Biologgers as a Tool to Investigate the Environmental Tolerances of Fishes

By

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Abstract

To date, a variety of methods have been used to determine the upper thermal tolerance of fishes, and knowledge of the physiology and behaviour of non-polar fishes at cold temperatures is limited. Thus, in this thesis, I used data storage tags (DSTs) to: (1) examine how measures of upper temperature and hypoxia tolerance differ in free-swimming Atlantic salmon as compared to those obtained from fish in respirometers and using a 'rapid screening tool' (performed on anesthetized fish); and (2) understand the impacts of winter temperatures on the behaviour, distribution and physiology of sea-caged salmon. Resting heart rates (f_{H}s) were significantly lower in free-swimming fish, and this resulted in greater values for the scope for f_{H} and for their critical thermal maximum (CT_{max}). There were large differences between the fish's core and water temperatures using the 'rapid screening tool', and this technique greatly underestimated thermal tolerance. These results highlight the limitations of commonly used methods of determining a fish's thermal tolerance, and suggests that scope for f_{H} is the most reliable predictor of this parameter. In Atlantic Canada, sea-caged Atlantic salmon experienced temperatures < 5°C for 5 months during the fall / winter, with a decline of 0.35 – 0.57°C week^{-1} from November (~ 8 – 10°C) to March (~1.1°C). The salmon's f_{H} closely followed water temperature, and while the fish mainly occupied the upper 5 m of the 30 m cage, there were frequent excursions to deeper depths. This novel study validates the use of data loggers for monitoring fish activity and physiology at cold winter temperatures.

General Summary

A fish's body temperature closely resembles water temperature. Thus, temperature extremes are very challenging, and understanding fish thermal biology is critical in the current era of climate change. While the upper thermal tolerance of fishes has received significant attention, the lab-based methods or protocols used vary considerably, and concerns have been raised about how values obtained using these methods reflect those in free-swimming fish both in the wild and in aquaculture sea farms. In addition, despite recent winter mortality events at Atlantic salmon sea-cages, our knowledge of the physiology, behaviour and distribution of these fish during the coldest months remains limited. In this thesis, I used implantable heart rate and temperature data storage tags (DSTs) to compare traditional methods of determining a fish's upper thermal tolerance with measurements on free-swimming fish, as well as monitored sea-caged Atlantic salmon over two fall / winter seasons. In the latter study, depth tags were also attached to assess the fish's behaviour and depth distribution.

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Co-Authorship Statement

The research contained in this M.Sc. thesis was performed by Rebeccah M. Sandrelli under the supervision of Dr. A. Kurt Gamperl. Rebeccah M. Sandrelli shared in the identification of the research topics and in the design of experiments and was primarily responsible for: the rearing and care of experimental animals in Chapter 2; conducting the described procedures and experiments; collecting and analyzing the data; and writing the first draft of all chapters and other sections of this thesis.

Dr. A. Kurt Gamperl: secured the grants which funded the research conducted as part of this thesis; helped identify the research topics and with experimental design; provided supervision and input throughout the experiments and during data collection and analysis; and contributed comments / feedback during the writing of this thesis and associated manuscripts. Dr. Andrew K. Swanson (Cooke Aquaculture Inc.) helped to coordinate the cage-site work and ensured access to fish at cage-sites in both 2020 and 2021 and provided edits for Chapter 3.

From this thesis I expect two manuscripts to be published:

Authorship for the publication derived from **Chapter 2** is: Rebeccah M. Sandrelli and A. Kurt Gamperl. This manuscript is titled 'The upper temperature and hypoxia limits of Atlantic salmon (*Salmo salar*) depend greatly on the method utilized' and was published in the *Journal of Experimental Biology* on September 26, 2023. doi:10.1242/jeb.246227

Authorship for the publication derived from **Chapter 3** is Rebeccah M. Sandrelli, Andrew K. Swanson, and A. Kurt Gamperl. This manuscript is in the final stages of preparation for submission to the journal *Aquaculture*.

