10°C. Oxygen levels and the photoperiod were ~100% air saturation and 12h light : 12h dark, respectively. The fish were fed to apparent satiation every day with a 3mm commercial pelleted diet (Signature Salmon, Northeast Nutrition, Truro NS. Canada).

4.2.2 Experimental protocols

4.2.2.1 Experiment #1

The salmon (280 fish; 175 ± 24 g, mean ± s.e.m.) were haphazardly distributed into eight 500 l tanks (35 fish $tank^{-1}$; **Figure S2**) supplied with 10 l min^{-1} of flow-through seawater (32 ppt salinity) at ~8°C and ~100% air saturation, and a 12h light : 12h dark photoperiod. The fish were acclimated to these tanks for two weeks before the start of the experiment. The 8 tanks were randomly separated into a control and an experimental treatment (4 tanks per treatment). Fish in the control tanks were maintained at a constant temperature of 8°C throughout the study, whereas fish in the experimental tanks were subjected to an incremental decrease in temperature of 1°C $week^{-1}$ from 8 to 1°C, followed by an additional week at 1°C. Feed was offered by hand to all tanks twice daily until apparent satiation was achieved. Fish were considered satiated when a few pellets accumulated at the bottom of the tank.

4.2.2.2 Experiment# 2

Atlantic salmon (225 fish; 343 ± 15 g) were haphazardly distributed into three 1000 l tanks (75 fish $tank^{-1}$; **Figure S12**) supplied with ~6°C flow-through seawater at ~100% air saturation at 7.5 l min^{-1} and a 12h light : 12h dark photoperiod. Feed was offered by hand to all tanks every other day until apparent satiation was achieved. The fish

were afforded three weeks of acclimation at ~6°C. The temperature was then decreased from 6 to 3°C over 10 days (0.3°C day^{-1}), and the fish were subsequently acclimated to 3°C for 5 weeks.

4.2.2.3 Experiment #3

Atlantic salmon (105 fish; 467 ± 31 g) were haphazardly separated into three 1000 l tanks (35 fish $tank^{-1}$; **Figure S12**) supplied with ~10°C flow-through seawater at ~100 % air saturation at 7.5 l min^{-1}, and a 12h light : 12h dark photoperiod. The fish were allowed 2 weeks of acclimation at ~10°C. The 3 tanks were subsequently haphazardly assigned to an acclimation temperature of 1, 4 or 8°C [the number of tanks limited by the capacity of a large glycol chiller (Technical Services, Memorial University) to maintain a temperature of 1°C], and temperature was decreased at 0.2°C day^{-1} until the target temperature was achieved. All tanks attained their assigned temperature after 5 weeks, and temperatures were maintained for an additional 8 weeks. Feed was offered by hand to all tanks every other day until apparent satiation.

4.2.3 Asymptomatic fish vs. those with 'early' and 'advanced' symptoms

A considerable number of fish were culled (i.e., euthanized with 0.4 g l^{-1} Syncaine (MS-222); Syndel Laboratories Ltd., Vancouver BC, Canada) during the three experiments mentioned in Section 2.2. To examine physiological changes in the affected fish, plasma samples were taken via caudal puncture (within 3 minutes of netting) from 27 salmon during Experiment #2 (Section 4.2.2.2) after 5 weeks at 3°C. These fish were separated into three groups. The first group were haphazardly sampled as apparently

healthy fish [designated as being 'asymptomatic' (n=9)]. The asymptomatic fish had no abnormal physical or behavioral symptoms. The symptomatic animals were sampled systematically to gather animals that had symptoms ranging in severity. The symptom range went from fish swimming lethargically and at the water's surface, to ones with extensive head ulceration. The symptomatic fish were subsequently divided into two groups (see below) using a scoring scheme adapted from Noble et al. (2018). All salmon were examined for three characteristics: snout/head damage, fin damage and skin lesions. Each characteristic was scored from 0-3, with 0 being an absence of damage, and 1 to 3 going from least to most severe (**Figure S4)**. Further, if fin damage was active, as opposed to healed, the fin damage score was doubled. The three scores for each fish were summed to give a total individual score. The 9 fish with the lowest scores were placed in the 'early' symptom group, and the 9 fish with highest scores were put in the 'advanced' symptom group.

4.2.4 Fish sampling

Blood samples (~1 ml) were transferred to 2 ml Eppendorf® tubes with 75 µl of saline containing lithium heparin (Sigma Chemical Co., Oakville, On, Ca; 1000 units ml^{-1}), shaken by inversion, and then centrifuged at 1,100 *xg* for 1 minute. The plasma was then distributed into a number of Eppendorf® tubes for the various analyses, and immediately frozen in liquid nitrogen prior to being stored at -80°C for later analyses.

The fish were then measured for weight, fork length and liver weight. Liver weight was used to calculate the fish's hepatosomatic index (HSI) using the following formula:

$$HSI = 100\ [W_L/W_t]$$

where W_t (g) was total body weight and W_L was liver weight.

The percentage of survival was calculated over the course of the 3 experiments according to the formula:

$$\text{Survival (\%)} = 100 - (M/I*100)$$

Where M was the cumulative number of mortalities (mortalities and culls) in a tank and I is the initial number of fish in the tank. Survival was recorded as the average of all tanks in an experiment/experimental group.

4.2.5 Plasma aspartate aminotransferase activity and hepatosomatic index

To better quantify the relationship between plasma aspartate aminotransferase (AST) levels and hepatosomatic index (HSI), Atlantic salmon from Experiment #2 (128 fish) had their blood sampled by caudal puncture after 5 weeks of acclimation at 3°C. The sampled fish were 'apparently healthy' and showed no signs of the condition(s) described in Section 3.1.

4.2.6 Plasma analyses

4.2.6.1 Spectrophotometry

Plasma samples were thawed, and diluted so that reaction rates could be accurately measured or that values were adequately covered by the standard curve (e.g., glucose, lactate, cortisol). All enzymatic activities were measured at 25°C using a SpectraMax M5e microplate reader (Molecular Devices, Sunnyvale, CA, USA). These assays were

developed for this experiment using established protocols on human plasma (Bergmeyer et al., 1978), fish plasma (Casillas et al., 1982), fish liver (Treberg et al., 2002) and fish muscle (Treberg et al., 2003).

Assay conditions were as follows:

Aspartate aminotransferase (AST; E.C. 2.6.1.1). 50 mM imidazole (pH 7.4), 200 mM aspartate, 0.2 mM NADH, 0.05 mM pyridoxal–5–phosphate and 14.5 U/ml malate dehydrogenase. The reaction was initiated with 7.0 mM α-ketoglutarate.

Lactate dehydrogenase (LDH; E.C. 1.1.1.27). 50 mM imidazole (pH 7.4) and 0.2 mM NADH. The reaction was initiated by the addition of 1 mM pyruvate.

Creatine kinase (CK; E.C. 2.7.3.2). 50 mM Imidazole (pH 7.4), 5.0 mM $MgCl_2$, 2 mM ADP, 0.8 mM NADP, 5 mM glucose, 10 mM AMP, 2 U/ml hexokinase, 2 U/ml glucose–6–phosphate dehydrogenase, and 10 μM P1,P5-Di(adenosine-5')-pentaphosphate. The reaction was initiated by the addition of 50 mM phosphocreatine.

Glucose. Samples were initially deproteinized in 1:4 0.6% protocatechuic acid. 250 mM Imidazole (pH 7.8), 5 mM $MgSO_4$, 10 mM ATP, 0.8 mM $NADP^+$ and 10 U/ml glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of 10 U/ml hexokinase.

Lactate. Samples were initially deproteinized in 1:4 0.6% protocatechuic acid. 200 mM glycine buffer solution and 25 mM NAD. The reaction was initiated by the addition of 400 U/ml of lactate dehydrogenase.

Cortisol levels in the plasma were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (NEOGEN Corp. Lexington, KY, USA).

4.2.6.2 Ion measurements

Plasma sodium (Na^+) and potassium (K^+) levels were analyzed using a PFP7 Flame Photometer (Jenway, Stone, Staffordshire, UK) following the manufacturer's methods for analysis. Chloride (Cl^-) and osmolality were measured according to manufacturer instructions using a ChloroChek Chloridometer (model 3400) and a VAPRO Vapor Pressure Osmometer (model 5600), respectively (ELITech Group, Logan, UT).

4.2.7 Statistical analyses

All data are reported as mean ± standard error of the mean (s.e.m.). The data were analyzed using Rstudio Version 1.4.1106 R 4.0.5 (R Core Team, 2020, https://www.r-project.org/). A Bonferroni outlier test was used to identify outliers before statistical analysis. Parameters measured were analysed by fitting linear models using the *lm* function. Statistical models were computed with 'treatment' and 'sampling' as variables. Each model fit was graphically examined (i.e., using histograms and qqplots) and residuals tested for normality (Shapiro–Wilk, $p<0.05$). Non-normally distributed data were power-transformed using a Box-Cox transformation (Atkinson et al., 2021) using the *boxcox* function implemented in the *EnvStats* (version 2.7.0) package in R (Millard, 2017) so that the data met the assumption of normality. Finally, when the model identified significant effects, least-squares means post-hoc tests with Bonferroni's *p*-value correction for multiple comparisons [using the *lsmeans* function in R (Lenth, 2016)] were used to identify significant differences between treatments at a particular sampling point, and between sampling points within a treatment. Plasma AST was $\log_{10}$-transformed using the *lm* function so that the relationship between this parameter and HSI became linear, and the

statistical model was computed with 'hsi' and 'group' as variables. Significant differences were further examined as mentioned above.

4.3 Results

4.3.1 Mortalities

Salmon in Experiments #1, #2 and #3 developed similar symptoms and experienced significant mortalities. In Experiment #1, mortalities began when the fish were lowered to 1°C, and reached 5% after 2 weeks at this temperature (**Figure 4-1**). In Experiment #2, there were few mortalities (2%) during the initial holding at 6°C (3 weeks), however, mortalities began when temperature was being lowered to 3°C, and was approximately 0.6 % day^{-1} when they were held at this temperature (**Figure 4-1**). During Experiment #3, mortalities reached 40, 33 and 30% by the end of the experiment in the tanks held at 8, 4 and 1°C respectively. The mortality rate was approximately 0.5, 0.5 and 0.8 % day^{-1} at the 3 temperatures (**Figure 4-1**). These mortalities all had similar symptoms. The first sign of any health and welfare related issue was abnormal swimming behavior, where the fish were unresponsive and lethargic, and swam at the surface of the water (often in a 'tail-down' position) (**Figure S13**). They would then develop ulcerations to the head and jaws that progressed rapidly and resulted in exposure of cranial/jaw bones only after a few days (**Figure S14**). In the later stages of the condition, fin rot was also observable on most affected fish, and skin lesions were recorded on the flanks of some salmon (**Figure S14**). When dissected, the moribund fish had large, pale and friable (i.e., easily broken apart) livers. These fish also had an enlarged spleen, enlarged gallbladder, and ascites (**Figure S14**). In contrast, the stock population used for the different experiments (held at 10°C)

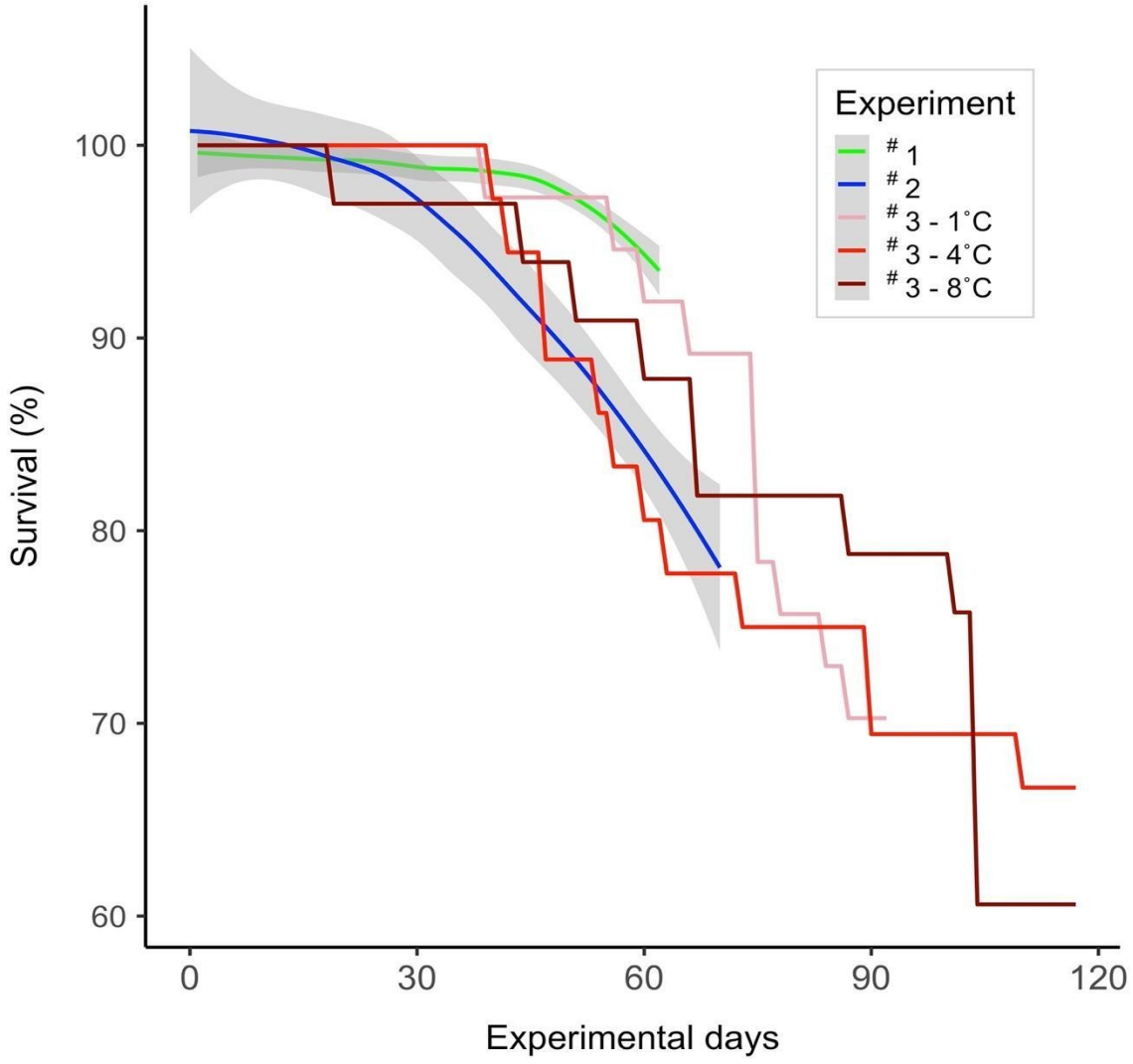

Figure 4-1. Survival (in %) over the course of the 3 experiments (#1, green; #2, blue; #3 – 1°C, pink; #3 – 4°C, red: and #3 – 8°C, dark red). Fish in Experiment #1 were exposed to a decline in temperature of 1°C week^{-1} from 8 to 1°C, and then maintained for an additional week at 1°C. Fish in Experiment #2 were held at 6°C for 21 days, then temperature was decreased for 10 days to 3°C and maintained at 3°C for 35 days. Fish in Experiment #3 were held for 14 days at 10°C, then temperature was decreased at a rate of 0.2°C day^{-1}. Forty-five days later, the fish were at their assigned temperature (1, 4 and 8°C) and held for 60 days. For Experiments #1 and 2, there were several tanks, and the plotted relationships with 95% confidence limits are shown (i.e., see the gray shaded areas). The equations for the two experiments are $y = 100 - 0.069x$ ($r^2 = 0.15$) and $y = 100 - 0.32x$ ($r^2 = 0.36$), respectively. No such fit was conducted for Experiment #3 as there was only one tank of fish at each temperature.

did not experience any of the described symptoms, and no mortalities were recorded in this group. The water source and husbandry procedures were the same / similar for all groups of fish.

4.3.2 Asymptomatic fish vs. those with 'early' and 'advanced' symptoms

The symptomatic fish (both 'early' and 'advanced') had significantly higher HSI values (2.5 ± 0.2 %) as compared to asymptomatic fish (1.6 ± 0.3 %) (**Figures 4-2 A and B**), and plasma cortisol values that were generally > ~100 ng ml^{-1} (**Figure 4-3**). Although there were no differences in plasma lactate levels between the three groups, salmon with 'advanced' symptoms had significantly lower levels of plasma glucose (1.95 ± 0.34 mM) as compared asymptomatic fish and fish with 'early' symptoms (3.94 ± 0.3 mM and 3.84 ± 0.44 mM, respectively; **Figure 4-3**).

Symptomatic fish had increased plasma sodium and chloride levels, and osmolality values (230 ± 11 mM, 188 ± 7 mM and 432 ± 24 mOsm kg^{-1}, respectively), as compared to asymptomatic fish (190 ± 2 mM, 136 ± 3 mM and 338 ± 6 mOsm kg^{-1}, respectively) (**Figure 4-4**). However, there were no significant differences in plasma potassium levels between fish in the 3 categories. Fish with 'advanced' symptoms also had significantly increased plasma LDH levels as compared to asymptomatic fish and those with 'early' symptoms (by ~2.8-fold; **Figure 4-5**), and greater plasma levels of CK and AST at $p < 0.1$ (by > 2 fold) as compared to both other groups. **Figure 4-6** shows the relationship between plasma AST levels and HSI for fish in Experiment #2, whereas **Figure 4-7** shows log_{10}-transformed plasma AST levels plotted against HSI. For the asymptomatic fish from Experiment #2 (128 in total), both relationships were negative (i.e., AST declined as

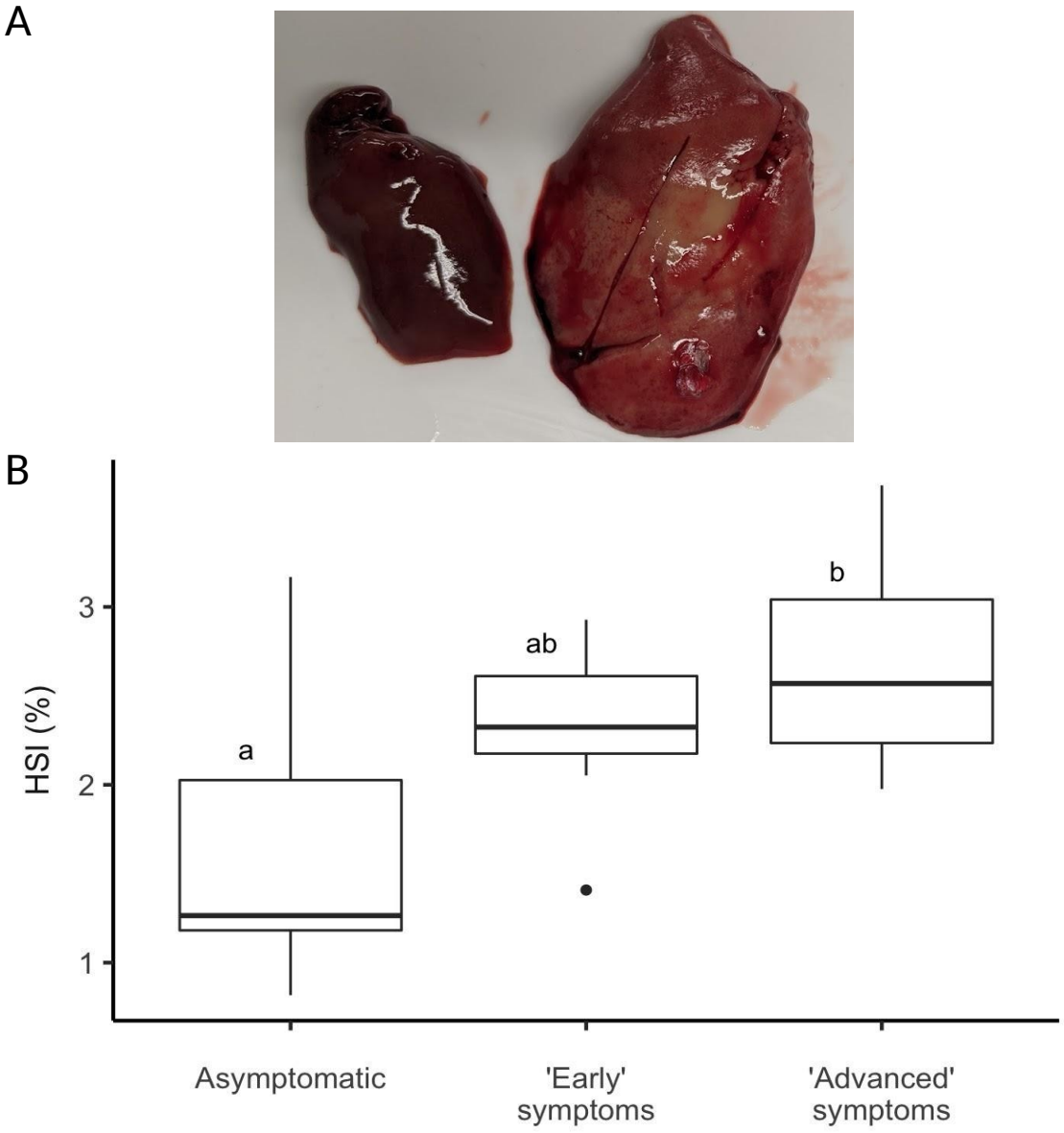

Figure 4-2. A: Pictures of the livers from an asymptomatic (left; 440.8 g; HSI of 1.3) and symptomatic (right; 451.7 g; HSI of 2.8) Atlantic salmon held at 3°C. **B**: Box-plots showing the hepatosomatic index (HSI) of asymptomatic fish, and fish with 'early' symptoms and fish with 'advanced' symptoms (n=9). The lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. Dissimilar letters indicate a significant difference ($p < 0.05$) between groups.

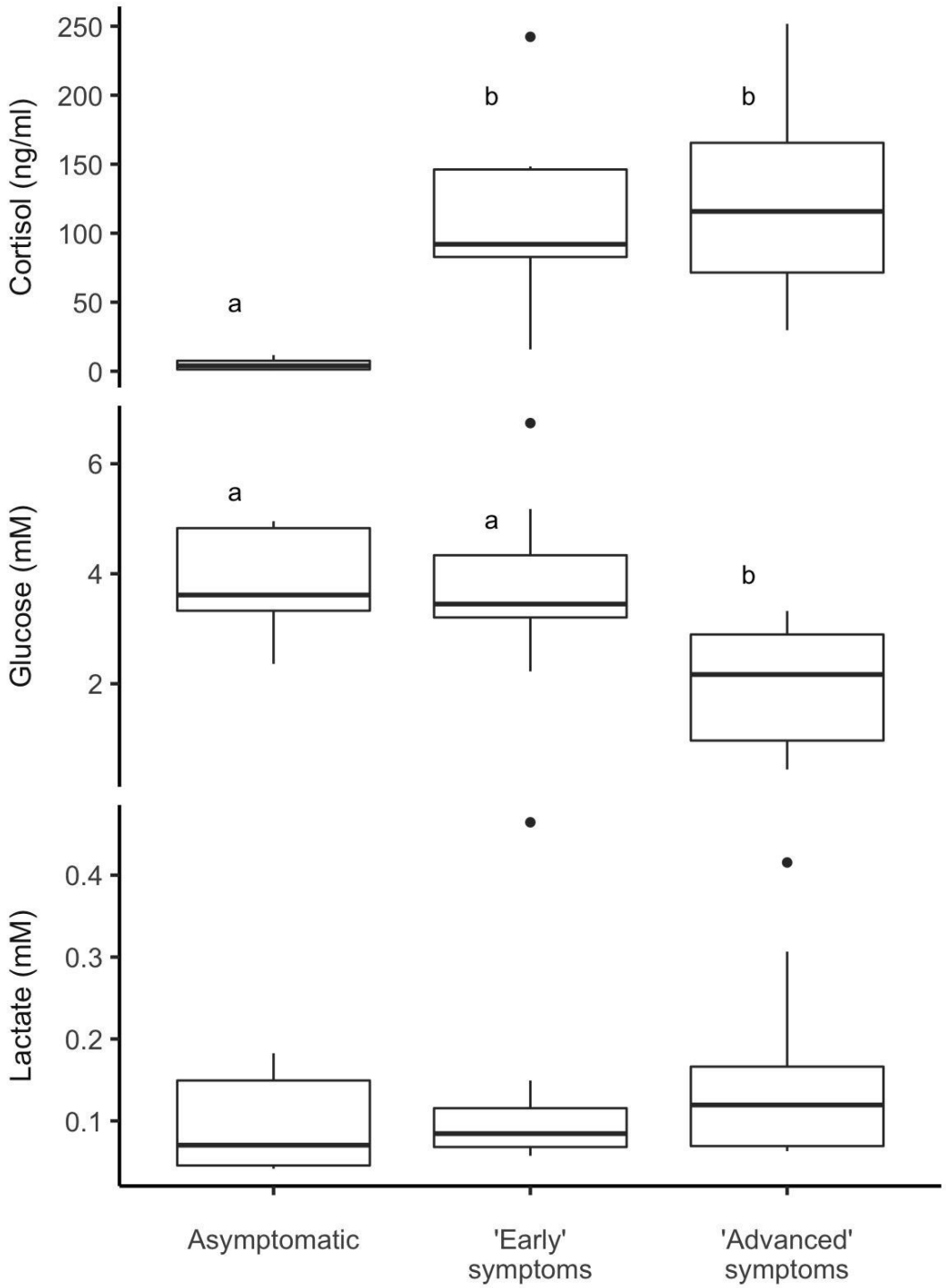

Figure 4-3. Plasma cortisol, glucose and lactate levels in asymptomatic fish, fish with 'early' symptoms and fish with 'advanced' symptoms (n=9). The lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. Dissimilar letters indicate a significant difference ($p < 0.05$) between groups.

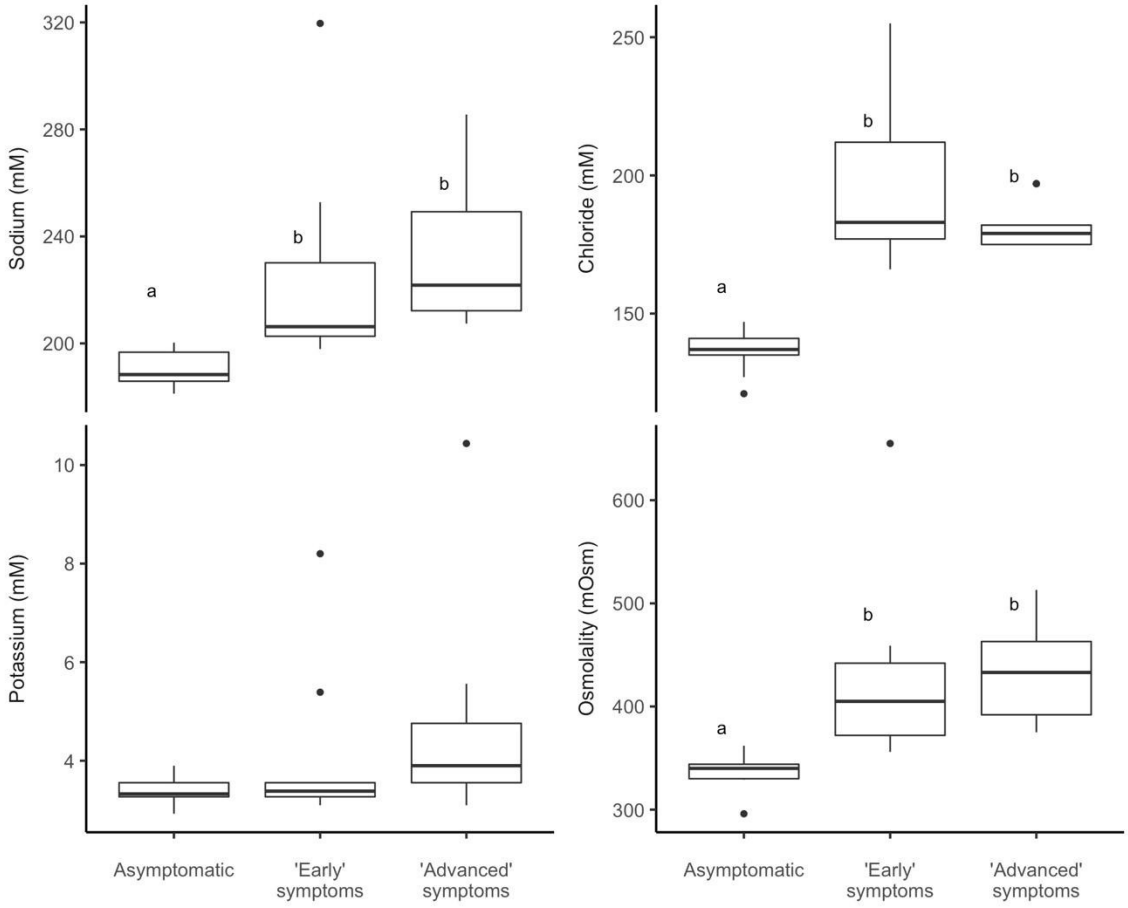

Figure 4-4. Plasma ion levels and osmolality in asymptomatic fish, fish with 'early' symptoms and fish with 'advanced' symptoms (n=9). The lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively (n=9 in each group). Filled circles show data points falling outside the 10th and 90th percentiles. Dissimilar letters indicate a significant difference ($p < 0.05$) between groups.

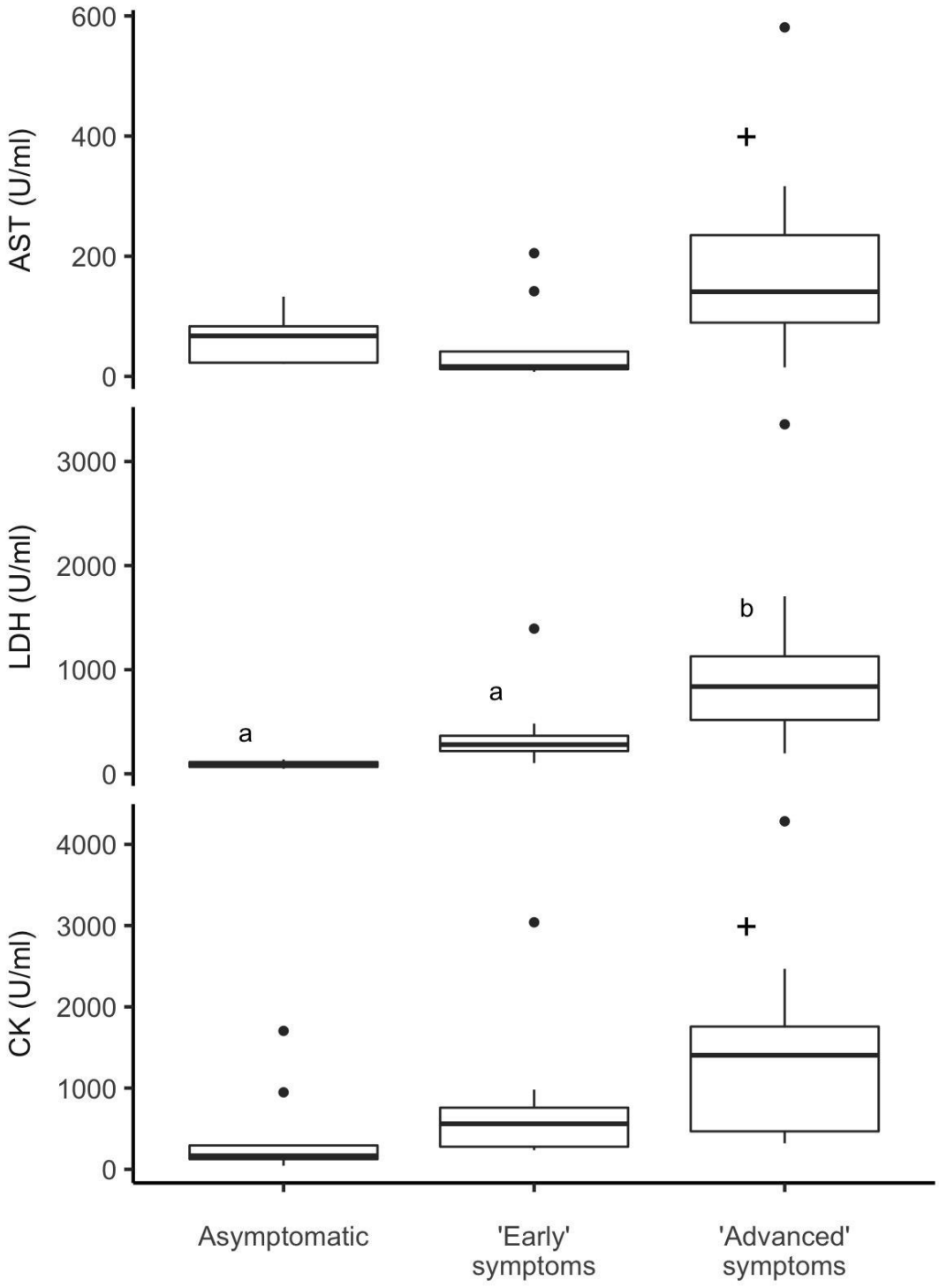

Figure 4-5. Plasma aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) activity in asymptomatic fish, fish with 'early' symptoms and fish with 'advanced' symptoms (n=9). The lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. Dissimilar letters indicate a significant difference ($p < 0.05$) between groups. A $^{+}$ symbol indicates a difference at $p < 0.1$.

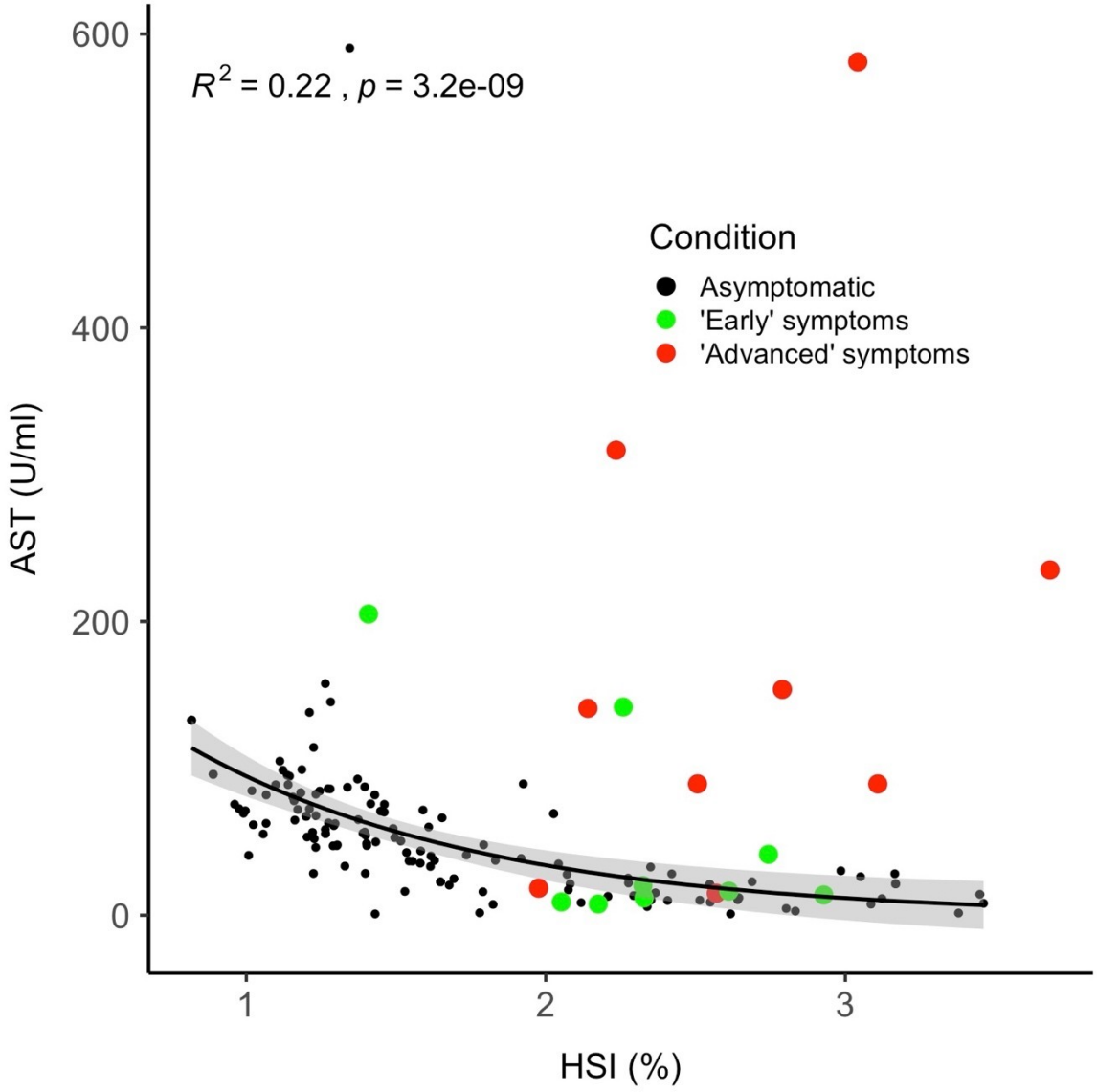

Figure 4-6. Relationship between hepatosomatic index (%; HSI) and plasma aspartate aminotransferase levels (U ml^{-1}; AST) in asymptomatic fish (black symbols, n=128) from Experiment #2, and fish sampled with 'early' symptoms (green symbols; n=9) and 'advanced' symptoms (red symbols; n=9). The line with 95% confidence limits shows the relationship between these two parameters for only the asymptomatic fish (black dots, $y= 260.81e^{-1.255x}$, $p = <0.001$, $R^2 = 0.22$).

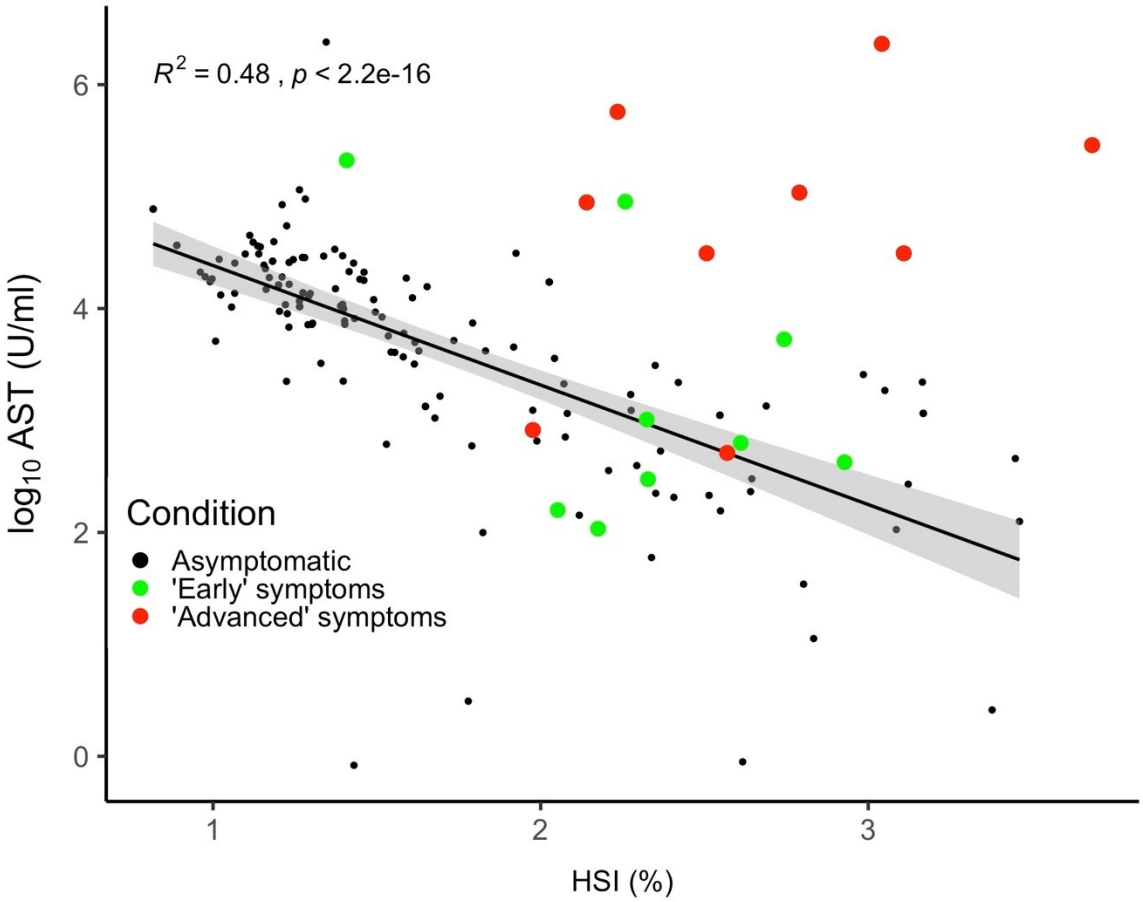

Figure 4-7. Relationship between hepatosomatic index (%; HSI) and log transformed plasma aspartate aminotransferase activity values (U ml^{-1}; AST) for asymptomatic fish (black symbols; n=128) from Experiment #2, and fish with 'early' symptoms (green; n=9) and 'advanced' symptoms (red; n=9). The line with 95% confidence limits shows the linear relationship between these two parameters for only the asymptomatic fish (black dots, y = 5.5 – 1.1x, p = <0.001, R^2 = 0.48).

salmon HSI increased), with the linear relationship having an $R^2 = 0.48$ ($p < 0.001$). Most of the fish classified as having 'early' symptoms (6 out of 9; **Figure 4-6 & 4-7**, green symbols) had values that were similar to those in asymptomatic fish. In contrast, 77% (7 of 9) of the fish in the 'advanced' symptoms group (**Figure 4-6 & 4-7**; red symbols) had values that were well above (average ~20-fold higher) those recorded in asymptomatic fish with similar HSI values.

4.4 Discussion

Some fish in the current study developed a chronic condition at 8°C and below, where the fish were first observed in a lethargic/unresponsive state swimming at the surface. Then head/jaw ulcerations would develop, with the addition of fin rot on most fins, and significant mortalities were recorded. Identification of the pathogen(s) associated with the above aetiology was not possible/part of this study, but the symptoms have many similarities to what is described in the literature for fish from Norwegian aquaculture companies that have been identified as having tenacibaculosis (i.e., an infection with *Tenacibaculum spp*.) (Nowlan et al., 2021; Småge et al., 2018, 2017; Spilsberg et al., 2022). The fish from these studies are most often sampled to identify the pathogens and the histopathological impact on the tissues, but no study has investigated how the disease affects the fish's physiology or vice versa. The symptomatic animals from the current study had abnormally large (and friable and pale) livers, and issues with liver function, osmoregulation and severe stress, in addition to head erosion / ulcers and fin rot. Large pale friable livers, and the physiological disturbances reported here, are well described at low temperatures in cultured gilthead sea bream affected by 'Winter Syndrome' (Ibarz et al., 2010b). This study provides a potential link (although more research is required)

between ‘Winter Syndrome’ and low temperature ulcers/infections in Atlantic salmon culture; where metabolic and osmoregulatory issues (including liver dysfunction/damage and lipid deposition) reduce the salmon’s overall health and robustness, and this may leave them vulnerable to opportunistic infections.