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List of Abbreviations and Symbols

3D	Three Dimensional
β	Beta
©	Copyright
°C	Degrees Centigrade
%	Percent
®	Registered Trademark
ABT	Arrhenius Breakpoint Temperature
ANOVA	Analysis of Variance
beats min^{-1}	Beats per Minute (Heart Rate)
bpm	Beats per Minute (Heart Rate)
CAI	Cooke Aquaculture Incorporated
CATC	Centre for Aquaculture Technologies Canada
cm	Centimeter
COM-BOX	Communication Box (Star Oddi)
$\mathrm{CT}_{\mathrm{max}}$	Critical Thermal Maximum
DST	Data Storage Tag
EA	External Acceleration
ECG	Electrocardiogram
Fig.	Figure
f_{H}	Heart Rate
f_{HABT}	Heart Rate at the Arrhenius Breakpoint Temperature
f_{Hcrit}	Oxygen Level When Bradycardia Occurred

f_{Hpeak}	Maximum Heart Rate During Temperature Challenge
f_{Hscope}	Difference Between Heart Rate at 10°C and Maximum Heart Rate
g	Gram
h	Hour
HRV	Heart Rate Variability
Hz	Hertz
Inc.	Incorporated
K	Kelvin
kg	Kilogram
l	Litre
LASCCR	Laboratory for Atlantic Salmon and Climate Change Research
LOE	Loss of Equilibrium
LME	Linear Mixed-Effects Statistical Model
ln	Natural Log
LOE	Loss of Equilibrium
Ltd.	Limited
m	Meter
ml	Milliliter
mg	Milligram
M_b	Body Mass
ms	Milliseconds
MICCSA	Mitigating the Impact of Climate-Related Challenges for Salmon Aquaculture
M.Sc	Master of Science Degree

M_v	Ventricle Mass
MT	Metric Tonnes
NaCl	Sodium Chloride
NS	Nova Scotia
NL	Newfoundland and Labrador
NSERC	National Sciences and Engineering Research Council of Canada
NST	Newfoundland Standard Time
OCLTT	Oxygen- and Capacity-Limited Thermal Tolerance
O_2	Oxygen
PQRS	Cardiac Electrical Activity Associated with Atrial and Ventricular Contraction
PEI	Prince Edward Island
$\dot{Q}$	Cardiac Output
Q-Q plot	Quantile-Quantile Graphical Technique to Determine Normal Distribution
QI	Quality Index
Q_{10}	The Fractional Change in a Rate Over a 10°C Range
$Q_{10preABT}$	The Factional Change in a Rate Up to ABT
R-R interval	Time Elapsed Between Two Successive R-waves in an ECG
RVM	Relative Ventricular Mass
s	Second
SEM	Standard Error of Mean
SST	Sea Surface Temperature

S_V	Stroke Volume
T_{arr}	Temperature at Which Arrythmias Begin
TD	Temperature and Depth
T_{fHpeak}	Temperature at Peak Heart Rate
T_{opt}	Optimum Temperature
TMS	Tricaine Methanesulfonate
T	Temperature
μg	Microgram
VAR	Variance in External Acceleration
VeDBA	Vectoral Sum Dynamic of Body Acceleration

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CHAPTER 1: General Introduction