4.4.1 Metabolic dysfunction and fatty liver

Elevated plasma glucose has been measured in fish acclimated to cold vs. warm temperatures (Atwood et al., 2003; Chang et al., 2020; Connors et al., 1978; Staurnes et al., 1994; Virtanen and Oikari, 1984; Woo, 1990), and cortisol has a major impact on glucose metabolism. Specifically, cortisol stimulates glucose release via glycogenolysis and gluconeogenesis (Aedo et al., 2021; Faught and Vijayan, 2016; Jerez-Cepa et al., 2019; Vargas-Chacoff et al., 2021), and results in increased circulating glucose levels (Barton, 2002; Laiz-carrión et al., 2003; Staurnes et al., 1994; Vargas-Chacoff et al., 2020). Surprisingly, the symptomatic fish in this study had high circulating levels of cortisol, but their glucose levels were not higher, and fish with ‘advanced’ symptoms had very low plasma glucose levels (**Figure 4-4**). Glucose is mostly produced and/or released by the liver (Polakof et al., 2012). However, low temperatures reduce the activity of the glycogenolysis pathway in fish in favor of gluconeogenesis from lipids and amino acids to maintain glucose levels (Melis et al., 2017; Seibert, 1985). Furthermore, Hochachka and Hayes (1962) demonstrated that low acclimation temperatures (4 vs 15°C) tended to promote lipid synthesis by liver and muscle homogenates instead of glycogen synthesis in brook trout (*Salvelinus fontinalis*). The observed increase in the size of the liver (HSI), and its transition to a pale colour (i.e., indicative of ‘fatty liver disease’; **Figure 4-2 and 4-3**)

are consistent with the above findings, and a drop in plasma glucose levels may well be associated with the salmon's erratic/lethargic swimming behavior and unresponsiveness. Glucose is the only energy source that can be utilized by the nervous system (Bélanger et al., 2011), and injection with high doses of insulin results in low plasma glucose and a comatose state in goldfish (*Carassius auratus*) that can be reversed when glucose is administered (Munford and Greenwald, 1974). In addition, injecting Atlantic salmon with a high dose of insulin-like growth factor I (IGF-I) resulted in aberrant swimming behavior, hypoglycemia and death (McCormick, 1996). Similar to the present study, gilthead sea bream affected by 'Winter Syndrome' have low glucose levels compared to asymptomatic fish, and the first symptoms of the condition is erratic/lethargic swimming (Gallardo et al., 2003). Disruptions in liver function are also associated with increased HSI (indicative of lipid deposition) that leads to metabolic collapse, and mortality (Ibarz et al., 2010b). Interestingly, Atlantic salmon given cortisol implants at 10°C had increased plasma glucose and decreased HSI after 14 days (Vargas-Chacoff et al., 2021), which is a completely opposite response as compared to what the symptomatic salmon experienced at 3°C with high cortisol in the current experiment. As most glucose to support metabolic function comes from/is produced by the liver, this is further evidence that 'Winter Syndrome' in Atlantic salmon is associated with disruptions in liver function.

Indeed, the fish from the current experiment had large, pale and friable livers. This change in liver size, color and structural integrity at low temperatures referred to as 'fatty liver' (Horn et al., 2018; Jia et al., 2006; Sissener et al., 2017), has been described in gilthead sea bream in association with 'Winter Syndrome' and ascribed to increased hepatic lipid deposition (Ibarz et al., 2010b; Melis et al., 2017; Sánchez-Nuño et al., 2018).

In Atlantic salmon, ‘paleness’ of the liver has been used as a scoring parameter where a darker color was associated with ‘apparently healthy’ fish, and paler colors (yellow to orange hue) were associated with ‘fatty liver’ (i.e., increased lipid content) (Katerina et al., 2020). Although liver size do vary in Atlantic salmon (primarily due to changes in lipid content) (Bou et al., 2017; Hundal et al., 2022; Sissener et al., 2017), HSI values similar to the ones measured in the current study for the symptomatic fish (~2.5) are almost unheard of in the literature. The accumulation of lipids in the liver has been linked to many different conditions in various fish species: high-lipid diets, low levels of dietary eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), starvation, parasites, bacterial infections and short day length (Fujisawa et al., 2021; Hundal et al., 2022; Nordgarden et al., 2002; SeppÄnen et al., 2009; Tao et al., 2018; Xu et al., 2021; Zhao et al., 2019; Zhou et al., 2020). Low temperatures have also been shown to lead to increased HSI values in juvenile white sturgeon (*Acipenser* transmontanus; Watson et al., 1998), European seabass (*Dicentrarchus labrax*; Islam et al., 2020) and gilthead sea bream (Melis et al., 2017). For Atlantic salmon, there are some indications that low temperatures can specifically increase hepatic lipid deposition (See Chapter 2 of current thesis; Vadboncoeur et al., submitted). Although, combinations of low temperature and continuous light (Døskeland et al., 2016), pathogens (Fjølstad and Heyeraas, 1985; Waagbø et al., 1988), an increase in dietary lipids (Dessen et al., 2021) and low dietary fish oil content (Ruyter et al., 2006) also result in increased lipid deposition and HSI. Importantly, high HSI values have been associated with a general decrease in a fish’s robustness, and increased mortality (Bou et al., 2017; Dessen et al., 2021; Hundal et al., 2022; Rorvik et al., 2003).

'Fatty liver' is similar to a common disease in humans called non-alcoholic fatty liver disease (NAFLD); zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are increasingly used as models to study this disease (Asaoka et al., 2014). Lipid accumulation in the liver (steatosis) associated with NAFLD induces hepatocyte oxidative stress, lipid peroxidation and cell death that promotes the inflammatory response in the liver (Shwartz et al., 2019). This inflammation, in combination with steatosis, leads to non-alcoholic steatohepatitis (NASH) and the later stages of the disease can be extremely detrimental to the health of affected patients (Schuster et al., 2018). If unresolved, the inflamed liver is unable to maintain normal functions (Robinson et al., 2016). NAFLD, especially in the later stages of the disease, can be associated with severe hypoglycemia as a result of a dysregulation in glucose metabolism (Lee et al., 2022). Consistent with the above, the high HSI in Atlantic salmon in this study (~2.5) appears to hinder the liver from fulfilling its normal physiological functions, which is consistent with gilthead sea bream affected by 'Winter Syndrome' and humans in the later stages of NAFLD.

'Winter Syndrome' has also been linked to metabolic depression, nutritional imbalance, immunosuppression and an increase in the incidence of infection by opportunistic pathogens in gilthead sea bream at sea-cages (Melis et al., 2017). The metabolic reorganization/dysregulation exhibited at low temperatures results in an increase in lipid deposition in the liver, because lipogenesis pathways in the liver are favoured /increased. However, this does not account for all of the lipid deposition in 'Winter Syndrome' (Ibarz et al., 2007). The Atlantic salmon in the current study experienced a similar increase in lipid deposition in the liver, and Atlantic salmon hepatocytes have been shown to increase fatty acid synthesis, but decrease the β-oxidation of fatty acids, at low

temperature (Fonseca-Madrigal et al., 2006). In this metabolic state associated with 'Winter Syndrome', which is exacerbated by high cortisol levels, lipids stored in the muscle and in the abdominal cavity are released into the bloodstream to be used for energy production. However, the imbalance between fatty acid β-oxidation and lipogenic pathways leads to a metabolic bottleneck in the liver, where lipids are deposited and stored at unhealthy levels instead of being oxidized for energy production.

4.4.2 Osmoregulatory disturbance

Most, but not all, symptomatic fish had ulcers. The fish in the worst condition had erosion/ulcers of their head/jaw, lesions on their flanks and fin rot on most fins (**Figure S14**). The ulcers would have certainly hindered the appropriate regulation of plasma [Na^+], [Cl^-] and osmolality by increasing the fish's permeability to external ions. For example, Atlantic salmon infected with sea lice have higher plasma [Na^+] and [Cl^-], and resulting osmolality, compared to control animals (Bowers et al., 2000; Fjelldal et al., 2020). However, the progression of fish from having 'early' to 'advanced' symptoms in the current experiment did not have a significant impact on plasma ion levels (**Figure 4-5**). This may be because the pathogen may have already been altering mucus production, and that this led to increased plasma osmolality (Shephard, 1994). However, the increase in plasma osmolality could also have been a response to the decrease in energy (glucose) availability. Indeed, glucose is the main source of energy for osmoregulation by the gills (Tseng and Hwang, 2008), and as discussed previously, reductions in liver function in the symptomatic fish led to a reduction in glucose production (circulating levels). Arctic, Antarctic and cold-acclimated temperate fish are also known to reduce the energy

requirements of osmoregulation by decreasing the gradient in osmolality between the animal and its environment (O'Grady and Devries, 1982; Pörtner et al., 1998). Thus, if the increased HSI resulted in liver dysfunction and limited glucose production, there may have been reductions in the energy expenditure associated with osmoregulation to preserve circulating glucose for more essential roles, and therefore, further imbalances in ions such as Na^+ and Cl^-.

Interestingly, no differences were found in plasma $[K^+]$. However, some symptomatic fish had high plasma $[K^+]$ values (**Figure 4-4**). Plasma $[K^+]$ is greater in cells than in the plasma (Palmer, 2015), and thus, the increase in plasma $[K^+]$ and enzymes of tissue damage (see below) were likely related to an increase in cell damage (and the release of cellular contents) in several tissues in fish with 'advanced' symptoms.

4.4.3 Tissue damage

There were no significant increases in plasma LDH, CK or AST in fish with 'early' symptoms. However, the activity of these enzymes in the plasma increased in fish with 'advanced' symptoms (**Figures 4-5, 4-6 & 4-7**), and this indicates that tissue damage was occurring as the condition progressed (Boyd, 1983). Plasma AST levels are used in human medicine to assess, in part, liver condition/function (Ono et al., 1995; Wang et al., 2019), and given that the symptomatic fish had visibly poor liver condition, there was an expectation that plasma AST activity would increase in the plasma as is described in humans and the different animal models affected by NAFLD (Mundi et al., 2020). Indeed, plasma AST levels were much higher in fish characterized as having 'advanced' symptoms (**Figures 4-5 & 4-7**), and this is similar to the results of Ye et al. (2019) who

fed different levels of rendered animal protein meal to hybrid grouper [*Epinephelus fuscoguttatus* (F) x *Epinephalus lanceolactus* (M)], and this resulted in hepatic steatosis, an increased in HSI and liver damage. Increases in plasma enzyme activity (e.g., CK, LDH and CK) are due to increased leakage from, and/or the apoptosis of, cells of certain tissues (Boyd, 1983; Klein et al., 2020). Cellular damage in the liver could have been related to oxidative stress, as reduced antioxidant enzyme activities and antioxidant gene expression, and increased lipid perioxidation, have been measured in cold-stressed gilthead seabream and yellow drum, (Ibarz et al., 2010a; Song et al., 2019). Hypoxia could similarly play a role in cellular damage in the liver due to reduced blood flow to this organ, and consequently, altered liver function. Hepatic steatosis decreases vascular compliance in humans affected by NAFLD (Solhjoo et al., 2011), creates hypertension in the portal vein (Francque et al., 2010, 2010; Van der Graaff et al., 2018), and this results in impairment of blood flow to the liver (Francque et al., 2012; van der Graaff et al., 2019). The cause of this phenomenon is primarily the swelling of hepatocytes that incorporate large lipid droplets, and the compression of sinusoids and restrictions in sinusoidal blood flow in the liver (van der Graaff et al., 2019). Drugs that reduce vasoconstriction in rats with early NAFLD lowered plasma enzyme activities and mitigated steatosis (van der Graaff et al., 2022). This reduction in blood flow to the liver could explain the reduction in plasma AST activity with increases in HSI (**Figure 4-6 & 4-7**) measured in 'apparently healthy' fish acclimated for 5 weeks at 3°C. Reduced blood flow to the liver could also result in increased plasma CK and LDH activities as enzymes released from other tissues/organs may not be properly filtered from the plasma. Alternatively, the reduction in plasma AST activity may be a mechanism to limit the liver's inflammatory response with increasing

HSI, since the release of cellular contents is one of the primary promoters of the inflammatory response (AnvariFar et al., 2017). We did not examine this relationship (i.e., its causes) further in this study. However, contrary to the present study on Atlantic salmon, NAFLD has not been linked with reduced plasma AST activity.

4.4.4 Plasma cortisol levels

Fish with 'early' and 'advanced' symptoms had high resting cortisol levels (generally > 100 ng ml^{-1}). High circulating levels of cortisol are commonly observed in Atlantic salmon infected with sea lice (Fjelldal et al., 2020) and infectious salmon anemia virus (Olsen et al., 1992); especially when the fish are in a moribund state. Cortisol is known to have depressive effects on the immune system, and high circulating levels of this glucocorticoid would limit the ability of the immune system to signal an infection or injury, or to delay or stop the inflammatory response (Coutinho and Chapman, 2011). Indeed, high circulating cortisol levels, in addition to the immunosuppressive effects of cold temperature itself (Abram et al., 2017), could be an important factor in the rapid development of the ulcers reported in the current experiment.

The liver is responsible for the clearance of plasma cortisol (Vijayan et al., 1991). Based on our hypothesis that high HSI values (here ~2.5) impede liver functions, it is possible/probable that cortisol may not have been properly/efficiently cleared from plasma, further reinforcing the metabolic state that symptomatic animals were experiencing. In this metabolic state, lipids are released from muscles and other tissues, and since fatty-acid oxidation and overall metabolic needs are reduced at low temperatures (Fonseca-Madrigal et al., 2006; Porter et al., 2022), there is a mismatch between the

amount of lipid released and that needed for energy production. These surplus lipids are deposited in the liver, increasing HSI. Cortisol has been identified in humans as a glycemic promoter when released during prolonged hypoglycemic stress (Tesfaye and Seaquist, 2010). Therefore, the combination of reduced cortisol clearance, and increased production due to hypoglycemia (**Figure 4-3**), would further exacerbate the physiological downward spiral that the fish is experiencing, and result in metabolic breakdown and liver failure.

4.4.5 Immune function and opportunistic infections

The fish in the current experiment, in addition to having symptoms that resembled 'Winter Syndrome', had head/jaw ulceration (erosion) similar to that observed at Norwegian sea-cages at low temperature and that were recently described as being attributed to tenacibaculosis (Småge et al., 2018, 2017; Spilsberg et al., 2022). It is hypothesized that physical damage to the head and jaw, due to rubbing against the nets of sea-cages, provides the necessary conditions for the proliferation of this pathogen in sea-cages (Spilsberg et al., 2022). This is consistent with the possible route of infection in the current study. The salmon were lethargic and swimming at the surface, often in close proximity to the outlet pipe of the tank's degasser (**Figure S13**) and the walls of the tank. The disease is associated with high variability in mortality rates between sites where it is identified, is linked with low temperature (< 8°C), and is more prevalent in smolts recently transferred to sea (Olsen et al., 2011; Spilsberg et al., 2022). 'Fatty liver' has not been reported to be associated with tenacibaculosis in Atlantic salmon. However, *Tenacibaculum spp.* infections have led to enlarged, pale and friable livers in black

damselfish (*Neoglyphidion melas*; Haridy et al., 2015), Picasso triggerfish (*Rhinecanthus assasi*; Haridy et al., 2015), European seabass (Khalil et al., 2018; Yardımcı and Timur, 2015) and Senegalese sole (*Solea senegalensis*; Mabrok et al., 2016). These studies, which focused on the bacteriology of the pathogen, did not quantify the change in liver size or liver lipid content. They only described the appearance of the liver. However, other pathogens or combinations of pathogens can result in fish with large, pale and friable livers. Shoemaker et al. (2015) described general bacterial infections in fish with the symptoms of: abnormal swimming, lethargy, fin erosion, ascites, swollen organs, pale organs, white nodules and hemorrhagic organs. Indeed, paleness and enlargement of the liver have been linked to hepatic steatosis in the later stages of *Aeromonas hydrophila* infection in channel catfish (*Ictalurus punctatus*; Grizzle and Kiryu, 1993), and *Pseudomonas plecoglossicida* infection in Barramundi (*Lates calcarifer*) led to steatosis 1 day post-infection and hepatic necrosis that resulted in 100% mortality at 5 days post-infection (Sun et al., 2020). Further, the HSI of year-old rainbow trout (150 ± 15g) infected with *Psychrobacter immobilis* was 2.97 as compared to 1.22 for apparently healthy fish (Hisar et al., 2002), data which are similar to that of asymptomatic and symptomatic fish in the current study.

In the late 1970's, 'Hitra-disease' or 'cold-water vibriosis' (CWV) (pathogen *Aliivibrio salmonicida*) caused 'liver lipoid degeneration' (later renamed 'fatty liver') in the winter in Norway (Egidius et al., 1984; Holm et al., 1985; Roald, 1976; Urbanczyk et al., 2007); the liver enlarged and changed in colour from brown to yellow in color (Fjølstad and Heyeraas, 1985; Waagbø et al., 1988). This condition is also associated with anemia (Poston et al., 1976; Smith, 1979; Waagbø et al., 1988), an enlarged spleen (Fjølstad and

Heyeraas, 1985; Roberts et al., 1979; Smith, 1979), and extensive hemorrhaging (i.e., petechiae in the skin, gills, liver, swim bladder, caeca and abdominal fat) (Egidius et al., 1984, 1981; Poppe et al., 1985). However, the absence of the latter condition (**e.g., see Figure S14G**) makes it unlikely that this pathogen played a major role in the symptoms displayed by fish in this study.

Given that a number of pathogenic infections are associated with 'fatty liver', in addition to 'Winter Syndrome', it is difficult to reconcile whether cold temperatures and 'Winter Syndrome' made the fish susceptible to opportunistic infections and mortality, or whether a pathogenic infection also contributed to the physiological disturbances reported in this study. For example, Ibarz et al. (2010) suggest that low temperatures are the initial cause of all these physiological disruptions in sea bream, and that these effects, combined with a cold-induced immune suppression, make the fish more susceptible to infection: this conclusion was based on the observation that a drop in temperature alone causes cold-induced fasting, thermal stress, and metabolic depression in this species; and that these immediate effects are related to an ionic imbalance caused by malfunctions of the gills and digestive system, and to a fatty liver which impacts liver function/metabolism and the blood's chemical composition. However, salmon mortalities in the same cages/regions/temperatures were greatly reduced after a CWV vaccination program was implemented (Kashulin et al., 2017), and Atlantic salmon can be acclimated to low temperatures for long periods with little to no mortalities (Arnesen et al., 1998; Døskeland et al., 2016; Madaro et al., 2018; Tang et al., 2022; Virtanen and Oikari, 1984). This latter information supports the hypothesis that the dramatic increase in lipid deposition/HSI,

liver dysfunction, metabolic breakdown and ionoregulatory disturbances observed in this study were induced by low temperature and exposure to a specific pathogen(s).

Irrespective of the causes(s) of salmon mortalities at cold temperatures in this study, and at Atlantic Canadian cage-sites (Pers. Comm.), research on sea bream suggests that impacts on production can be overcome. Production losses associated with 'Winter Syndrome' in gilthead sea bream have decreased over the past 20 years due to better management and feed formulations (Ibarz et al., 2010b). These winter feeds include higher proportions of fish meal, decreased plant proteins and inclusion of micronutrients like vitamin C, vitamin E, taurine and betaine (Richard et al., 2016; Schrama et al., 2017; Silva et al., 2014; Teodósio et al., 2021). Feeds with low EPA/DHA can also limit mortalities in Atlantic salmon during outbreaks of furunculosis and CWV (Rorvik et al., 2003). This is because DHA (from marine oils) decreases salmon liver inflammation and adipocyte lipid deposition as compared to fish fed oleic acid (OA; i.e., vegetable oils) (Bou et al., 2020).

4.5 Conclusion

Atlantic salmon, at temperatures of 8°C and below, developed severe head and jaw ulcers in 3 lab-based experiments that had many similarities to what has been described in Norwegian salmon aquaculture as tenacibaculosis. These fish also had large, pale and friable livers, and the hallmarks of metabolic collapse, ionoregulatory disturbance and liver dysfunction as described for 'Winter Syndrome'/'Disease' in cultured gilthead sea bream and yellow drum. Thus, it appears that these multiple facilitating factors may make Atlantic salmon with 'Winter Syndrome' walk a 'physiological tightrope' that leads to hepatic lipid deposition, an increase in liver size (HSI), and eventually liver failure.

However, it is still difficult to discern with certainty what triggers the increase in lipid deposition and liver size. It appears that winter-rearing conditions result in not a single factor, but a combination of factors that favor the development of 'fatty liver', including bacterial infection(s). Thus, additional studies need to be conducted to understand the prevalence of 'Winter Syndrome' at sea-cages in Atlantic Canada, to identify the pathogens associated with disease outbreaks at sea-cages in winter (at cold temperatures), and to identify/develop management strategies (including vaccines and diet formulations) that can be used to reduce losses of fish and production at cage-sites in Atlantic Canada.

4.6 References

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CHAPTER 5: Summary, Research Limitations and Perspectives

5.1 Temperature, stress and mortality

High temperatures are stressful for Atlantic salmon (*Salmo salar*) (Beemelmanns et al., 2021; Gamperl et al., 2020; Jeffries et al., 2014). However, the literature on how low temperatures (to 0-1°C) impact many aspects of this species' physiology is not as clear (Liu et al., 2020; Sandnes et al., 1988). The results from Chapter 2 demonstrate that an incremental (1°C per week) decrease in water temperatures leads to the reduction (at ~5-6°C), then cessation (at ~1°C) of feeding. Starting at 4-5°C, increases and decreases in plasma Cl^- and K^+ levels, respectively, and an increase in plasma osmolality were measured. With temperatures approaching 1°C, the fish experienced mild to moderate stress. Further, Chapter 3 shows that rapid exposure to 0°C from 3°C for 4 or 24 hours (i.e. a 'cold-shock') has few effects on salmon, with physiological changes limited to mild stress, and interestingly, an increase in gill Na^+-K^+-ATPase activity. This latter result is likely key to this species maintaining ion homeostasis following an event of this nature. Overall, these data show that Atlantic salmon have the capacity to adjust their physiology so that they can tolerate temperatures close to their lower thermal limit. This conclusion may not be surprising as salmon in the wild are found at low temperatures in rivers (Caissie et al., 2014) and off the coast of Norway (i.e., down to as low as -0.5°C; Strøm et al., 2020). However, significant mortality was recorded in every experiment where salmon were held for long periods below 8°C (Chapter 4), and this is consistent with the scientific literature (Spilsberg et al., 2022) and reports (CBC News, 2020; Stundin, 2022; The Fish Site, 2022) of losses of salmon in sea-cages. In my experiments, 5 – 30% of the fish developed enlarged livers (had increased HSI), had elevated levels of AST which suggests liver damage, and developed severe ulceration/erosion of the head and snout that was

often accompanied with fin rot in the later stages of this condition (Chapter 4). It is likely that this is related to their duration of exposure to cold temperatures, and possibly the diets currently fed to salmon.

Cultured salmon are fed a diet produced with increasing proportions of plant protein and lipid, and they cannot migrate to more favorable thermal environments to ensure that exposure to cold temperatures is of limited duration (see Appendices Figure S1). Further, it is possible that selection for specific traits desirable for a cultured fish species (i.e., high growth, late maturity, feed conversion efficiency) may also play a role in the development of health issues at low temperature. The current research shows that the development of skin ulcers, usually but not exclusively to the head and jaw, is accompanied by an increase in HSI and the accumulation of lipids in the liver, and that this is concomitant with the release of cellular markers of this tissues' damage (e.g., AST). The literature identifies many facets of winter rearing conditions that depress lipid metabolism in the liver and that would encourage lipid accumulation (Dessen et al., 2021; Døskeland et al., 2016; Fjølstad and Heyeraas, 1985; Ruyter et al., 2006), and higher lipid content and/or liver size has been linked to reduced overall robustness in fishes (Bou et al., 2017; Dessen et al., 2021; Hundal et al., 2022; Rorvik et al., 2003). Thus, the effects of low temperatures, in combination with specific diet formulations, could explain the aetiology observed in salmon in the current study, and when combined with opportunistic infections could be the main cause of winter-related mortalities in cage-sites in cold temperate regions (Spilsberg et al., 2022).

5.2 Mortality in lab-based experiments vs. winter mortality events

Significant mortalities (5 – 30%) were recorded during the experiments described in this master's thesis, and are also reported by the salmon aquaculture industry at cage-sites in winter (CBC News, 2020; Stundin, 2022; The Fish Site, 2022). From the information available, the mortalities experienced at sea-cages are mostly acute in nature, since they are reported after storms or other significant events, with many thousands of fish dying (Huffman, 2019; Willick, 2019). According to industry reports, the fish either freeze when ambient temperature drops below ~-0.7°C (Fletcher et al., 1988; Huffman, 2019; Hurst, 2007), or develop severe ulcers during harsh winter conditions (Spilsberg et al., 2022; Stundin, 2022). However, it is important to mention that mortalities at cage-sites are collected via water pumps at the bottom of the cages or manually by divers, and that the frequency of the recovery of mortalities is limited during the winter months. Further, it is possible that mortalities are underreported, as producers in some areas (e.g., Newfoundland) are not required to report mortality events (the numbers of mortalities) when they are of less than 10% of total fish on a site (Government of Newfoundland and Labrador, 2021). Thus, little information exists related to the development of skin and head ulcers after harsh winter conditions in the industry. This makes it difficult to compare mortality rates at cage-sites in winter with those that were observed in the current experiments and to identify specific causes of mortality [i.e., what is the role of diet vs. temperature alone (Dessen et al., 2021), vs. temperature-induced immune suppression and metabolic suppression (Abram et al., 2017; Melis et al., 2017)].

5.3 Experimental limitations

5.3.1 ‘Cold-shock’

As discussed in Chapter 3, there are no standardized methods for conducting ‘cold-shock’ protocols, whereas high temperature challenges are more consistent (e.g., CT_{max} and IT_{max} assessments of upper thermal tolerance) (Bartlett et al., 2022; Elliott and Elliott, 1995; Ignatz et al., 2021), and thus, it is difficult to compare studies as the selected endpoints are often different. The ‘cold-shock’ experiments currently described in the literature have typically emulated real-world events (Foss et al., 2012; Panase et al., 2018), and this is one shortcoming of the study performed in Chapter 3. For example, although there are limited temperature data available for salmon cage-sites in winter, one might expect temperatures to be at or near 0°C for several days, and fish in the current study were exposed my salmon to a maximum ‘cold-shock’ of 24 hours due to technical limitations in holding these temperatures for prolonged periods. Nonetheless, the data presented in Chapter 3 strongly suggests that rapid declines in temperature to 0°C, on their own, will not lead to major fish losses in the Atlantic Canadian salmon industry.

5.3.2 ‘Winter Syndrome’

The identification of a condition similar to ‘Winter Syndrome’ [see Ibarz et al. (2010) for a review], accompanied by the development of ulcers, was a novel finding in this thesis, and further research on these conditions and how to mitigate them, could be of benefit for the industry. However, it is also possible that the development of these symptoms may have impacted the results reported in Chapters 2 and 3. The goal of this master’s thesis was to document physiological changes in Atlantic salmon reared at

temperatures similar to those observed at cage-sites in Atlantic Canada, more specifically in Newfoundland, during the winter. This information is crucial for the aquaculture industry given recent winter mortality events. For example, it is well established that salmonids reared in seawater have an absolute lower limit that is just above the freezing point of seawater (Fletcher et al., 1988; Hurst, 2007), and accounts of severe ulcers in Atlantic salmon have been reported as a cause of winter mortality events (Stundin, 2022). Given that the experiments detailed in previous chapters were conducted in land-based tank systems with controlled environments, there was no possibility of the fish 'freezing'. However, severe ulcers were observed in some fish when held at 8°C and below, and these eventually resulted in significant mortalities (i.e., fish having to be culled). This condition has not been previously described in salmon held in tanks, however, in all but Chapter 2, the water entering the tanks was not UV-sterilized, and this may have allowed for pathogens that cause ulcers to enter the system. Interestingly, some studies have encouraged the development of low temperature ulcers by scaring the fish or using bath challenges with high doses of bacteria (Olsen et al., 2011; Småge et al., 2018). Nonetheless, they mention that the spread of ulcers is not 'straightforward' (Spilsberg et al., 2022), and there was no indication that tank conditions on their own would lead to the development of ulcers.

The research presented in this thesis does not conclude that a single factor is responsible for the development of ulcers and the poor health and welfare of Atlantic salmon at cold temperatures. The fish in the early stages of the development of ulcers already had enlarged livers (a greater HSI), but some fish had no signs of the disease and had enlarged livers. Chapter 4 of the current thesis attempts to shed some light on the

relationship(s) between liver enlargement and ulcers, but the analysis is somewhat problematic as the experiments that the samples were taken from were not specifically designed to observe the physiological impacts of cold on the prevalence and severity of ulcers. Further, the comparison between the 'asymptomatic' and 'symptomatic' fish has to be taken with 'a grain of salt' because the 'asymptomatic' fish were only 'apparently healthy' (i.e., did not show external signs of the condition) and they were held in the same tanks as the 'symptomatic' fish and cannot be considered a true 'control' group.

5.3.3 Winter feed formulations

In Chapter 4, it is hypothesized that feed composition may play an important role in the development of 'Winter Syndrome' and dermal ulcers due to the accumulation of lipids in the liver, liver damage, and the reduction of the overall robustness of the fish reared under cold temperatures (Bou et al., 2017; Dessen et al., 2021; Hundal et al., 2022). The use of feeds not specifically designed for cold temperatures may lead to unwanted physiological responses that, while associated with low temperatures, may not be prevalent if diets more optimal for cold temperatures were developed.

5.4 Future research

Given the reported development of ulcers at sea-cages and the associated mortalities in Atlantic Canada (Pers. Commun.; cage-site managers NL), and our findings which suggest that this may be related to problems with liver function (i.e., 'Winter Syndrome'), there is an urgent need to further our understanding of low temperature effects on the physiology and health of Atlantic salmon at cold temperatures.

5.4.1 Low temperature physiology

As discussed in Chapter 2, the Atlantic salmon's physiology is affected by declining temperatures, and these changes may be related to the fish's need to maintain homeostasis under such conditions. Further studies should be conducted on the Atlantic salmon's osmoregulatory physiology at low temperature, and how it may impact cell volume, and potentially induce osmotic stress. Such research, including that conducted at cage-sites themselves, could lead to the identification of fish with better tolerance of cold temperatures.

The primary goal of aquaculture is to produce the maximum number of healthy fish with as little feed as possible. This thesis clearly shows that salmon stop feeding at 1-2°C. However, there are no studies on the physiological changes that Atlantic salmon go through when fasting occurs at such low temperature, and how this affects the potential compensatory growth once feeding resumes in association with rising temperatures in the spring (Rørvik et al., 2018). When thermal conditions improve, fish will consume more feed and regain lost energy reserves. Given that feeding at low temperature may be problematic for liver metabolism, it may be important to look at reducing rations before winter, and modifying diets going into winter, so that the fish are better able to perform under such conditions and 'Winter Syndrome' is avoided.

5.4.2 Winter feed formulations

The amount and composition of feed, and the energy reserves required to overwinter, are important considerations as temperatures experienced by Atlantic salmon in the winter may be < 2°C for several months in Atlantic Canada and Iceland (see

Björnsson et al., 2007; **Figure S1**). The formulation of feeds specifically for cold periods of the year has proven extremely beneficial in gilthead sea bream aquaculture with regard to survival, health and welfare during colder months (Richard et al., 2016; Schrama et al., 2017; Silva et al., 2014; Teodósio et al., 2021), and similar benefits could be realized in the salmon aquaculture industry. Specifically, the replacement of fish oils with vegetable oils in salmon diets (the latter known to generally have lower levels of polyunsaturated fatty acids, most importantly EPA and DHA; Francis et al., 2007; Kowalska et al., 2011; Monge-Ortiz et al., 2018), may be detrimental when Atlantic salmon are reared at low temperatures. This is based on the fact that salmon are known to accumulate greater proportions of saturated lipids in the liver (Ruyter et al., 2006), and that increasing the level of fat in salmon diets at cold temperatures has significant effects on this species' survival and overall robustness (Dessen et al., 2020). However, this is probably not the only adjustment that should be made to salmon diets formulated for the period of decreasing temperatures. Experiments have shown that modifying vitamin C and E levels, and increasing the inclusion levels of taurine and betaine can have significant positive effects with regard to fish health, production and 'Winter Syndrome' (Barros et al., 2015; El-Sayed and Izquierdo, 2021; Espe et al., 2012; Richard et al., 2016; Schrama et al., 2017; Silva et al., 2014). In fact, such studies are currently being planned and should begin early in 2023.

5.4.3 'Winter-Syndrome' / 'Fatty Liver' and opportunistic infections

In the context of aquaculture and the industry's desire for maximum growth, liver function appears to be a limiting factor in Atlantic salmon culture at low temperatures

(Døskeland et al., 2016; Ruyter et al., 2006; Sissener et al., 2017). In Atlantic salmon and other cultured species, 'fatty liver' refers to an abnormally high lipid content in liver cells (Horn et al., 2018; Jia et al., 2006; Sissener et al., 2017). It is similar to non-alcoholic fatty liver disease (NAFLD) that affects millions of humans around the world and can cause significant health issues (Schuster et al., 2018; Shwartz et al., 2019). The development of ulcers (to the head/jaws) in some fish described in previous chapters were always linked with an abnormally large liver. The link between lipid liver content and the fish's overall robustness has been hinted at by the literature (Bou et al., 2017; Dessen et al., 2021; Hundal et al., 2022; Rorvik et al., 2003), but there are no definitive physiological explanations and the relationship between 'fatty liver' and skin infections at these temperatures is not clear. Is it simply cold-induced suppression of the salmon's innate and specific immunity (Abram et al., 2017) that makes them susceptible to infections of the dermis at cold temperatures, or do the physiological and behavioral changes associated with 'fatty liver'/'Winter Syndrome' have a direct and/or synergistic effect. For example, one could suggest that 'Winter Syndrome' makes the fish lethargic and causes them to swim erratically, that this results in them bumping into objects in tanks (or rubbing against the nets of sea-cage aquaculture pens; Ćirić, 2020), and that abrasion of the dermis caused by such activity makes them susceptible to pathogens that have the capacity to cause disease/ulcers at cold temperatures (Olsen et al., 2011).

The current thesis is the only study that provides specific evidence with regards to salmon developing 'fatty liver' and 'Winter Syndrome' at cold temperatures, and to suggest that this condition is related to the development of dermal ulcers in this species at

low temperature. Clearly, additional studies involving measures of liver fatty acid levels, the level of hepatic peroxidation and the activities of key enzymes in lipid metabolism, and the examination of liver histology are required before we can definitively say that Atlantic salmon can develop 'Winter Syndrome'/'fatty liver'. These possible findings would allow more certainty with regard to developing effective management strategies (e.g., modifying feeding regimes and diet formulations) to improve the survival, health and welfare of salmon at cold temperatures. Clearly, this should be a goal of researchers and the industry in the short-term as the cage-culture of salmon in Atlantic Canada and Iceland is expected to continue at a large scale for the foreseeable future, and winter-related losses threaten the sustainability and profitability of the industry, and its 'social licence' and support amongst the public.

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Appendix

Table S1. Weekly average seawater temperatures (°C) in the cold-exposure and control (8°C) tanks.

	Control	**Cold-exposure**
	Temperature °C	**Temperature °C**
Week 0	8.1 ± 0.2	8.2 ± 0.2
Week 1	8.4 ± 0.1	7.0 ± 0.7
Week 2	8.4 ± 0.1	6.0 ± 0.2
Week 3	8.4 ± 0.1	5.2 ± 0.4
Week 4	8.5 ± 0.0	4.2 ± 0.4
Week 5	8.4 ± 0.0	3.2 ± 0.4
Week 6	8.4 ± 0.1	2.2 ± 0.5
Week 7	8.4 ± 0.1	1.5 ± 0.2
Week 8	8.5 ± 0.0	1.2 ± 0.1

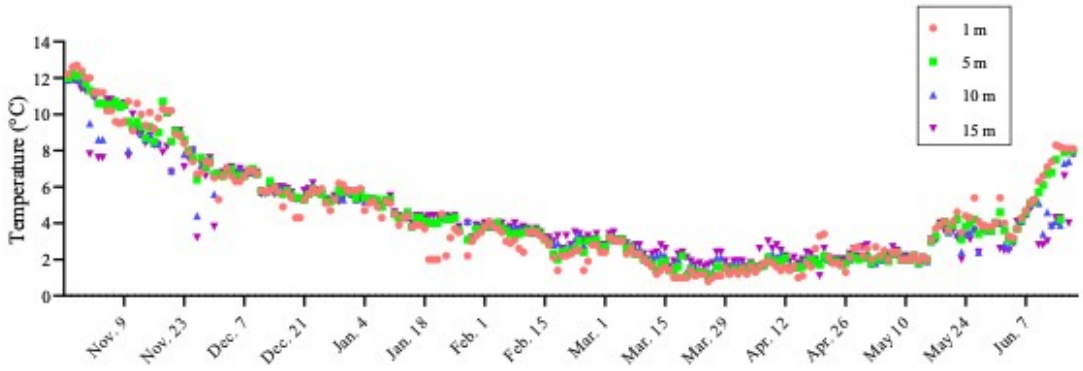

Figure S1. Salmon cage-site temperatures at 1, 5, 10 and 15 meters depth from approx. Nov. 1st of 2020 to June 15th of 2021 at a location on the south coast of Newfoundland. Sandrelli and Gamperl (In Prep.).

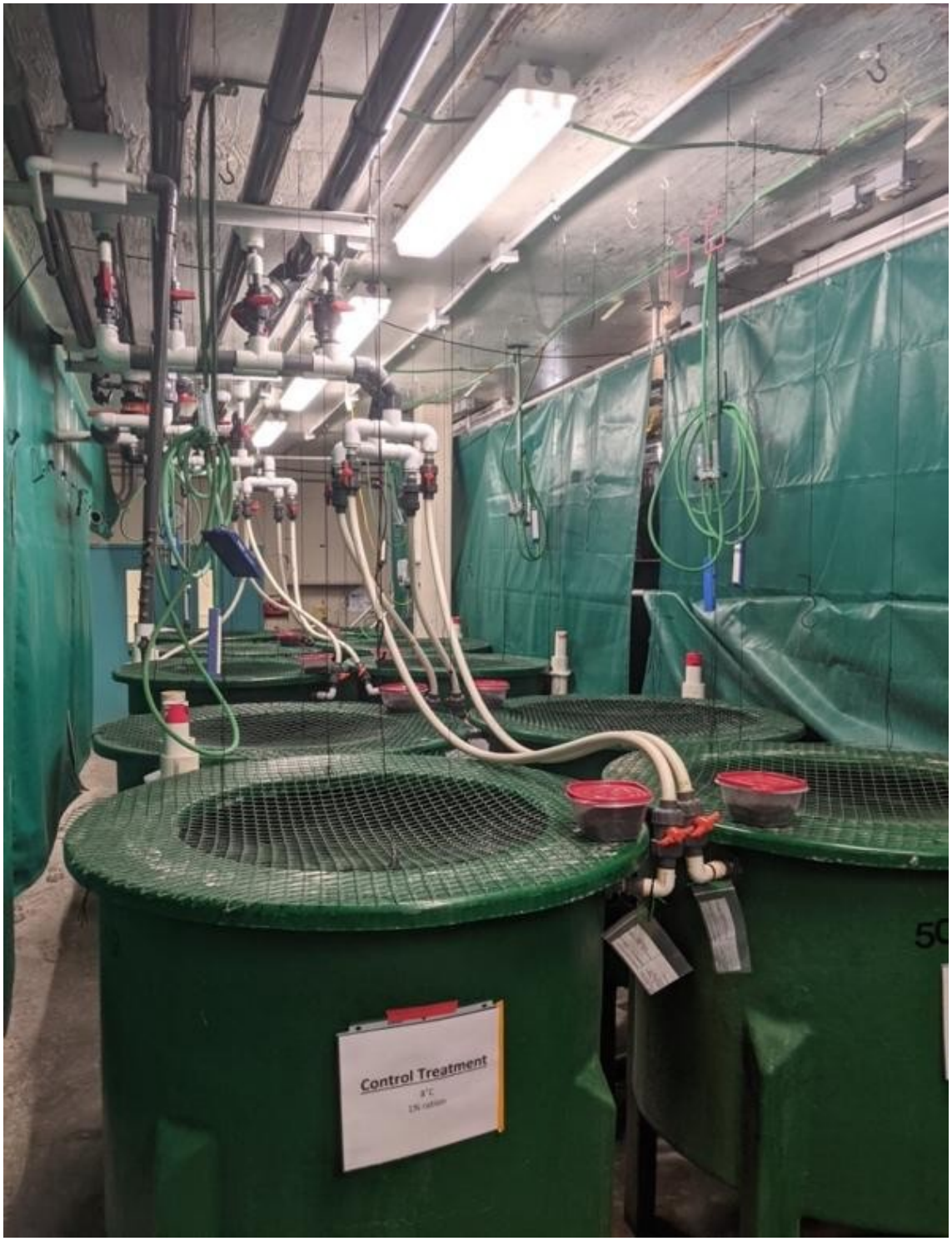

Figure S2. Tanks used in the experiment conducted in Chapter 2. All 8 500 L tanks were connected to 2 flow-through seawater sources (8°C and ambient) so that specific water temperatures could be obtained. Photoperiod was 12h light : 12h dark. Curtains were used on both sides of the tanks to minimize stress disturbance.

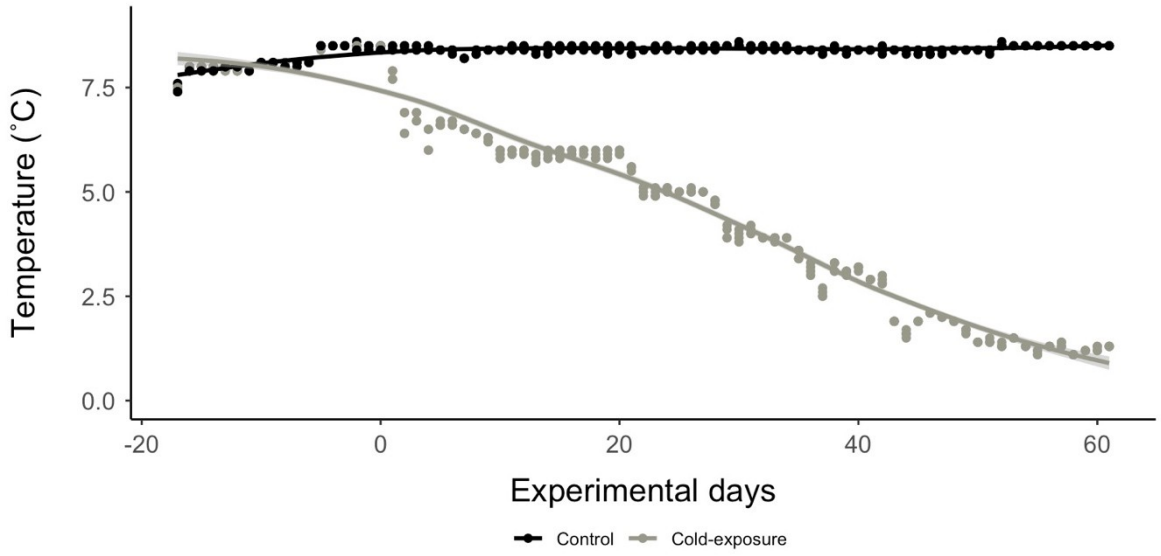

Figure S3. Water temperatures in the control (black) and cold-exposure tanks (grey) for each day of the experiment. Day 0 represents the date of the initial sampling. 'Negative' days indicate the acclimation period at 8°C.