1.1 Fish Thermal Biology and Climate Change

Temperature has been described as the ‘abiotic master factor’ in fishes (Brett, 1971) as it controls / limits behaviour and physiological functions (Fry, 1947). Given that fish are ectotherms, and that their body temperature is largely dependent on external water temperatures, changes in temperature impact nearly all aspects of their biology, health, and welfare (Alfonso et al., 2021; Brett, 1971; Gamperl et al., 2020; Oppedal et al., 2011; Pörtner and Farrell, 2008; Tromp et al., 2018; Wade et al., 2019). Global sea surface temperatures (SSTs) have increased and are expected to rise by ~1.5°C by the year 2052 (IPCC, 2018; IPCC, 2022), and will have a significant impact on many marine ectotherms, including fishes (Caputi et al., 2016; Callaway et al., 2012; Gamperl et al., 2020; Genin et al., 2020; Pörtner and Knust, 2007; Reid et al., 2019). In addition, the frequency and severity of storms and weather events (Bender et al., 2010) will increase temperature variability (Solomon et al., 2007; Szekeres et al., 2016), and these temperature fluctuations will present challenges for both wild and farmed finfish, including the economically important Atlantic salmon (*Salmo salar*). Fish have a preferred thermal range at which growth and performance are maximum, and large deviations from this range have detrimental impacts, including decreased appetite and growth, increased stress, and temperature-related mortality (Alfonso et al., 2021; Burke et al., 2020; Gamperl et al., 2020; Reid et al., 2019). The optimal rearing temperatures for post-smolt Atlantic salmon are 12-18°C (Handeland et al., 2008; Hevrøy et al., 2013; Jobling, 1981; Sambraus et al., 2018). However, sea-cage temperatures in Atlantic Canada and in Northern Europe vary from ~ 0 to 20°C annually (Burt et al., 2012; Gamperl et al., 2021; Johansson et al., 2006, 2007; Strom et al., 2020), and the frequency of occurrence and duration of high temperature events have, and are expected to, increase with climate change (e.g. Calado et al., 2021; Frölicher et al., 2018; Oliver et al., 2019; Stehfest et al., 2017; Vasseur et al., 2014; Wade et al., 2019).

1.2 Understanding Upper Thermal and Hypoxia Tolerances

Reductions in performance beyond a species-specific thermal optimum (T_{opt}) have been linked to oxygen limitation via the widely recognized, but highly controversial (Clark et al., 2013a, 2013b; Jutfelt et al., 2014; Lefevre, 2016; Norin et al., 2014), oxygen- and capacity-limited thermal tolerance (OCLTT) theory that was originally described by Pörtner (2002, 2010 and Pörtner et al., 2017), but based on results from Fry (1947). Thermal challenges associated with an acute temperature increase are associated with cardiovascular adjustments to promote increased oxygen delivery to the tissues, and thus, limitations in cardiac function are a key determinant of a fish's upper thermal tolerance (Farrell, 2009; Wang and Overgaard, 2007). With regards to meeting the demands of increased temperature, no change or adjustment in cardiac function is more important than an increase in heart rate (f_H). Increases in cardiac output ($\dot{Q}$; the amount of blood pumped by the heart per minute) with temperature are achieved solely by increases in f_H as stroke volume (S_V; the amount of blood pumped per heartbeat) is largely unchanged (Clark et al., 2008; Farrell and Smith, 2017; Gollock et al., 2006; Mendonça and Gamperl, 2010; Steinhausen et al., 2008; Stevens et al., 1972).

Further, as a result of rising water temperatures, coastal upwelling and eutrophication, global oceanic oxygen content continues to decline and regional hypoxic episodes are becoming both more severe and more frequent (Altieri and Gedan, 2015; Breitburg et al., 2018; Sampaio et al., 2021; Schmidtko et al., 2017). This is concerning for fish which inhabit nearly all aquatic habitats and must extract oxygen from the water (Zhang and Farrell, 2022).