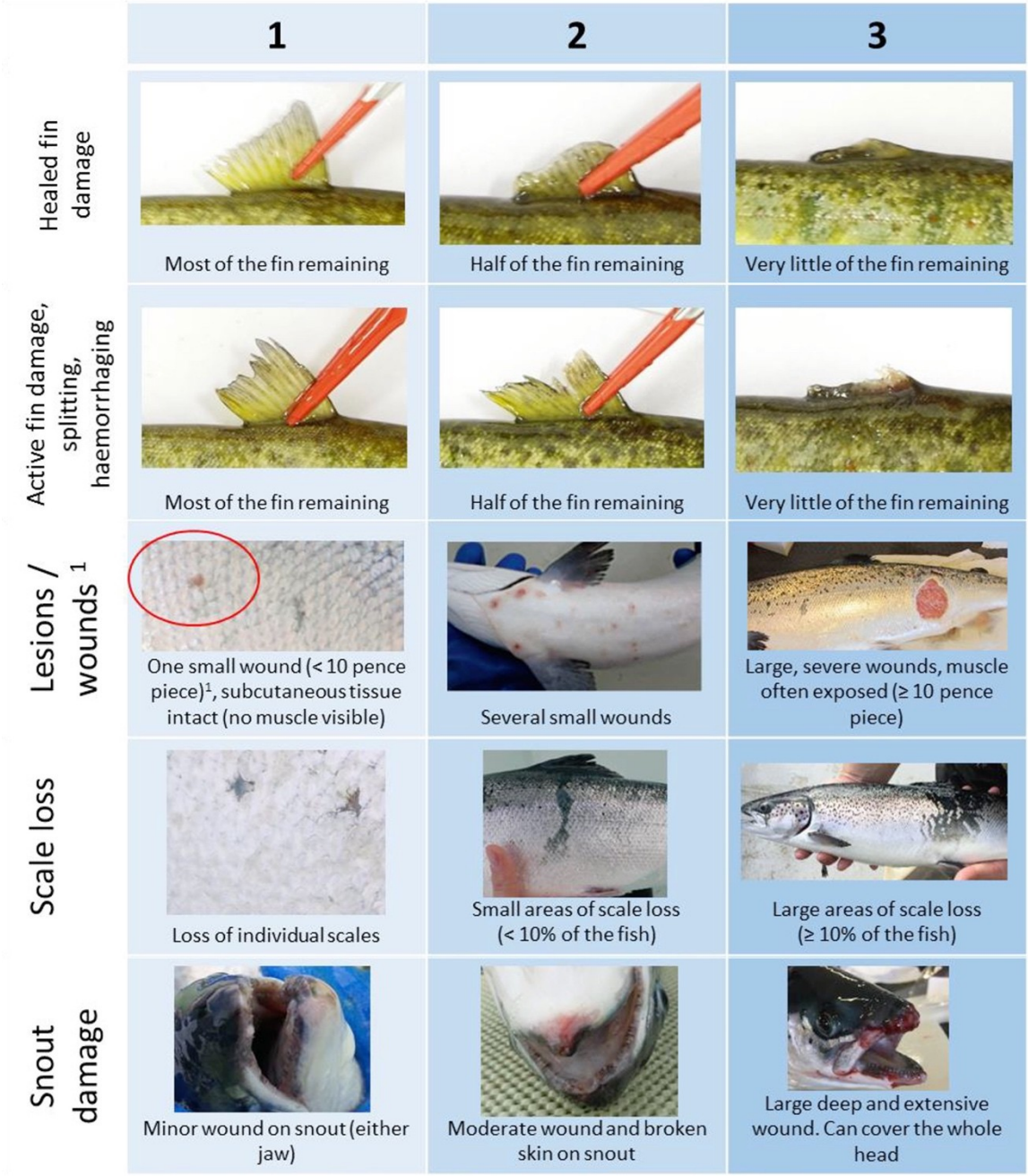

Figure S4. Scheme used to score the external condition of Atlantic salmon (adapted from Nobles et al., 2018).

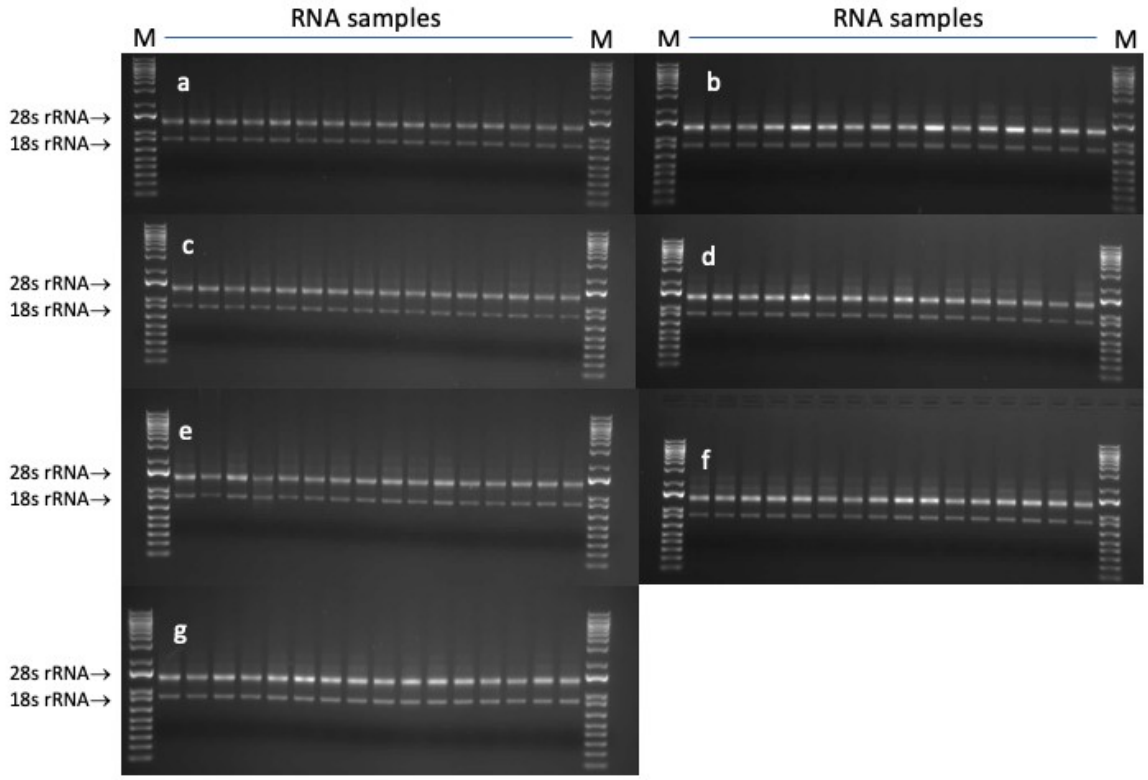

Figure S5. Integrity of TRIzol-extracted total RNA samples following phenol:chloroform:isoamyl alcohol extraction, DNase-treatment and column-purification. RNA samples were electrophoretically separated on 1% agarose gels (alongside the 1 kb Plus DNA Ladder (M, Invitrogen/Thermo Fisher Scientific), and visualized under UV light following ethidium bromide staining. All RNA samples were of high integrity (i.e., tight 28S and 18S ribosomal RNA bands, with 28S being approximately twice as intense as 18S). Sample order is (a: 1-16, b: 17-32, c: 33-48, d: 49-64, e: 65-80, f: 81-96 and g: 97-112) (4 control, 8 cold-exposure and 4 control then next sampling (x7)).

Table S2. Identity (%) between nucleotide sequences of four cytosolic heat shock protein 90 paralogue cDNAs (coding sequence only) analyzed in the qPCR study.

% identity	*hsp90aa1a*	*hsp90aa1b*	*hsp90ab1a*	*hsp90ab1b*
hsp9a0a1a	100			
hsp90aa1b	92	100		
hsp90ab1a	83	82	100	
hsp90ab1b	81	81	93	100

Table S3. Identity (%) between nucleotide sequences of four heat shock protein 70 paralogue cDNAs (coding sequence only) analyzed in the qPCR study.

% identity	*hsp70a*	*hsp70b*	*hsp70c*	*hsp70d*
hsp70a	100			
hsp70b	79	100		
hsp70c	84	76	100	
hsp70d	81	77	82	100

Table S4. Primers used in the qPCR studies.

Gene Name (symbol) (GenBank Acc. No.)	Nucleotide sequence (5'-3')	Amplicon size (bp)	[a]Efficiency (%)	Reference
cold inducible RNA binding protein *(cirbp)* (BT059171)	F: TTGAGTACACAGCGGTGAATT R: ACCAATCTGATGCTATGACGAGA	132	98.3	Beemelmanns et al., 2021b
serpin peptidase inhibitor, clade H, member 1 *(serpinh1)* **alias heat shock protein 47** (*hsp47)* (XM_014214963)	F: GACCATTCAAAAATCAACCTCA R: CATGGCTCCATCAGCATTCT	129	97.7	Beemelmanns et al., 2021b
heat shock 70 kDa protein a *(hsp70a)* (BT043589)	F: GTGGTGAGCGATGGTGGCAA R: GACAGCATTGTTCACTGGCTT	150	100	[b]Caballero-Solares et al., 2020
heat shock 70 kDa protein b *(hsp70b)* (BT046112)	F: GTGGTCAGCGACGGAGGAAA R: GACTGCATTGGACACCTTCTG	150	97.5	[b]Unpublished
heat shock 70 kDa protein c *(hsp70c)* (BT045715)	F: AGTGATCAACGACTCGACACG R: CACTGCATTGGTTATAGTCTTG	151	93.6	[b]Beemelmanns et al., 2021b
heat shock 70 kDa protein d *(hsp70d)* (BT058774)	F: GTTATCAATGATTCTACTCGGCC R: CTGCATTGTTGACAGTTTTTCC	148	90.8	[b]Unpublished
heat shock protein 90 alpha family class A member 1a *(hsp90aa1a)* (KC150878)	F: TAGGCTCAGACGAGGAGGATGA R: GTTACGGGTCCACAGGGGTT	134	97.5	[c]Unpublished
heat shock protein 90 alpha family class A member 1b *(hsp90aa1b)* (KC150879)	F: CACGATGGCGCATGTGGG R: GGGTTACGAGTCCAAAGGGG	110	[d]101.7	[c]Unpublished
heat shock protein 90 alpha beta 1a *(hsp90ab1a)* (KC150882)	F: GCTCTGATGACGAGGAGGAC R: TGATGTCGTCAGGGTTTCTG	129	101.8	[e]This study
heat shock protein 90 alpha beta 1b *(hsp90ab1b)* (KC150883)	F: CTGGACCAGGAACCCTGATG R: CTTGACTGCAAGGTGGTCCT	94	97.6	[e]This study
Candidate Normalizers				
60S ribosomal protein L32 (*rpl32*) (BT043656)	F: AGGCGGTTTAAGGGTCAGAT R: TCGAGCTCCTTGATGTTGTG	119	98.8	Xue et al., 2015
β-actin (*actb*) (BG933897)	F: CCAAAGCCAACAGGGAGAAG R: AGGGACAACACTGCCTGGAT	91	97.8	Xue et al., 2015
elongation factor 1-alpha 1 (*ef1a1*) (AF321836)	F: TGGCACTTTCACTGCTCAAG R: CAACAATAGCAGCGTCTCCA	197	95.0	Caballero-Solares et al., 2018
elongation factor 1-alpha 2 (*ef1a2*) (BT058669)	F: GCACAGTAACACCGAAACGA R: ATGCCTCCGCACTTGTAGAT	132	92.8	Katan et al., 2019
[f]**eukaryotic translation initiation factor 3 subunit D** (*eif3d*) (GE777139)	F: CTCCTCCTCCTCGTCCTCTT R: GACCCCAACAAGCAAGTGAT	105	97.1	Caballero-Solares et al., 2017
[f]**polyadenylate-binding protein 1** (*pabpc1*) (EG908498)	F: TGACCGTCTCGGGTTTTTAG R: CCAAGGTGGATGAAGCTGTT	108	95.1	Xu et al., 2013

[a] Amplification efficiencies were calculated using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of input total RNA, with the exception of *cirbp and serpinh1* which were calculated using a 5-point 1:3 dilution series starting with cDNA representing 50 ng of input total RNA. See methods for details.

[b] Primers were designed by Dr. Jennifer Hall as part of the Genomic Applications Partnership Program [GAPP # 6604: Biomarker Platform for Commercial Aquaculture Feed Development project funded by the Government of Canada through Genome Canada and Genome Atlantic, and Cargill Innovation (formerly EWOS Innovation) and awarded to Dr. Matthew Rise].

[c] Primers were designed by Dr. Xi Xue as part of the Genomic Applications Partnership Program [GAPP #6607: Integrated Pathogen Management of Co-infection in Atlantic salmon (IPMC) project funded by the Government of Canada through Genome Canada and Genome Atlantic, and Cargill Innovation (formerly EWOS Innovation) and awarded to Dr. Matthew Rise]. The IPMC project was also funded by the Government of Newfoundland and Labrador through the Department of Tourism, Culture, Industry and Innovation (Leverage R&D award #5401-1019-108).

[d] Amplification efficiency for *hsp90aa1b* was calculated from pooled fish at 3 and 1°C as expression levels were too low in the pooled fish at 8°C.

[e] Primers were designed by Dr. Jennifer Hall, Aquatic Research Cluster, CREAIT Network.

[f] Expression levels of the transcripts of interest were normalized to expression levels of these two transcripts

Table S5. qPCR analysis of transcript expression levels for six candidate normalizer genes. C_T values were obtained for 3 individuals from each group at 8°C and for 4 samples from each group at all of the other sampling temperatures and analyzed using geNorm. For each gene, the C_T values for each group are presented (mean □ s.e.m.), along with the geNorm M values. Eif3d and rpl32 were selected as the normalizers for the qPCR study.

C_T value	***actb***	***ef1a1***	***ef1a2***	***eif3d***	***pabpc1***	***rpl32***
8°C						
Control	18.98 ± 0.61	19.24 ± 0.32	19.36 ± 0.21	22.62 ± 0.28	21.03 ± 0.28	19.88 ± 0.16
Cold-exposure	19.29 ± 0.08	19.36 ± 0.21	19.31 ± 0.15	23.03 ± 0.27	21.43 ± 0.19	20.09 ± 0.18
6°C						
Control	19.20 ± 0.19	18.96 ± 0.00	18.99 ± 0.01	22.73 ± 0.02	21.14 ± 0.04	19.86 ± 0.06
Cold-exposure	19.58 ± 0.20	19.00 ± 0.10	19.10 ± 0.07	22.87 ± 0.03	21.43 ± 0.07	20.10 ± 0.05
5°C						
Control	19.46 ± 0.13	19.47 ± 0.03	19.19 ± 0.01	23.13 ± 0.06	21.58 ± 0.04	20.05 ± 0.09
Cold-exposure	20.04 ± 0.20	19.62 ± 0.25	19.48 ± 0.22	23.26 ± 0.21	21.65 ± 0.12	20.33 ± 0.15
4°C						
Control	19.89 ± 0.08	19.79 ± 0.11	19.47 ± 0.05	23.51 ± 0.04	21.59 ± 0.03	20.39 ± 0.03
Cold-exposure	19.61 ± 0.16	19.10 ± 0.10	19.15 ± 0.12	22.77 ± 0.09	21.22 ± 0.06	20.01 ± 0.08
3°C						
Control	19.35 ± 0.16	19.40 ± 0.12	19.09 ± 0.07	23.16 ± 0.11	21.62 ± 0.13	20.33 ± 0.09
Cold-exposure	19.41 ± 0.20	19.14 ± 0.12	19.18 ± 0.08	23.00 ± 0.14	21.29 ± 0.13	20.20 ± 0.17
2°C						
Control	19.28 ± 0.18	19.06 ± 0.11	18.92 ± 0.13	22.83 ± 0.16	21.33 ± 0.16	20.04 ± 0.16
Cold-exposure	19.56 ± 0.11	18,85 ± 0.16	19.08 ± 0.16	22.71 ± 0.15	21.21 ± 0.07	19.91 ± 0.20
1°C						
Control	19.33 ± 0.15	18.92 ± 0.22	18.80 ± 0.25	22.93 ± 0.19	21.32 ± 0.18	20.20 ± 0.33
Cold-exposure	19.16 ± 0.14	18.82 ± 0.13	18.99 ± 0.10	22.67 ± 0.10	21.04 ± 0.05	20.00 ± 0.12
geNorm M value	**0.263**	**0.202**	**0.214**	**0.173**	**0.178**	**0.183**

Table S6. C_T values (mean ± s.e.m.) obtained for the TOIs and normalizers (eif3d and rpl32) in the 5 experimental qPCR studies. For each qPCR study, 2 TOIs and the 2 normalizers were assessed across 4 linked plates.

	8°C		6°C		5°C		4°C	
	Control	**Cold-exposure**	**Control**	**Cold-exposure**	**Control**	**Cold-exposure**	**Control**	**Cold-exposure**
Study 1								
hsp90aa1a	29.7 ± 0.2	30.6 ± 0.1	29.7 ± 0.1	29.4 ± 0.1	30.0 ± 0.1	29.1 ± 0.2	30.2 ± 0.1	27.3 ± 0.2
hsp90aa1b	29.3 ± 0.1	30.6 ± 0.2	29.6 ± 0.2	29.0 ± 0.2	30.2 ± 0.1	28.6 ± 0.2	30.2 ± 0.2	27.5 ± 0.2
eif3d	22.1 ± 0.1	22.6 ± 0.1	22.3 ± 0.1	22.3 ± 0.0	22.7 ± 0.0	22.4 ± 0.1	22.6 ± 0.1	22.1 ± 0.1
rpl32	19.8 ± 0.1	20.0 ± 0.0	19.8 ± 0.1	19.8 ± 0.0	20.0 ± 0.0	20.3 ± 0.4	20.0 ± 0.1	19.6 ± 0.0
Study 2								
hsp70c	24.8 ± 0.1	25.8 ± 0.2	24.3 ± 0.1	24.3 ± 0.1	25.0 ± 0.1	23.8 ± 0.1	24.6 ± 0.1	23.0 ± 0.1
hsp70d	18.8 ± 0.1	19.2 ± 0.1	18.7 ± 0.1	18.9 ± 0.1	19.0 ± 0.0	18.7 ± 0.1	19.0 ± 0.1	18.3 ± 0.1
eif3d	22.7 ± 0.1	23.2 ± 0.1	22.8 ± 0.1	22.9 ± 0.1	23.4 ± 0.1	23.1 ± 0.1	23.0 ± 0.1	22.7 ± 0.1
rpl32	19.9 ± 0.1	20.0 ± 0.1	19.8 ± 0.1	19.8 ± 0.0	20.0 ± 0.0	20.0 ± 0.1	19.9 ± 0.1	19.6 ± 0.0
Study 3								
hsp70a	27.1 ± 0.1	28.2 ± 0.2	27.6 ± 0.1	28.2 ± 0.1	27.7 ± 0.1	27.7 ± 0.2	28.4 ± 0.1	28.1 ± 0.1
hsp70b	28.3 ± 0.2	29.1 ± 0.1	27.8 ± 0.1	27.3 ± 0.1	28.8 ± 0.1	27.7 ± 0.1	28.1 ± 0.1	27.0 ± 0.1
eif3d	21.1 ± 0.1	21.6 ± 0.1	21.3 ± 0.1	21.4 ± 0.0	21.6 ± 0.0	21.4 ± 0.1	21.6 ± 0.1	21.1 ± 0.1
rpl32	18.6 ± 0.1	18.8 ± 0.0	18.8 ± 0.1	18.8 ± 0.1	19.2 ± 0.3	18.8 ± 0.1	19.0 ± 0.1	18.6 ± 0.0
Study 4								
hsp90ab1a	19.4 ± 0.1	20.1 ± 0.1	19.6 ± 0.1	19.8 ± 0.1	19.9 ± 0.0	19.6 ± 0.1	19.9 ± 0.1	19.4 ± 0.1
hsp90ab1b	22.0 ± 0.1	22.5 ± 0.1	22.0 ± 0.1	22.1 ± 0.1	22.4 ± 0.0	21.9 ± 0.1	22.2 ± 0.1	21.6 ± 0.1
eif3d	22.9 ± 0.4	23.1 ± 0.1	22.8 ± 0.1	23.0 ± 0.0	23.0 ± 0.0	22.7 ± 0.1	23.0 ± 0.1	22.4 ± 0.1
rpl32	19.9 ± 0.1	20.1 ± 0.0	20.0 ± 0.1	20.1 ± 0.1	19.9 ± 0.0	19.8 ± 0.1	20.0 ± 0.1	19.7 ± 0.0
Study 5								
cirbp	30.5 ± 0.1	31.0 ± 0.1	30.5 ± 0.1	30.1 ± 0.1	31.3 ± 0.1	30.6 ± 0.1	30.8 ± 0.1	29.6 ± 0.0
serpinh1	30.2 ± 0.2	30.8 ± 0.2	27.6 ± 0.3	28.6 ± 0.2	31.0 ± 0.1	29.9 ± 0.1	27.9 ± 0.2	28.5 ± 0.2
eif3d	20.5 ± 0.1	21.0 ± 0.1	20.7 ± 0.1	20.7 ± 0.0	21.1 ± 0.0	20.8 ± 0.1	21.0 ± 0.1	20.4 ± 0.1
rpl32	17.9 ± 0.0	18.1 ± 0.0	18.0 ± 0.1	18.0 ± 0.0	18.1 ± 0.0	18.1 ± 0.1	18.2 ± 0.1	17.8 ± 0.0

Table S6. continued

	3°C		2°C		1°C	
	Control	**Cold-exposure**	**Control**	**Cold-exposure**	**Control**	**Cold-exposure**
Study 1						
hsp90aa1a	27.1 ± 0.4	28.6 ± 0.1	30.4 ± 0.2	26.4 ± 0.3	30.1 ± 0.1	25.7 ± 0.4
hsp90aa1b	27.6 ± 0.3	28.4 ± 0.1	30.3 ± 0.2	26.8 ± 0.2	30.0 ± 0.1	26.3 ± 0.2
eif3d	22.1 ± 0.1	22.3 ± 0.0	22.7 ± 0.3	22.0 ± 0.1	22.5 ± 0.1	22.2 ± 0.1
rpl32	19.8 ± 0.1	20.0 ± 0.1	20.4 ± 0.2	19.7 ± 0.1	20.3 ± 0.1	20.0 ± 0.1
Study 2						
hsp70c	23.1 ± 0.2	23.6 ± 0.2	24.8 ± 0.3	22.4 ± 0.2	24.2 ± 0.1	22.2 ± 0.2
hsp70d	18.3 ± 0.1	18.6 ± 0.1	19.2 ± 0.2	18.0 ± 0.1	18.7 ± 0.1	18.1 ± 0.1
eif3d	22.6 ± 0.1	22.8 ± 0.1	23.3 ± 0.2	22.6 ± 0.1	23.1 ± 0.1	22.8 ± 0.1
rpl32	19.6 ± 0.1	19.8 ± 0.1	20.2 ± 0.2	19.5 ± 0.1	20.1 ± 0.1	19.9 ± 0.1
Study 3						
hsp70a	27.9 ± 0.1	28.3 ± 0.1	28.0 ± 0.3	27.9 ± 0.1	27.6 ± 0.1	28.1 ± 0.1
hsp70b	26.4 ± 0.3	27.2 ± 0.1	28.6 ± 0.3	26.1 ± 0.2	27.9 ± 0.1	26.5 ± 0.4
eif3d	21.0 ± 0.1	21.2 ± 0.1	22.3 ± 0.5	21.2 ± 0.2	21.6 ± 0.1	21.6 ± 0.3
rpl32	18.6 ± 0.1	18.8 ± 0.1	19.7 ± 0.3	18.7 ± 0.1	19.1 ± 0.1	20.2 ± 0.8
Study 4						
hsp90ab1a	19.1 ± 0.1	19.3 ± 0.1	19.9 ± 0.2	18.9 ± 0.1	19.3 ± 0.1	18.9 ± 0.1
hsp90ab1b	21.2 ± 0.1	21.6 ± 0.1	22.2 ± 0.2	21.2 ± 0.1	21.9 ± 0.1	21.2 ± 0.1
eif3d	22.3 ± 0.1	22.6 ± 0.1	23.1 ± 0.3	22.3 ± 0.1	22.7 ± 0.1	22.4 ± 0.1
rpl32	19.5 ± 0.1	19.7 ± 0.1	20.2 ± 0.2	19.4 ± 0.1	19.8 ± 0.1	19.6 ± 0.1
Study 5						
cirbp	29.2 ± 0.1	29.5 ± 0.1	31.2 ± 0.3	29.7 ± 0.1	31.3 ± 0.1	30.4 ± 0.1
serpinh1	28.9 ± 0.3	28.8 ± 0.2	28.9 ± 0.2	29.3 ± 0.3	30.5 ± 0.1	29.4 ± 0.3
eif3d	20.3 ± 0.1	20.5 ± 0.1	21.1 ± 0.2	20.3 ± 0.1	21.0 ± 0.1	20.7 ± 0.1
rpl32	17.7 ± 0.1	17.9 ± 0.1	18.6 ± 0.2	17.8 ± 0.1	18.4 ± 0.1	18.2 ± 0.1

```
hsp90aa1a       ATGCCGGAAGCCCATGACACCCCGATGGAGGAAGAGGCAGAGACCTTTGCCTTCCAGGCT  60
hsp90aa1b       -------------------------ATGGAGGAGGAGGCAGAGACCTTTGCCTTCCAGGCT  36
hsp90ab1a       ------ATGCCTGAAGAAATGCGCCAAGAGGAGGAGGCTGAGACCTTTGCCTTCCAGGCA  54
hsp90ab1b       ------ATGCCTGAAGAAATGCGCCAAGAGGAGGAGGCTGAGACCTTTGCCTTCCAGGCA  54
                                     ***** ***** ********************

hsp90aa1a       GAGATCGCCCAGCTGATGTCCCTGATCATCAACACTTTCTACTCAAACAAAGAGATTTTC  120
hsp90aa1b       GAGATCGCCCAGCTGATGTCCCTGATCATCAACACATTCTACTCCAACAAAGAGATCTTC  96
hsp90ab1a       GAGATCGCTCAGCTCATGTCCCTGATCATCAACACCTTTTATTCCAACAAGGAAATCTTC  114
hsp90ab1b       GAGATTGCTCAGCTCATGTCCCTGATCATCAACACCTTTTATTCCAACAAGGAAATCTTT  114
                ***** ** ***** ********************** ** ** ** ***** ** ** **

hsp90aa1a       CTTAGGGAGCTCATCTCCAACTCTTCAGATGCTCTAGACAAAATCCGCTATGAGAGCCTC  180
hsp90aa1b       CTCAGGGAGCTCATCTCTAACTCCTCAGATGCTCTGGACAAAATCCGCTATGAGAGCCTG  156
hsp90ab1a       CTCAGGGAGTTGATTTCCAATGCATCTGATGCTTTGGACAAAATCCGATACGAAAGTCTG  174
hsp90ab1b       CTCAGGGAGTTGATCTCCAATGCCTCTGATGCTTTGGACAAAATCCGCTATGAAAGTCTG  174
                ** ****** * ** ** **  * ** ****** * *********** ** ** ** **

hsp90aa1a       ACAGACCCATCCAAGATGGACTCTGGCAAGGACCTGAAGATCGAGGTCATTCCCAACAAG  240
hsp90aa1b       ACAGACCCGACCAAGATGGACTCTGGCAAGGACCTGAAGATCGAGGTCATTCCCAACAAG  216
hsp90ab1a       ACGGACCCCACCAAGCTGGACAACGGCAAGGAACTGAAGATTGACGTCATCCCCAATGTG  234
hsp90ab1b       ACGGACCCCACCAAGCTGGACAATGGCAAGGAATTGAAGATCGACATCATCCCCAATGTG  234
                ** *****  ***** *****   ********  ******* **  **** *****   *

hsp90aa1a       GAGGAGCGCACCCTGACCCTGGTTGACACCGGCATCGGCATGACCAAGGCCGACCTAATC  300
hsp90aa1b       GAGGAGCGCACCCTGACCTTGGTTGACACCGGCATCGGCATGACCAAGGCTGACCTCATC  276
hsp90ab1a       GAGGAGCGCACCCTGACCCTAATCGACACTGGAATTGGCATGACCAAAGCTGACCTCATC  294
hsp90ab1b       GAGGACCGTACCCTGACCCTCATCGACACTGGAATTGGCATGACCAAAGCTGACCTCATC  294
                ***** ** ********* *  * ***** ** ** *********** ** ***** ***

hsp90aa1a       AACAACCTGGGAACCATTGCAAAGTCTGGCACCAAGGCCTTCATGGAGGCCCTGCAGGCT  360
hsp90aa1b       AACAACCTTGGAACCATCGCCAAGTCTGGCACCAAGGCCTTCATGGAGGCCCTGCAGGCT  336
hsp90ab1a       AACAACCTGGGAACCATCGCGAAGTCTGGCACCAAGGCCTTCATGGAGGCCCTGCAGGCT  354
hsp90ab1b       AACAACCTGGGAACTATTGCCAAGTCTGGCACCAAGGCCTTCATGGAGGCCCTGCAGGCT  354
                ******** ***** ** ** ************************************

hsp90aa1a       GGAGCAGACATCTCCATGATCGGACAGTTCGGTGTGGGTTTCTACTCTGCCTACCTGGTG  420
hsp90aa1b       GGAGCTGACATCTCTATGATTGGGCAGTTCGGAGTGGGTTTCTACTCCGCCTACCTGGTG  396
hsp90ab1a       GGAGCTGACATCTCCATGATTGGGCAGTTTGGTGTGGGTTTCTACTCTGCCTACCTGGTG  414
hsp90ab1b       GGAGCTGACATCTCCATGATTGGGCAGTTCGGTGTGGGATTCTACTCTGCCTACCTGGTG  414
                ***** ******** ***** ** ***** ** ***** ******** ************

hsp90aa1a       GCTGAGAGGGTGACTGTCATCACCAAGCACAATGATGATGAGCAGTACATCTGGGAATCA  480
hsp90aa1b       GCTGAGAGGGTGACTGTCATCACCAAGCACAACGATGATGAGCAGTACATCTGGGAATCC  456
hsp90ab1a       GCCGAGAGGGTGACTGTCATCACTAAGCACAACGATGATGAGCAGTACATCTGGGAGTCC  474
hsp90ab1b       GCAGAAAGAGTGACCGTCATCACCAAGCACAATGATGACGAGCAGTACATCTGGGAGTCT  474
                ** ** ** ***** ******** ******** ***** ***************** **

hsp90aa1a       TCCGCTGGCGGATCCTTCACCGTCAAAGTCGACACGTCAGCTGAGTCTATTGGTCGTGGG  540
hsp90aa1b       TCTGCTGGCGGATCCTTCACCGTCAAAGTCGACACGTCAGCTGAGTCTATTGGTCGTGGC  516
hsp90ab1a       TCAGCCGGAGGCTCCTTCACAGTCAAGGTCGACACTGGG---GAGCCCATGTTGCGTGGA  531
hsp90ab1b       TCAGCCGGAGGCTCATTCACAGTGAAGGTCGACAGTGGG---GAGCCCATGTTGCGTGGA  531
                ** ** ** ** ** ***** ** ** *******        *** * **    *****

hsp90aa1a       ACCAAGGTGATTCTGTACCTGAAGGAAGACCAGACAGAATACTGTGAGGAGAAACGGGTC  600
hsp90aa1b       ACCAAGGTGATTCTGTACCTGAAGGATGACCAGACAGAATATTGTGAGGAGAAACGGGTC  576
hsp90ab1a       ACTAAGGTGATTCTGCACATGAAGGAGGACCAGACGGAGTATGTTGAGGAAAAAAGGGTC  591
hsp90ab1b       ACTAAGGTGATTCTACACATGAAGGAGGACCAGACTGAGTATGTTGAGGAGAAGAGGGTC  591
                ** ***********  ** ******* ******** ** **   ****** **  *****
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hsp90aa1a       AAAGAGATCGTGAAGAAGCACTCCCAGTTCATCGGATATCCCATCACACTCTTTGTGGAG 660
hsp90aa1b       AAAGAAATTGTGAAGAAGCACTCCCAGCTCATCGGATACCCCATCACACTCTTTGTGGAG 636
hsp90ab1a       AAGGAGGTGGTCAAGAAGCACTCTCAGTTCATTGGATATCCCATCACCCTCTTCGTTGAG 651
hsp90ab1b       AAGGAGGTGGTCAAGAAGCACTCTCAGTTCATTGGATATCCCATCACACTTTTCGTTGAA 651
                ** **  * ** ********** *** **** ***** ******** ** ** ** **

hsp90aa1a       AAGGAGCGTGACAAGGAAGTGAGTGACGATGAGGCAGAGGAGGAAGAGGAGAAGGAGAAG 720
hsp90aa1b       AAGGAGCGTGACAAGGAAGTGAGCGATGATGAGGCGGAGGAGGAGGAGAAGGAGAAGAAG 696
hsp90ab1a       AAGGAGCGTGAAAAGGAGATCAGTGATGATGAGGAGGAGAAGGCAGAGGAGGAGAAAGAG 711
hsp90ab1b       AAGGAGCGTGAAAAAGAGATCAGTGATGATGAGGAGGAGGAGACAGAGGAGGAGAAGGAG 711
                *********** ** **  * ** ** *******  *** **   *** ** ** *  **

hsp90aa1a       GATGGGGAAGAAGGAGAGAAGAGAGAGGTTGACAAACCCGAGATCGAGGACGTAGGCTCA 780
hsp90aa1b       GATGGGGAAGGAGAGGA-----------GGACAAACCTGATATCGAGGACGTAGGCTCG 744
hsp90ab1a       G---AGAAGGAGG-----------CAGAGGACAAGCCCAAGATCGAGGACGTTGGCTCT 756
hsp90ab1b       GAAAAGGCGGAGG-----------CTGAGGACAAGCCCAAAATTGAGGACGTGGGCTCA 759
                *    *   *  *               ***** **  * ** ******** *****

hsp90aa1a       GACGAGGAGGATGACCATGACCATGACAG------CGCATGC---GGTGACAAGAAGAAG 831
hsp90aa1b       GATGAGGAGGATGACCATGATCATGGCCACGATGGCGCATGTGGGGACAAGAAGAAGAAG 804
hsp90ab1a       GATGACGA----------------------GGAGGACTCCAAAGACAAGGACAAGAAA 792
hsp90ab1b       GATGATGA----------------------GGAGGATTCCAAAGACAAGGACAAGAAG 795
                ** ** **                        *  *    *   *  * *****

hsp90aa1a       AAGAAGAAGAAGATCAAGGAGAAATACATTGACCAGGAGGAGCTGAACAAGACCAAACCC 891
hsp90aa1b       ACGAGGAAGAAGATCAAGGAGAAGTACATTGACCAGGAGGAGCTGAACAAGACCAAGCCC 864
hsp90ab1a       AAGACCAAGAAGATCAAGGAGAAGTACATTGACCAAGAGGAGCTGAACAAGACCAAGCCC 852
hsp90ab1b       AAGACTAAGAAGATCAAGGAGAAGTACATTGACCAGGAGGAGCTGAACAAGACCAAGCCC 855
                * **  ***************** *********** ******************** ***

hsp90aa1a       CTGTGGACCCGTAACCCTGATGACATCACCAACGAGGAGTATGGAGAGTTCTACAAGAGT 951
hsp90aa1b       CTTTGGACTCGTAACCCTGATGACATCACCAACGAGGAGTACGGAGAGTTCCACAAGAGC 924
hsp90ab1a       ATTTGGACCAGAAACCCTGACGACATCACCATGGAGGAGTACGGAGAGTTCTACAAGAGC 912
hsp90ab1b       ATCTGGACCAGGAACCCTGATGACATCACCATGGAGGAGTACGGGGAGTTCTACAAGAGC 915
                 * *****  * ******** **********  ******** ** ****** *******

hsp90aa1a       CTGACCAACGACTGGGAAGAACACCTGGCTGTCAAGCACTTCTCAGTGGAGGGCCAGCTG1011
hsp90aa1b       CTGACCAATGACTGGGAGGAACACCTGACTGTCAAGCATTTCTCAGTGGAGGGCCAGCTG 984
hsp90ab1a       CTGACCAACGACTGGGAGGAACACCTGGCTGTCAAGCACTTTTCAGTGGAGGGCCAGCTG 972
hsp90ab1b       CTGACCAATGACTGGGAGGACCACCTTGCAGTCAAGCACTTCTCAGTGGAGGGCCAGCTG 975
                ******** ******** ** *****  * ******** ** ******************

hsp90aa1a       GAGTTCCGTGCCCTGCTCTTTGTGCCTCGCCGTGCACCCTTTGACCTCTTTGAAAACAAG1071
hsp90aa1b       GAGTTCCGTGCATTGCTCTTTGTGCCTCGCCGTGCACCCTTTGACCTCTTTGAGAACAAG1044
hsp90ab1a       GAGTTCCGTGCTCTCCTCTTTATCCCCCGCCGTGCACCCTTTGACCTCTTTGAGAACAAG1032
hsp90ab1b       GAGTTCCGCGCCCTGCTCTTTATCCCACGCCGCGCGCCCTTTGACCTCTTTGAGAACAAA1035
                ******** **  * ****** * ** ***** ** ***************** *****

hsp90aa1a       AAGAAGAAGAACAATATCAAGCTGTATGTCAGGAGGGTCTTCATCATGGACAACTGTGAT1131
hsp90aa1b       AAGAAGAAGAACAACATCAAGCTGTACGTCAGGAGGGTCTTCATCATGGACAACTGTGAC1104
hsp90ab1a       AAGAAGAAGAACAACATAAAGCTGTATGTCAGGAGGGTCTTCATCATGGACAGCTGTGAG1092
hsp90ab1b       AAGAAGAAGAATAACATCAAGCTGTATGTCAGGAGGGTCTTCATCATGGACAGCTGTGAA1095
                *********** ** ** ******** ************************ ******

hsp90aa1a       GATCTTATCCCTGAGTACCTCAACTTCATCAAGGGTGTGGTGGACTCTGAGGATCTCCCC1191
hsp90aa1b       GATCTGATCCCTGAGTACCTCAACTTCATCAAGGGTGTGGTGGACTCTGAGGATCTCCCC1164
hsp90ab1a       GAGCTCATCCCAGAGTACCTGAATTTTGTGCGTGGTGTGGTAGACTCTGAGGATCTCCCC1152
hsp90ab1b       GAGCTCATCCCGGAGTACCTGAGTAATGTGCGTGGTGTGGTGGACTCTGAGGATCTCCCC1155
                ** ** ***** ******** *      *    ******** ******************
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hsp90aa1a       CTGAACATCTCCAGAGAGATGCTGCAGCAGAGCAAGATCCTCAAGGTGATCCGCAAGAAC1251
hsp90aa1b       CTGAACATCTCCAGAGAGATGCTGCAGCAGAGCAAGATCCTCAAGGTGATCCGCAAGAAC1224
hsp90ab1a       CTGAACATCTCCCGAGAGATGCTGCAGCAGAGCAAGATCCTCAAGGTGATCCGCAAGAAC1212
hsp90ab1b       CTGAACATCTCCCGAGAGATGCTGCAACAGAGCAAGATCCTCAAGGTCATCCGCAAGAAC1215
                ************ ************* ******************* ************

hsp90aa1a       CTGGTCAAGAAGTGTATAGAGCTCTTCACTGAGCTCTCAGAGGACAAAGAAAACTACAAG1311
hsp90aa1b       CTGGTCAAGAAGTGTATAGAGCTTTTCACAGAACTCTCAGAGGACAGAGATAACTACAAG1284
hsp90ab1a       ATCGTCAAGAAGTGTATGGAGCTGTTCGGTGAGCTGGCAGAGGACAGGGAGAACTACAAC1272
hsp90ab1b       ATCGTCAAGAAGTGTATGGAGCTGTTCGGTGAGCTGGCAGAGGACAAGGAAAACTACAAG1275
                 * ************** ***** ***   ** **  *********  ** ********

hsp90aa1a       AAGTACTACGAGCAGTTCTCCAAGAACATCAAGCTGGGGATCCATGAGGACTCTCAGAAC1371
hsp90aa1b       AAGTACTACGAGCAGTTCTCAAAGAACATCAAGCTGGGTATCCATGAGGACTCTCAGAAC1344
hsp90ab1a       AAGTTCTATGATGGCTTCTCCAAGAACCTCAAGCTGGGCATCCACGAGGACTCCCAGAAC1332
hsp90ab1b       AAGTTTTACGATGGCTTCTCCAAGAACCTCAAGCTGGGGATCCACGAGGACTCCCAAAAC1335
                ****  ** **    ***** ****** ********** ***** ******** ** ***

hsp90aa1a       CGTAAGAGGCTGTCAGACATGCTGCGCTACTACACCTCAGCCTCAGGGGACGAGATGGTC1431
hsp90aa1b       CGTAAGAGGCTGTCAGACATGCTGCGCTACTACACCTCAGCCTCAGGGGACGAGATGGTT1404
hsp90ab1a       CGCAAGAAGCTGTCGGAGCTGCTGCGCTACCACAGTTCTCAGTCCGGAGATGAGCTGACC1392
hsp90ab1b       CGCAAGAAGCTGTCCGAGCTGCTGCGCTACCACAGCTCCCAGTCCGGAGATGAGCTGACC1395
                ** **** ****** **  ********** ***  **    ** ** ** *** **

hsp90aa1a       TCCCTCAAAGACTACGTCACACGCATGAAGGAAACCCAGAAACATATCTACTACATCACT1491
hsp90aa1b       TCCCTGAAGGACCACGTCACACGCATGAAGGACACCCAGAAACACATCTACTACATCACT1464
hsp90ab1a       TCCCTCACGGAGTACCTCACCCGCATGAAGGACAACCAGAAATCCATCTATTACATCACT1452
hsp90ab1b       TCCCTCACAGAGTACCTCACCCGCATGAAGGACAATCAAAAATCCATCTACTATATAACC1455
                ***** *  **  ** **** ********** *  ** ***   ***** ** ** **

hsp90aa1a       GGCGAGACCAGAGACCAGGTGGCTAACTCTGCATTCGTGGAACGCCTTCGAAAGGCCGGC1551
hsp90aa1b       GGCGAGACCAAAGACCAGGTGGCCAACTCTGCGTTCGTGGAGCGCCTTCGCAAGGCCGGC1524
hsp90ab1a       GGCGAGAGCAAGGACCAGGTGGCCAACTCTGCCTTTGTGGAGCGTGTGCGCAAGCGAGGA1512
hsp90ab1b       GGTGAGAGCAAGGACCAGGTGGCCAACTCCGCCTTTGTGGAACGTGTGCGCAAGCGTGGC1515
                ** **** **  ********** ***** ** ** ***** **  * ** ***   **

hsp90aa1a       CTGGAAGTAATCTACATGATTGAGCCCATTGATGAGTACTGTGTCCAGCAGCTGAAGGAG1611
hsp90aa1b       CTGGAGGTGATCTACATGATTGAGCCCATTGATGAGTACTGTGTCCAGCAGCTGAAGGAG1584
hsp90ab1a       TTCGAGGTCCTGTACATGACGGAGCCCATTGACGAGTACTGTGTCCAGCAGCTGAAGGAG1572
hsp90ab1b       TTCGAGGTCCTGTACATGACGGAGCCCATCGACGAGTACTGCGTCCAGCAGCTGAAGGAG1575
                 * ** **  * *******  ******** ** ******** ******************

hsp90aa1a       TATGACGGCAAGACCCTTGTCTCTGTGACCAAGGAGGGTCTGGAGCTGCCTGAGGACGAG1671
hsp90aa1b       TACGATGGCAAGAACCTGGTCTCTGTGACCAAGGAGGGTCTGGAGCTGCCTGAGGATGAG1644
hsp90ab1a       TTTGACGGCAAGACCTTGGTCTCCGTGACCAAGGAGGGCCTGGAGCTGCCGGAGGACGAG1632
hsp90ab1b       TTTGATGGTAAAACCCTGGTCTCTGTAACCAAGGAGGGCTTGGAGCTGCCTGAGGATGAG1635
                *  ** ** ** * * * ***** ** ***********  ********** ***** ***

hsp90aa1a       GACATGAAGAAGAGGCATGAAGAGCAGAAATCTCAGTTTGAGAACCTCTGCAAGATCATG1731
hsp90aa1b       GGCATGAAGAAGAGACAGGAAGAGCAGAAGTCTCAGTTTGAGAACCTCTGCAAGATCATG1704
hsp90ab1a       GAGGAGAAGAAGAAGATGGATGAGGACAAGACAAAGTTCGAGAACCTCTGCAAGCTCATG1692
hsp90ab1b       GAGGAGAAGAAGAAGATGGAGGAGGACAAGACGAGGTTTGAGAACCTCTGCAAGCTCATG1695
                *    ********     ** *** * **  *   *** ************** *****

hsp90aa1a       AAGGACATCTTGGAGAAGAAAGTGGAGAAGGTGACAGTGTCCAACCGCCTGGTCTCCTCT1791
hsp90aa1b       AAGGACATCCTGGAGAAGAAAGTTGAGAAGGTGACAGTGTCCAACCGCCTGGTCTCCTCC1764
hsp90ab1a       AAGGAGATTCTGGACAAGAAAGTAGAGAAGGTGACCGTGTCAAACAGGCTGGTGTCGTCG1752
hsp90ab1b       AAGGAGATCCTGGACAAGAAAGTAGAGAAGGTAACTGTGTCAAACAGGCTGGTGTCATCG1755
                ***** **  **** ******** ******** ** ***** *** * ***** ** **
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hsp90aa1a       CCCTGCTGCATCGTGACCAGCACCTATGGCTGGACGGCCAACATGGAGAGGATAATGAAG1851
hsp90aa1b       CCCTGCCGCATCGTGACCAGCACCTACGGCTGGACGGCCAACATGGAGAGGATCATGAAG1824
hsp90ab1a       CCCTGCTGCATTGTGACCAGCACGTATGGCTGGACGGCCAACATGGAGAGGATCATGAAG1812
hsp90ab1b       CCCTGCTGCATCGTGACCAGCACGTATGGCTGGACGGCCAACATGGAGCGCATCATGAAG1815
                ****** **** ********** ** ********************* * ** ******

hsp90aa1a       GCCCAGGCCCTGAGGGACAATTCCACCATGGGCTACATGGCTGCCAAGAAGCACCTGGAG1911
hsp90aa1b       GCCCAGGCCCTAAGGGACAACTCTACCATGGGCTACATGGCTGCCAAGAAGCACCTGGAG1884
hsp90ab1a       GCACAGGCCCTGAGGGACAACTCCACCATGGGCTACATGATGGCCAAGAAACACCTGGAG1872
hsp90ab1b       GCACAGGCCCTGAGGGACAACTCCACAATGGGCTACATGATGGCCAAGAAGCATCTGGAG1875
                ** ******** ******** ** ** ************   ******** ** ******

hsp90aa1a       ATCAACCCAGACCACCCCATCGTGGAGACCCTGAGGCAGAAGGCAGAGGCTGATAAGAAT1971
hsp90aa1b       ATCAACCCAGACCACCCAATCGTGGAGACCCTGAGGAAGAAGGCAGAGGCTGATAAGAAT1944
hsp90ab1a       ATCAACCCGGACCACCCCATCGTGGAGACCCTGAGGCAGAAGGCTGACCTGGACAAGAAT1932
hsp90ab1b       ATCAACCCAGACCACCCCATTGTGGAGACCCTAAGGCAGAAGGCTGACCTGGACAAGAAT1935
                ******** ******** ** ********** *** ******* **    ** ******

hsp90aa1a       GATAAGTCAGTGAAGGACCTGGTCCTTCTGCTGTTTGAAACGGCTCTGTTGTCCTCTGGG2031
hsp90aa1b       GATAAGTCTGTGAAGGACCTGGTCATTCTGCTGTTTGAGACGGCTCTGCTGTCCTCTGGG2004
hsp90ab1a       GACAAGGCGGTGAAGGACCTGGTAATCCTGCTTTTCGAGACCGCTCTCCTCTCCTCTGGC1992
hsp90ab1b       GACAAGGCGGTGAAGGACTTAGTCATCCTATTGTTCGAGACGGCATTGCTCTCTTCGGGC1995
                ** *** * ********* * **  * **  * ** ** ** **  *  * ** ** **

hsp90aa1a       TTCACCTTGGATGACCCTCAGACACACTCCAACCGCATCTACAGAATGATCAAGCTAGGA2091
hsp90aa1b       TTCACCTTGGATGACCCTCAGACACACTCCAACCGAATCTACCGGATGATTAAGCTAGGC2064
hsp90ab1a       TTCAGCCTGGATGACCCTCAAACTCACTCCAACCGCATCTACAGGATGATCAAGCTGGGC2052
hsp90ab1b       TTCAGCCTGGATGACCCCCAGACTCACTCCAACCGCATCTACAGGATGATCAAGCTGGGC2055
                **** * ********** ** ** ********** ****** * ***** ***** **

hsp90aa1a       CTGGGTATTGACGAGGATGACCTGACCCCCGAGGAGCCAACCTTAGCCCCTGTGGAGGA-2150
hsp90aa1b       CTGGGTATTGACGAGGATGAGCAGACCCCTGAGGAGCCAACTTCAGCCCCCGTCGAGGG-2123
hsp90ab1a       CTGGGAATCGATGATGACGAAGTGATCCCTGAGGAGCCCACCTCTGCACCTGCCCCAGAT2112
hsp90ab1b       CTGGGAATCGATGATGATGAAGTGATCCCTGAGGAGCCCACCTCTGCACCCGCCCCAGAT2115
                ***** ** ** ** ** **   ** *** ******** ** *  ** ** *     *

hsp90aa1a       --CATGCCTCCCCTGGAGGGAGACGAGGACACATCCAGGATGGAGGAGGTTGACTAG   2205
hsp90aa1b       --CATGCCTCCACTAGAGGGAGACGAGGACACATCCAGGATGGAGGAGGTTGACTAG   2178
hsp90ab1a       GAGATCCCACCTCTAGAGGGAGATGACGATGCGTCACGCATGGAGGAAGTGGATTAG   2169
hsp90ab1b       GAGATCCCACCTCTAGAGGGAGATGATGACGCTTCACGCATGGAGGAAGTGGATTAG   2172
                   ** ** ** ** ******** ** **  * **  * ******** ** ** ***
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Figure S6. Alignment of the coding sequences for the four cytosolic heat shock protein 90 paralogue cDNAs from Atlantic salmon included in the qPCR studies (hsp90aa1a, hsp90aa1b, hsp90ab1a, and hsp90ab1b). Nucleotide sequences were aligned using the multiple sequence alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Identical nucleotide pairs in all four sequences at a given position are identified by an asterisk (*), and gaps are indicated by a dash (-). The sequences of the primers used in the qPCR studies are highlighted in yellow (forward) and in blue (reverse).