Cardiovascular adjustments are critical to a fish's ability to maintain arterial blood oxygen content and to survive at decreased oxygen levels. Hypoxic or reflex bradycardia is a slowing of heart rate, that improves the rate of oxygen diffusion from the water into the blood, and is an important hypoxia-related mechanism in fish (Farrell, 2007; Randall and Shelton, 1963). While the significance of hypoxic bradycardia is not fully understood, several cardiac benefits resulting from this reflex have been proposed, including increased time for diffusion of oxygen into the myocardium, reduced cardiac oxygen demands, increased coronary blood flow during the prolonged diastolic period, and improved cardiac contractility (Farrell, 2007; Joyce et al., 2016; McKenzie et al., 2009). Although it can be difficult to compare between studies given variations in experimental methods, Leeuwis et al. (2019) reported a difference of ~20% air saturation in the hypoxia tolerance of comparable sized Atlantic salmon (*S. salar*) and sablefish (*Anoplopoma fimbria*), with the latter being more hypoxia tolerant. The sensitivity to hypoxia is both species and temperature dependent. (Farrell, 2007; Rogers et al., 2016; Wood, 2018). In fact, recent studies show that while increases in f_H are critical to the survival of fish at high temperatures, the induction of bradycardia at low oxygen levels (i.e., when they are experiencing hypoxia) may in fact reduce their thermal tolerance (Gamperl et al., 2021; Leeuwis et al., 2021)

Given concerns about the effects of accelerated climate change on average ocean temperatures, deoxygenation (hypoxia), and the severity and frequency of heat waves on fish (Lefevre et al., 2021; Little et al., 2020; Pörtner and Knust, 2007; Pörtner et al., 2017; Sinclair et al., 2016; van der Walt et al., 2021), the scientific community has been determining the upper temperature and hypoxia tolerance of many fish species, and examining how these parameters relate to their oxygen consumption and heart function (Anttila et al., 2014;

Casselman et al., 2012; Ern et al., 2016; Hansen et al., 2017; Leeuwis et al., 2019; Mignucci et al., 2021; Motyka et al., 2017; Muller et al., 2020). The most widely used method to determine the acute upper thermal tolerance of fishes is the critical thermal maximum (CT_{max}; Becker and Genoway, 1979) test. This assessment of thermal tolerance involves holding fish at an acclimation temperature, and then heating the water at a constant rate (°C h^{-1}) until loss of equilibrium (LOE); LOE defined as the inability to maintain normal dorsal-ventral orientation (Brauner and Richards, 2020). However, the rate of temperature increase varies greatly in the literature, with values ranging from 18°C h^{-1} (Antilla et al., 2013; Becker and Genoway, 1979; Casselman et al., 2012) to ~1-2°C h^{-1} (Leeuwis et al., 2019; Penney et al., 2014; Zanuzzo et al., 2019); the latter the maximum that would be considered ecologically relevant under typical conditions. Such tests have traditionally been performed in respirometers, with heart function assessed by implanting flow probes around the ventral aorta (Gamperl et al., 2011; Gollock et al., 2006; Keen and Gamperl, 2012; Mendonça and Gamperl, 2010). However, a 'rapid screening tool' for determining the thermal tolerance of fish was recently developed by Casselman et al. (2012). In this test, fish are anesthetized and placed supine in a water bath, injected with atropine (to block cholinergic tone on the heart) and isoproterenol (to ensure maximum adrenergic cardiac stimulation), and f_H is measured using subdermal ECG electrodes as water temperature is increased. However, there have been concerns about how accurately f_H –temperature relationships and indices used to estimate a species' thermal tolerance (Arrhenius breakpoint, ABT; the temperature at maximum heart rate, Tf_{Hpeak}) under these highly controlled / manipulated and unnatural conditions reflect those in free-swimming fishes (Moytka et al., 2017; van der Walt et al., 2021).

Data storage tags (DSTs) have recently been used to determine cardiac breakpoint temperatures in anesthetized Roman seabream, *Chrysoblephus laticeps* (Skeeles et al., 2020) and Cape white sea bream, *Diplodus capansis* (van der Walt et al., 2021) following the procedures used by Casselman et al. (2012), and these studies indicate that there are limitations in using such manipulated conditions for estimating cardiac parameters. Further, Mignucci et al. (2021) recently compared the f_H of gilthead sea bream, *Sparus aurata,* that were implanted with DSTs while free-swimming in a tank vs. recovered in a respirometer. These authors found that fish in the respirometers had higher resting values of f_H and suggested that DSTs (biologging) provide more reliable insights into the cardiac and behavioural responses of fish to environmental stressors. Clearly, more research needs to be conducted in this area so that we can understand the constraints of highly manipulated measurements of fish cardiac function, and how they relate to measures of cardiac function and thus, of thermal and hypoxia tolerance of various species under ‘real world’ conditions. Such information is critical to ensuring that conservation and / or management strategies implemented to protect fishes in nature, or aquaculture management procedures, are based on accurate and reliable information about their thermal biology.

1.3 Impacts of Cold Temperatures

Climate change will also result in more climate variability, and periodic reductions in ocean water temperatures during the winter months (Szekeres et al., 2016). A rapid decline in water temperature decreases the fish’s body temperature, and this can result in a cascade of physiological and behavioural responses characterized by ‘cold shock’ (Donaldson et al., 2008; Liu et al., 2020; Reid et al., 2022). Atlantic Canada’s aquaculture industry has experienced several mass mortality (‘winter chill’) events [i.e., in 2014, 2015, 2019 and 2020 (*Harsh weather linked to die-off at Newfoundland salmon farm*, 2020; Huffman, 2019; Pennel, 2014; The Canadian Press*,* 2015)]; which appeared to have been caused by very low seawater temperatures during winter storms. Although, it is presumed that ‘winter chill’ mortality is associated with below 0°C temperatures which cause blood and tissues to freeze, other factors such as disease and overcrowding (Círíc, 2020) may also be contributing factors. Despite the concern expressed by aquaculture companies and regulatory agencies given the economic and fish welfare impacts of such events, with the exception of two recent papers (Vadboncoeur 2023a, 2023b), there has been very little research on the lethal and sub-lethal consequences of acute or prolonged exposure of Atlantic salmon, or other cultured finfish species, to such temperatures.

Monitoring fish welfare, behavior and physiology at aquaculture sites, particularly in winter, can be challenging given the number of fish in a cage, sea ice cover, rough seas and winter storm events. However, valuable insights can be gained from the long-term, continuous, monitoring of animals that are exposed to natural and aquaculture-related stressors. Recent advancements in the development of DSTs (also known as biologgers) have allowed for the monitoring of a variety of environmental and physiological parameters in aquaculture-reared salmonids including depth, temperature, heart rate and acceleration / swimming speed (Føre et al., 2018; Gamperl et al., 2021). Further, their use has provided valuable information on the stress / physiological responses induced by various aquaculture procedures (e.g., crowding, brailing, transport and euthanasia: Brijs et al., 2018, 2019; Føre et al., 2021; Hvas et al., 2020), and the behavioural and physiological responses of fish (salmon) to extremes in summer water temperatures (Gamperl et al., 2021). However, to date, there have been no investigations on how winter conditions in the North Atlantic affect the physiology and behaviour of salmon in sea-cages. This research has recently been made more feasible by the development of DSTs that can record ECGs for prolonged periods (e.g., at 100 Hz for 15 seconds; https://www.star-oddi.com; Garðabær, Iceland), and thus, are capable of accurately determining heart rate (f_H) at cold temperatures (i.e., at very low heart rates). Further, such research is critical given the recent challenges (mortalities) faced by the salmon aquaculture industry during the winter in Atlantic Canada, and contradicting data / information on how exposure to winter water temperatures affects the physiology and health of Atlantic salmon (e.g., Liu et al., 2020 vs. Sandnes et al., 1988). Understanding the distribution, physiology and behaviour of farmed salmon during periods of cold exposure could provide the industry with valuable information with regard to how to mitigate the impacts of these events on fish health, welfare and production.

1.4 Research Objectives

In the current era of climate change, ocean temperatures are increasing in variability due to more frequent storms, and extreme high and low temperatures will continue to influence aspects of fish distribution, physiology and health. In this thesis, I used DSTs (biologging) to investigate the effects of climate change-related environmental challenges on salmon behaviour and physiology, with the goals of: 1) furthering our understanding of how measures of temperature and hypoxia tolerance determined in lab-based experiments relate to those in free-swimming fishes; and 2) examining how fall / winter temperatures impact the physiology, behaviour and distribution of Atlantic salmon when raised in sea-cages in Newfoundland.