```
hsp70a      ------ATGTCTAAAGGACCAGCAGTAGGCATTGACCTGGGGACCACATACTCCTGTGTG    54
hsp70b      ATGTCATCAGCTAAAGGCCCGTCTATCGGCATTGACCTGGGCACCACCTACTCCTGTGTG    60
hsp70c      ------ATGTCAAAGGGACCAGCAGTTGGCATTGACCTGGGCACCACCTACTCCTGTGTG    54
hsp70d      ------ATGTCTAAGGGACCAGCAGTCGGCATCGATCTCGGGACCACCTACTCCTGTGTG    54
                     * ** ** **  *  * ***** ** ** ** ***** ************

hsp70a      GGCATCTTTCAGCATGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACAGAACCACA    114
hsp70b      GGGGTGTTCCAGCATGGCAAAGTGGAGATCATCGCCAACGACCAGGGCAACAGGACCACA    120
hsp70c      GGTGTGTTTCAGCATGGCAAAGTGGAGATCATAGCCAACGACCAGGGAAACAGGACCACA    114
hsp70d      GGTGTGTTCCAGCATGGCAAGGTTGAAATCATTGCCAACGACCAGGGCAACAGGACCACT    114
            **  * ** *********** ** ** ***** ************** ***** *****

hsp70a      CCCAGCTATGTGGCCTTCACCGACACGGAGAGGCTGATCGGTGACGCCGCCAAGAACCAG    174
hsp70b      CCCAGCTATGTGGCCTTCACAGACACCGAGAGACTCATCGGAGACGCAGCAAAGAACCAG    180
hsp70c      CCCAGTTATGTCGCCTTCACAGACACAGAAAGGCTGATTGGGGACGCAGCCAAGAACCAA    174
hsp70d      CCAAGCTACGTTGCCTTCACTGACTCTGAGAGGCTAATCGGGGATGCTGCTAAGAATCAG    174
            ** ** ** ** ******** *** * ** ** ** ** ** ** ** ** ***** **

hsp70a      GTGGCCATGAACCCCACCAACACTGTGTTCGATGCCAAGCGTCTAATTGGTCGTAAGTTT    234
hsp70b      GTGGCCATGAACCCCAACAACACCGTTTTTGACGCCAAACGCCTGATTGGCCGAAAGTTC    240
hsp70c      GTGGCCATGAACCCCACAAACACAGTGTTTGATGCTAAGCGGCTGATAGGGCGGAAGTTT    234
hsp70d      GTTGCCATGAACCCCTGCAACACAGTATTCGATGCTAAGAGACTGATTGGCCGCAGGTTT    234
            ** ************   ***** ** ** ** ** **  * ** ** ** ** * ***

hsp70a      GATGATGCCGTGGTACAGTCGGACATGAAGCACTGGCCCTTCACAGTGGTGAGCGATGGT    294
hsp70b      AACGATCAGGTCGTGCAAGCCGACATGAAGCACTGGCCCTTCAAGGTGGTCAGCGACGGA    300
hsp70c      GACGACAGTGTCGTCCAGGCAGACATGAAACACTGGCCGTTTACAGTGATCAACGACTCG    294
hsp70d      GATGATGGAGTTGTTCAATCGGACATGAAGCATTGGCCCTTTGAAGTTATCAATGATTCT    294
             * **    ** ** **  * ******** ** ***** **    **  * *  **

hsp70a      GGCAAGCCCAAGATGGAGGTAGAGTACAAGGGAGAGAGGAAGACCTTTTTCCCTGAGGAG    354
hsp70b      GGAAAGCCTAAAGTTCAGGTAGATTACAAAGGTGAGAACAAATCCTTCAACCCAGAGGAG    360
hsp70c      ACACGGCCCAAGGTCCAAGTGGAGTACAAGGGAGAGACCAAGGCCTTCTACCCAGAGGAG    354
hsp70d      ACTCGGCCTAAGCTCCAAGTTGAATACAAAGGCGAGACTAAGTCATTCTACCCAGAAGAA    354
                 *** **  *  * ** ** ***** ** ****  **  * **   *** ** **

hsp70a      GTGTCCTCCATGGTGCTCACCAAAATGAAGGAGATCTCTGAAGCTTATCTGGGCAAGCCA    414
hsp70b      ATCTCCTCCATGGTCCTGGTGAAGATGAGGGAGATCGCTGAGGCTTACCTGGGCCAGAAG    420
hsp70c      ATCTCCTCCATGGTGCTGGTCAAGATGAAGGAGATTGCAGAGGCCTACCTGGGCAAGACT    414
hsp70d      ATTTCATCCATGGTTCTGGTCAAGATGAAGGAGATTGCTGAGGCCTACCTTGGAAAAACT    414
             * ** ******** **    ** **** ******  * ** ** ** ** **  *

hsp70a      GTGAACAATGCTGTCATCACAGTCCCTGCCTACTTCAACGACTCCCAGCGCCAAGCAACC    474
hsp70b      GTGTCCAATGCAGTCATCACAGTCCCTGCCTACTTCAACGATTCACAGAGACAGGCCACT    480
hsp70c      ATAACCAATGCAGTGGTCACTGTGCCAGCTTACTTCAACGACTCTCAGCGCCAGGCCACC    474
hsp70d      GTCAACAATGCAGTTGTTACCGTACCTGCCTACTTCAACGACTCCCAGCGCCAGGCAACC    474
             *   ****** **  * ** ** ** ** ********** ** *** * ** ** **

hsp70a      AAAGATGCTGGAGTGATCTCTGGACTCAACGTCCTGCGCATCATCAACGAGCCCACCGCT    534
hsp70b      AAGGACGCTGGAGTGATCGCTGGGCTGAATGTGCTGAGAATCATCAACGAGCCCACTGCG    540
hsp70c      AAAGATGCAGGGACTATCTCAGGACTCAACGTACTCCGCATCATCAATGAGCCAACTGCT    534
hsp70d      AAAGATGCTGGTACCATCTCGGGGCTGAATGTGCTGCGTATCATCAATGAGCCAACTGCT    534
            ** ** ** **    *** * ** ** ** ** **  * ******** ***** ** **

hsp70a      GCAGCCATCGCCTATGGCCTGGACAAGAAGGTCGGGGGTGAGCGCAACGTCCTGATCTTC    594
hsp70b      GCAGCCATCGCCTACGGCATGGACAAAGGCATGTCCAGGGAACGCAACGTCCTGATTTTT    600
hsp70c      GCTGCTATCGCCTATGGCCTGGACAAGAAGGTGGGAGTGGAGAGAAACGTCCTAATCTTT    594
hsp70d      GCTGCCATTGCCTACGGCTTGGACAAGAAGGTCGGTGCTGAAAGGAATGTCCTTATCTTT    594
            ** ** ** ***** *** *******     *       **  * ** ***** ** **
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hsp70a      GACCTGGGCGGCGGCACCTTTGACGTGTCCATCCTGACCATCGAGGATGGCATCTTTGAG    654
hsp70b      GACCTGGGTGGGGGCACCTTTGACGTGTCCATCCTGACCATCGAGGATGGGATCTTTGAG    660
hsp70c      GACCTAGGCGGAGGTACGTTTGATGTGTCTATCCTGACCATCGAAGACGGGATCTTTGAG    654
hsp70d      GATCTGGGTGGCGGCACCTTTGACGTGTCCATCCTGACCATCGAGGATGGCATCTTTGAG    654
            ** ** ** ** ** ** ***** ***** ************** ** ** *********

hsp70a      GTGAAGTCCACTGCTGGTGACACCCACCTGGGAGGCGAGGACTTCGACAACCGCATGGTC    714
hsp70b      GTGAAGGCCACGGCTGGAGACACTCACCTGGGCGGGGAGGACTTTGACAACCGCCTGGTT    720
hsp70c      GTGAAGTCCACGGCCGGAGACACCCATCTTGGAGGAGAGGACTTCGACAACCGCATGGTC    714
hsp70d      GTCAAGTCCACTGCCGGAGACACCCATCTGGGTGGAGAAGACTTTGACAACCGCATGGTC    714
            ** *** **** ** ** ***** ** ** ** ** ** ***** ********* ****

hsp70a      AACCACTTCATCGGTGAGTTCAAGCGTAAATTCAAGAAGGACATCAGCGGCAACAAGCGT    774
hsp70b      AGTCACTTTGTGGAGGAGTTCAAGAGGAAACACAAGAAGGACATCAGCCAGAACAAGCGG    780
hsp70c      AACCACTTCATCTCTGAGTTCAAGCGCAAATACAAGAAGGACATCAGCGATAACAAGAGG    774
hsp70d      AACCACTTCATCGCGGAGTTCAAGCGCAAGTACAAGAAAGACATCAGCGACAACAAGAGG    774
            *  *****  *    ********* * **   ****** *********   ****** *

hsp70a      GCGGTGCGGCGCCTGCGCACTGCCTGTGAGCGGGCCAAGCGCACCCTCTCCTCCAGCACC    834
hsp70b      GCTCTGAGGAGGCTGAGGACAGCCTGCGAGAGGGCCAAGAGAACACTGTCCTCCAGCTCC    840
hsp70c      GCCGTGCGTCGTCTGCGCACCGCCTGTGAACGTGCCAAGCGCACCCTGTCCTCCAGTACC    834
hsp70d      GCTGTTCGCCGTCTCCGTACCGCATGTGAGAGGGCAAAGCGCACCCTGTCCTCCAGCACC    834
            **  *  *  * **  * ** ** ** **  * ** *** * ** ** ********  **

hsp70a      CAAGCCAGCATCGAGATTGACTCCCTCTATGAGGGTGCCGACTTCTACACCTCCATTACC    894
hsp70b      CAGGCCAGCATTGAGATTGACTCCCTCTTTGAGGGCATCGACTTCTACACCTCCATCACC    900
hsp70c      CAGGCCAGCATTGAGATTGACTCTCTGTATGAGGGCGTCGACTTCTACACCTCCATCACC    894
hsp70d      CAGGCCAGCATCGAGATCGACTCTTTGTACGAGGGAATTGACTTCTACACCTCCATCACC    894
            ** ******** ***** *****  * *  *****    ***************** ***

hsp70a      AGGGCTCGCTTTGAGGAGCTCAACGCTGACCTGTTCCGCGGTACCCTGGAACCCGTGGAG    954
hsp70b      AGGGCTCGTTTTGAGGAGATGTGTTCCGACCTCTTCAGGGGAACCCTGGAGCCTGTGGAG    960
hsp70c      AGGGCTCGCTTTGAGGAGCTGAACGCTGACCTGTTCAGAGGCACCCTGGACCCCGTGGAG    954
hsp70d      AGGGCTCGCTTTGAGGAGCTCAATGCAGACCTTTTCCGTGGCACCCTTGACCCAGTGGAG    954
            ******** ********* *     * ***** *** * ** ***** ** ** ******

hsp70a      AAGTCTATGAGAGATGCCAAAATGGACAAGGCCCAGATACACGACATCGTCCTGGTGGGA   1014
hsp70b      AAAGCCCTCGGGGATGCCAAGATGGACAAGGCCCAAATCCACGACGTCGTCCTGGTCGGA   1020
hsp70c      AAGTCTCTGAGGGACGCCAAGATGGACAAGGCCCAGGTTCACGACATCGTCCTAGTGGGA   1014
hsp70d      AAATCCCTCCGCGATGCCAAGATGGACAAAGCCCAGGTGCACGACATCGTTCTGGTCGGA   1014
            **  *  *  * ** ***** ******** *****  * ****** **** ** ** ***

hsp70a      GGCTCCACACGTATCCCCAAGATCCAGAAGCTTCTGCAGGACTTATTCAATGGCCGAGAC   1074
hsp70b      GGCTCCACCCGGATCCCCAAGGTCCAGAAGCTCCTGCAGGACTTTTTCAACGGCCGAGAG   1080
hsp70c      GGCTCCACACGCATCCCCAAGATCCAAAAGTTGCTGCAGGACTTCTTCAATGGGAAGGAG   1074
hsp70d      GGCTCCACTCGTATCCCCAAGATCCAGAAACTGCTCCAAGATTTCTTCAACGGCAAAGAG   1074
            ******** ** ********* **** **  * ** ** ** ** ***** **    **

hsp70a      CTCAACAAGAGCATCAACCCTGATGAGGCTGTGGCTTATGGTGCCGCTGTGCAGGCTGCC   1134
hsp70b      CTAAACAAGAGCATCAACCCAGACGAGGCGGTGGCCTACGGCGCTGCCATCCAGGCGGCC   1140
hsp70c      CTCAACAAGAGCATCAACCCTGATGAGGCGGTTGCCTATGGTGCAGCTGTTCAGGCGGCC   1134
hsp70d      CTCAACAAGAGCATCAACCCCGACGAAGCTGTGGCGTATGGCGCAGCTGTCCAGGCAGCC   1134
            ** ***************** ** ** ** ** ** ** ** ** ** * ***** ***

hsp70a      ATCTTAGCCGGTGACAAGTCAGAGAACGTGCAGGACCTGCTGCTGCTGGACGTCACACCC   1194
hsp70b      ATCTTGTCTGGCGACAAGTCTGAGAACGTCCAGGACCTGCTGCTGCTGGATGTGGCTCCC   1200
hsp70c      ATCTTGTCCGGAGACAAGTCTGAGAACGTGCAGGACCTGCTGCTGCTGGACGTCACGCCA   1194
hsp70d      ATACTTTCAGGTGACAAGTCTGAGAATGTCCAGGACCTGCTTTTGCTGGACGTCACACCC   1194
            **  *  * ** ******** ***** ** ***********  ******* **  * **
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hsp70a      CTGTCCCTGGGTATTGAGACAGCTGGAGGGGTCATGACCGTGCTCATCAAGAGGAACACC   1254
hsp70b      CTGTCCCTGGGCATCGAAACCGCCGGAGGGGTCATGACCGCCCTGATCAAACGCAACACC   1260
hsp70c      CTGTCCCTGGGCATTGAGACGGCCGGAGGGGTCATGACCGTGCTCATCAAGAGGAACACC   1254
hsp70d      CTGTCCCTGGGTATTGAGACCGCTGGAGGTGTCATGACCGTCCTGATCAAACGTAACACC   1254
            *********** ** ** ** ** ***** **********  ** *****  * ******

hsp70a      ACCATCCCCACCAAGCAGACCCAGACCTTCACAACATACTCAGAAAACCAACCCGGAGTG   1314
hsp70b      ACCATCCCATCCAAACAGACCCAGACCTTCACCACTTACTCCGACAACCAGCCCGGGGTC   1320
hsp70c      ACCATCCCCACCAAGCAGACGCAGACCTTCACAACATACTCAGACAACCAGCCTGGGGTG   1314
hsp70d      ACCATCCCAACCAAGCAGACCCAGACCTTCACCACCTACTCAGACAACCAGCCTGGTGTG   1314
            ********  **** ***** *********** ** ***** ** ***** ** ** **

hsp70a      CTCATCCAGGTGTATGAGGGGGAGAGAGCCATGACCAAGGACAACAATATCCTTGGCAAG   1374
hsp70b      ATGATCCAGGTCTATGAGGGAGAGAGAGCCATGACCAAGGACAACAACCTCCTGGGGAAG   1380
hsp70c      CTCATTCAGGTATATGAGGGGGAAAGAGCCATGACCAAAGACAACAACCTACTGGGGAAG   1374
hsp70d      CTCATTCAGGTGTATGAGGGTGAGAGGGCCATGACCAAGGACAACAACCTGTTGGGCAAG   1374
             * ** ***** ******** ** ** *********** ********  *  * ** ***

hsp70a      TTTGAGCTCACCGGAATCCCCCCTGCCCCCAGGGGCGTCCCCCAGATCGAGGTGACCTTT   1434
hsp70b      TTTGAGCTCTCTGGGATCCCCCCTGCCCCACGAGGAGTCCCTCAGATCGAGGTGACCTTT   1440
hsp70c      TTTGAGTTGTGTGGGATTCCACCAGCCCCCCGGGGTGTGCCTCAGATCGAGGTCACCTTT   1434
hsp70d      TTTGAGCTGACTGGAATCCCCCCTGCACCTCGCGGTGTTCCTCAGATTGAGGTCACATTC   1434
            ****** *    ** ** ** ** ** **  * ** ** ** ***** ***** ** **

hsp70a      GACATTGACGCCAACGGCATCCTCAATGTGTCTGCGGTGGACAAGAGCACCGGCAAGGAG   1494
hsp70b      GACATCGACGCCAACGGCATTCTGAACGTATCAGCGGTGGACAAGAGTACGGGCAAAGAG   1500
hsp70c      GACATTGACGCCAACGGCATCATGAACGTGTCTGCCGCTGACAAGAGCACCGGCAAGGAG   1494
hsp70d      GACATCGATGCTAACGGCATCATGAATGTGTCTGCTGCTGACAAAAGCACTGGGAAGGAG   1494
            ***** ** ** ********  * ** ** ** ** *  ***** ** ** ** ** ***

hsp70a      AACAAGATCACCATCACCAATGACAAAGGACGTCTGACCAAGGAGGACATTGAGCGCATG   1554
hsp70b      AACAAGATCACCATCACCAACGACAAGGGCCGGCTCAGCAAAGAGGATATTGAGAGGATG   1560
hsp70c      AATAAGATCACCATCACCAATGACAAAGGTCGTCTGAGTAAGGAGGACATAGAGCGCATG   1554
hsp70d      AACAAGATCACCATCACCAATGATAAGGGTCGTCTGAGCAAGGAGGACATTGAGCGCATG   1554
            ** ***************** ** ** ** ** ** *  ** ***** ** *** * ***

hsp70a      GTGCAAGAGGCTGACCAGTACAGGGCTGAGGACGAGGCTCAGAAGGAGAAGGTCACAGCT   1614
hsp70b      GTGCAGGACGCTGACAAATACAAAGCTGAGGATGAAGCACAGAGGGAGAAGATAGCAGCC   1620
hsp70c      GTCCAGGAGGCTGAACAGTACAAAGCTGCGGACGATGTCCAGAGGGACAAGGTGGCGTCC   1614
hsp70d      GTCCAGGAGGCTGAGAAGTACAAGTGTGAGGATGATGTGCAGCGTGACAAGGTCTCTTCT   1614
            ** ** ** *****  * ****    ** *** ** *  ***   ** *** *  *  *

hsp70a      AAGAACTCACTGGAGTCCCTGGCCTTCAACATGAAGAGCACCGTGGATGACGAGAAGCTC   1674
hsp70b      AAGAACTCCCTGGAGTCGTACGCCTTCAATATGAAGAGCAGCGTGGAGGACGACAACATG   1680
hsp70c      AAGAATGGCCTGGAGTCATACGCCTTCAACATGAAGTCCACCGTGGAGGACGAGAAGCTC   1674
hsp70d      AAGAACTCACTAGAGTCCTATGCTTTCAACATGAAGTCTACTGTGGAGGATGAGAAACTG   1674
            *****    ** *****    ** ***** ******   *  ***** ** ** **  *

hsp70a      GCGGAAAAGATCAGCCCGGAGGACAAGAAGACCATCGTGGACAAGTGCAACGAAGTGATC   1734
hsp70b      AAAGGCAAGATCAGCGAGGAGGACAAAAAGAAAGTGGTGGACAGGTGCGACCAGACCATT   1740
hsp70c      AAGGGCAAGCTCAGCGACGAGGACAAACAGAAGATCCTGGACAAATGCAATGAGGTCATC   1734
hsp70d      CAGGGAAAGATCAGTGATGAGGACAAGACAAAGATTCTGGAGAAGTGCAACGAGGTCATC   1734
               *  *** ****    ********    *   *  **** *  *** *  *    **

hsp70a      TCCTGGCTGGACAGGAACCAGACAGCAGAGAAGGATGAGTATGAGCACCAGCAGAAGGAG   1794
hsp70b      TCCTGGTTGGAGAACAACCAGCTGGGTGACAAAGAGGAGTATGAGCATCAGCTAAAGGAG   1800
hsp70c      AGCTGGCTGGACAAGAACCAGTCTGCAGAGAAGGAGGAGTTTGAGCACCACCAGAAGGAG   1794
hsp70d      GGGTGGCTGGACAAGAACCAGACTGCTGAGAAGGAAGAGTACGAGCACCACCAGAAGGAG   1794
               *** **** *  ******   *  ** ** ** ****  ***** ** *  ******
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hsp70a      CTGGAGAAGGTGTGTAACCCCATCATTACCAATCTGTATCAGGGTACCGGAGGCCCACCA   1854
hsp70b      CTGGAGAAAGTATGCCAGCCTATAATTACCAAGCTGTACCAGCAGGGAGGGATGCCCACA   1860
hsp70c      CTGGAGAAGGTCTGTAACCCCATCATCACCAAGCTGTACCAGGGTGCTGGGGGGATGCCT   1854
hsp70d      CTGGAGAAGGTGTGCAACCCCATCATCACCAAGCTATACCAGGGTGCAGGTGGGATGCCT   1854
            ******** ** **  * ** ** ** ***** ** ** ***      **        *

hsp70a      GGAGGTATGCCAGGCGGTATGCCAGGCGGTATGCCTGGAGGCTTCCCTGGAGGTGCTGGG   1914
hsp70b      GGTTGCTGTGGAGATCAGG---CACGGACCAGCTCTGGTGCCAGCTCCCAAG--------   1909
hsp70c      GGAGGGATGCCTGGAGGGATGCCTGGAGGGATGCCTGGAGGGATGCCTGGGGGGATGCCT   1914
hsp70d      GGCGGTATGCCTGAGGGCATGGCTGGCGGATTCCCTGGA----------------------   1893
            **  *       *         *  *        ****

hsp70a      GCTGGCT-------------------------------------CTTCCTCTGGTCCAACC   1938
hsp70b      ----------------------------------------------------GCCCAACC   1917
hsp70c      GGAGGGATGCCCGGGGCTGGTGGTGCCGCACCCGGAGGAGGTGGATCCTCTGGACCAACA   1974
hsp70d      ---------------GCTGGTGGTGCTGCTCCTGGAGGTGGTGGATCATCTGGACCAACC   1938
                                                                * *****

hsp70a      ATCGAGGAGGTTGACTAA    1956
hsp70b      ATTGAAGAGGTTGACTGA    1935
hsp70c      ATTGAGGAAGTCGACTAA    1992
hsp70d      ATTGAGGAAGTCGACTAA    1956
            ** ** ** ** **** *
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Figure S7. Alignment of the coding sequences for the four heat shock protein 70kDa-like cDNAs from Atlantic salmon included in the qPCR studies (hsp70a, hsp70b, hsp70c, and hsp70d). Nucleotide sequences were aligned using the multiple sequence alignment tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Identical nucleotides in all four sequences at a given position are identified by an asterisk (*), and gaps are indicated by a dash (-). The sequences of the primers used in the qPCR studies are highlighted in yellow (forward) and in blue (reverse).

Figure S8. Examples of moribund fish from the last two weeks of the experiment at 1°C. All of these fish had similar symptoms: ulcers to the head/jaw (A, B, D, F), fin rot (A, B, C, E) and scale loss (A, B).

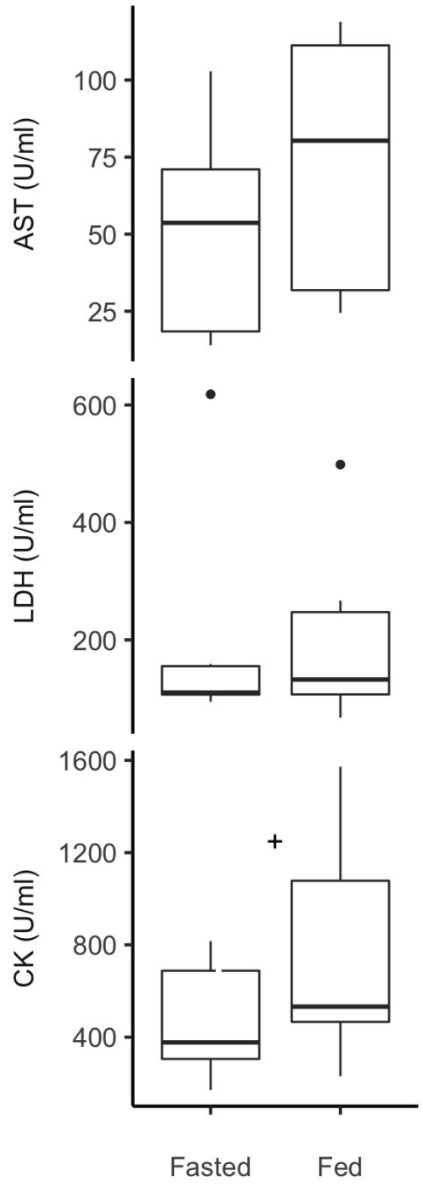

Figure S9. Plasma activity of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) in salmon maintained at 6°C and fasted for 24 h before sampling (Fasted) or sampled at 6 h post-feeding (Fed). + indicates a significant difference at a p value < 0.1. Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. The experiment was conducted at ~6°C and n=9.

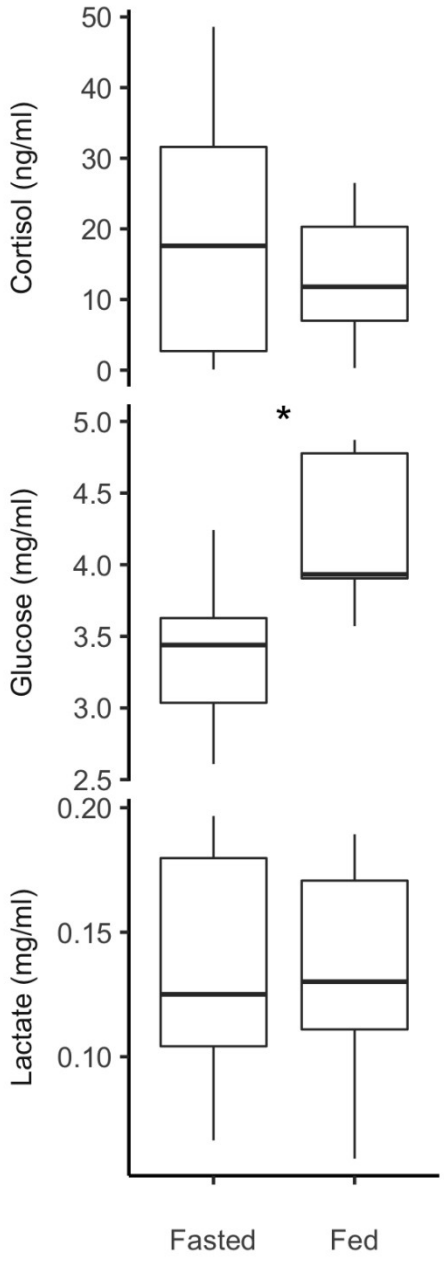

Figure S10. Plasma cortisol, glucose and lactate in salmon maintained at 6°C and fasted for 24 h or sampled at 6 h post-feeding (Fed). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. * Indicates a significant difference at $p < 0.05$. The experiment was conducted at ~6°C and n= 9.

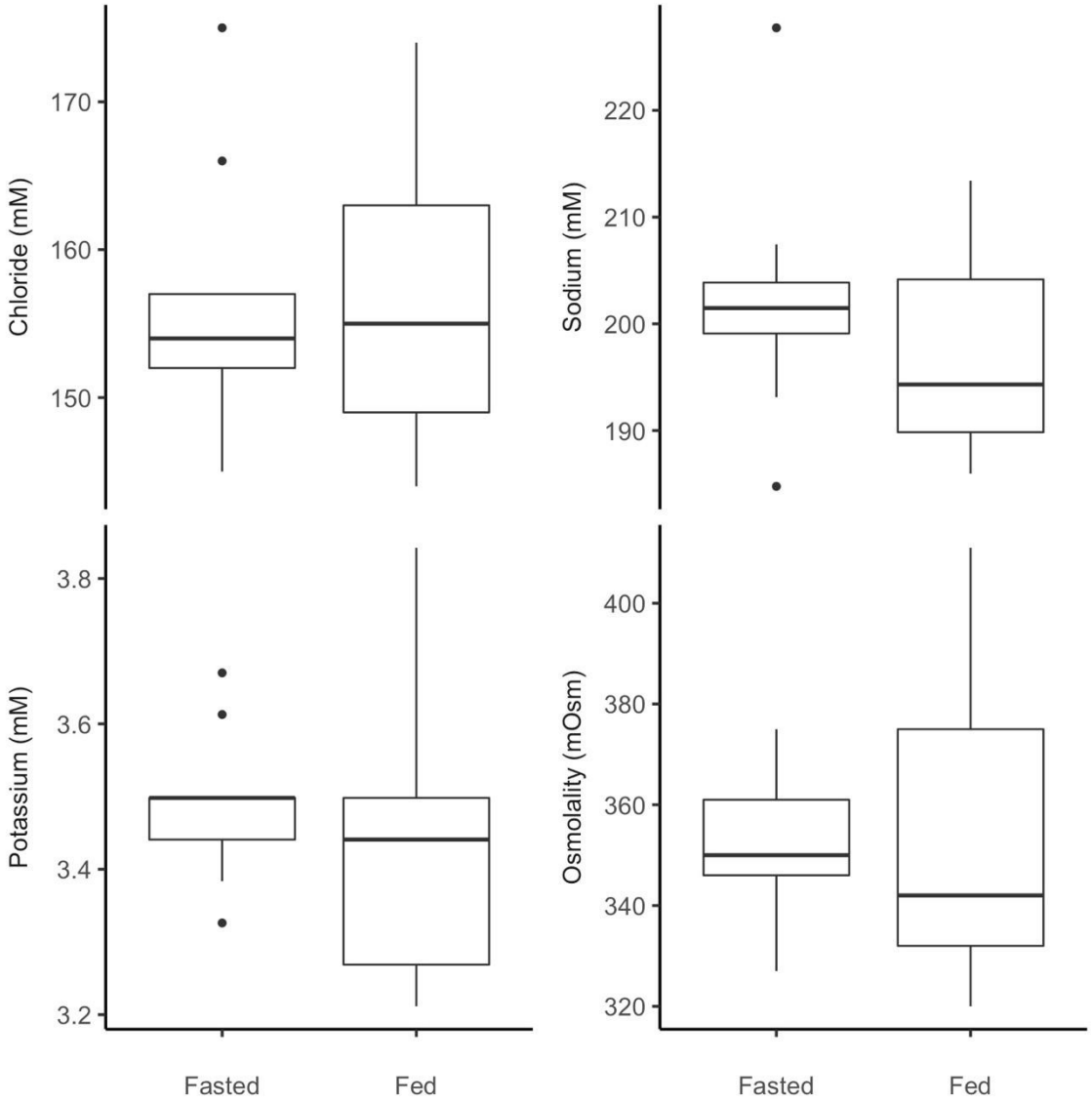

Figure S11. Plasma chloride, potassium, sodium and osmolality values in salmon maintained at 6°C and fasted for 24 h before sampling (Fasted) or sampled at 6 h post-feeding (Fed). Lower and upper box boundaries indicate the 25th and 75th quartiles, respectively, the line inside the box is the median value, and the vertical lines delimit the 10th and 90th percentiles, respectively. Filled circles show data points falling outside the 10th and 90th percentiles. The experiment was conducted at ~6°C and n=9. No values were significantly different between the two groups.

Figure S12. Tank set-up (A, C) for Chapters 3 & 4 including the large capacity glycol chiller (B) used to hold temperatures at 0˚C. The fish were held in three 1000 l-tanks (75 and 35 fish per tank for Experiments #2 and #3 respectively). The tanks were in a flow through configuration and supplied with 32 ppt seawater at 7 l min-1. Each tank was used for a different acclimation group in Experiment #3.

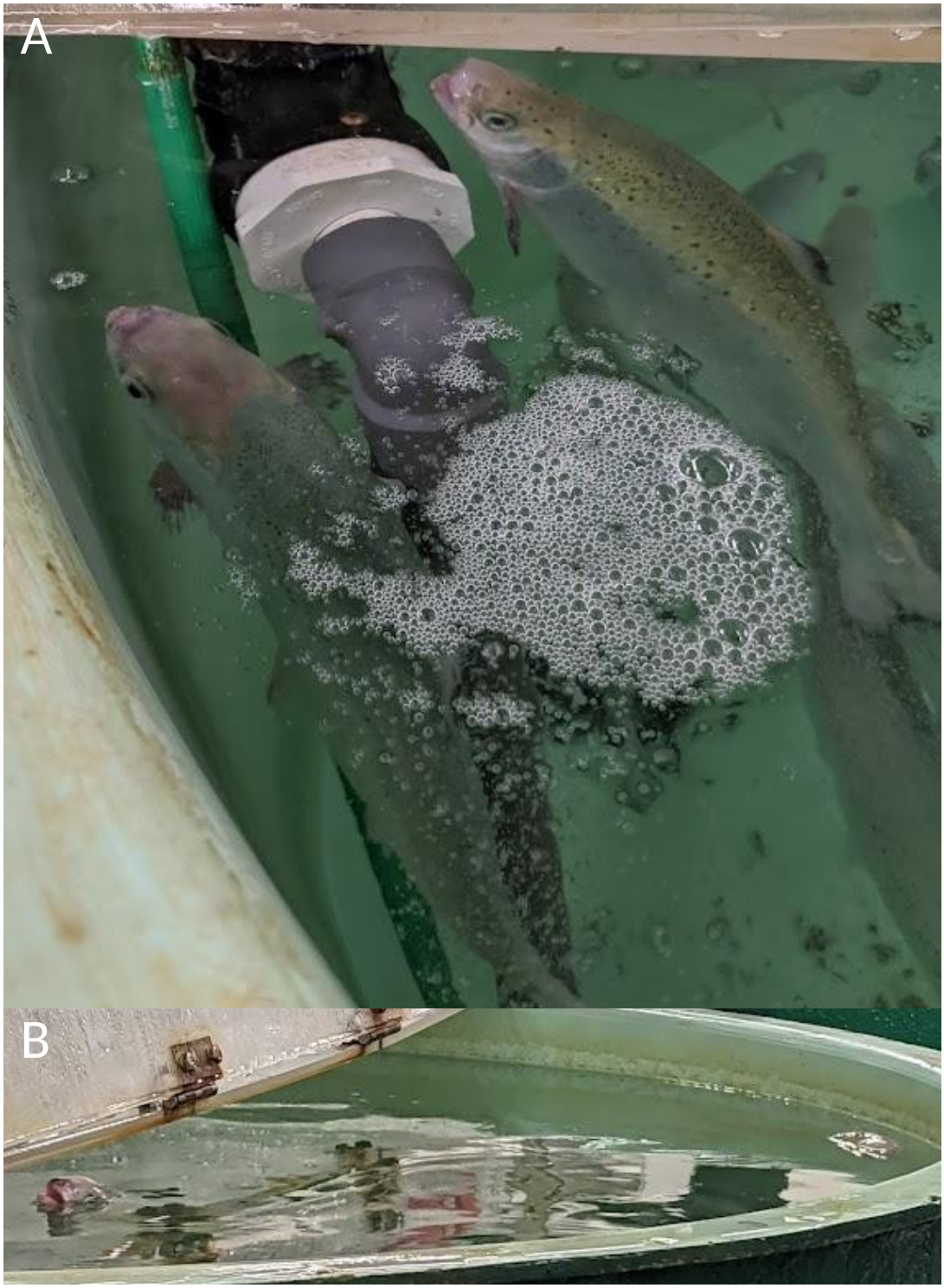

Figure S13. 'Early' symptomatic fish in their experimental tanks. Fish were lethargic / unresponsive to external stimuli, and swimming at the surface of the water (A, B), and often found in a 'tail-down' position (A).

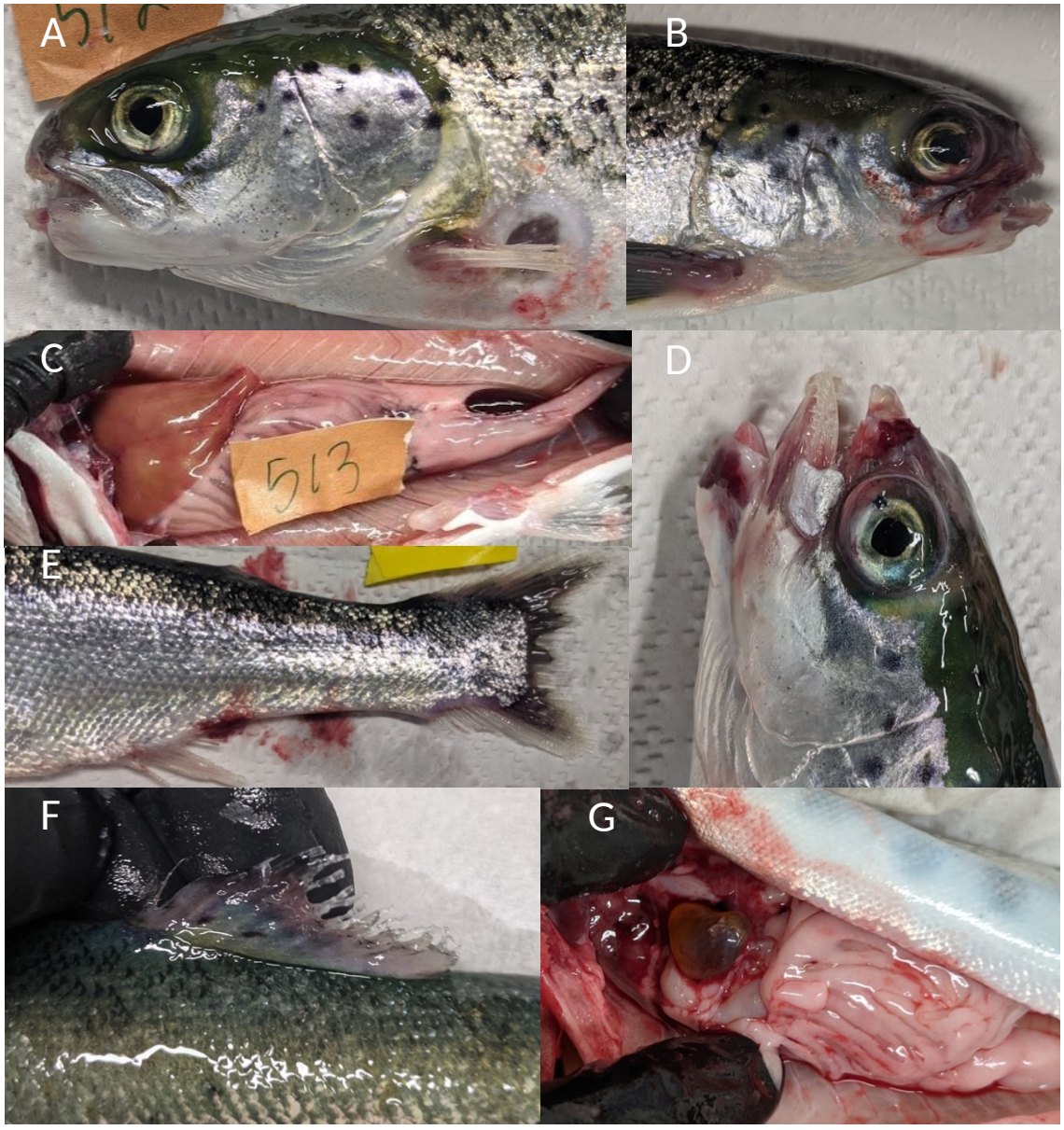

Figure S14. Symptomatic fish with head/jaw ulcerations (B, D), fin rot (A,D and E), and skin lesions (A). When dissected, these fish also had large pale livers (C), ascites, and often an enlarged spleen (G).