Specifically, in Chapter 2, I compared measures of upper thermal (critical thermal maximum, CT_{max}) and hypoxia tolerance using heart rate (f_H) measurements in free-swimming fish in a tank implanted with a DST and given 4-weeks of recovery, with those: i) fitted with Doppler® flow probes in a respirometer; and ii) exposed to the 'rapid screening tool' of Casselman et al. (2012) which involves anesthetizing and pharmacologically stimulating fish prior to a rapid heating protocol. The latter two protocols have been used to make ecological predictions about the effects of increasing temperatures on fish populations, however, there are concerns about the reliability and accuracy of using these highly manipulative methods, and how values obtained using them relate to those of free-swimming fish. Advances in biologger technology and battery life now enable scientists to record the electrocardiograms (ECGs) and heart rate of free-swimming fish after extended periods (weeks) of post-surgical recovery. Thus, it is likely that physiological data obtained from fish using DSTs (biologging) more accurately represent values that would be expected from fish in the wild or in aquaculture settings.

In Chapter 3, I implanted and attached data storage tags to Atlantic salmon reared in sea-cages in late October / early November in two consecutive years. This allowed for long-term monitoring of Atlantic salmon (*S. salar*) depth distribution, heart rate, temperature, and activity / swimming speed at cage-sites during the winter. These data greatly improve our understanding of how this species is affected by declining water temperatures and several months of exposure to temperatures < 5°C.

These experiments were performed on Atlantic salmon because it is a eurythermal fish whose environmental tolerances have been reported in several studies (e.g., Anttila et al., 2013; 2014; Gallant et al. 2017; Hvas et al., 2017; Leeuwis et al., 2019; Penney et al. 2014), and it is an important aquaculture species world-wide (including Canada) that is experiencing challenging temperatures and hypoxic conditions at cage-sites (Burke et al., 2020; Burt et al., 2012; Círíc, 2020; Gamperl et al., 2021; *Harsh weather linked to die-off at Newfoundland salmon farm*, 2020; Huffman, 2019; Oldham et al., 2017; Oppedal et al., 2011; Pennel, 2014; Stehfest et al., 2017; The Canadian Press*,* 2015).

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CHAPTER 2: The Upper Temperature and Hypoxia Limits of Atlantic Salmon (*Salmo salar*) Depend Greatly on the Method Utilized

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Abstract

In this study, Atlantic salmon were: (i) implanted with heart rate (f_H) data storage tags (DSTs), pharmacologically stimulated to maximum f_H, and warmed at 10°C h^{-1} (i.e., tested using a 'rapid screening tool'); (ii) fitted with Doppler® flow probes, recovered in respirometers and given a critical thermal maximum (CT_{max}) test at 2°C h^{-1}; and (iii) implanted with f_H DSTs, recovered in a tank with conspecifics for 4 weeks and had their CT_{max} determined at 2°C h^{-1}. Fish in respirometers and those free-swimming were also exposed to a stepwise decrease in water oxygen level (100% to 30% air saturation) to determine the oxygen level at which bradycardia occurred. Resting f_H was much lower in free-swimming fish than in those in respirometers (~ 49 versus 69 beats min^{-1}) and this was reflected in their scope for f_H (~104 versus 71 beats min^{-1}) and CT_{max} (27.7 versus 25.9°C). Further, the Arrhenius breakpoint temperature and temperature at peak f_H for free-swimming fish were considerably greater than for those tested in the respirometers and given a 'rapid screening tool' (18.4, 18.1 and 14.6°C; and 26.5, 23.2 and 20.2°C, respectively). Finally, the oxygen level at which bradycardia occurred was significantly higher in free-swimming salmon than in those in respirometers (~62% versus ~53% air saturation). These results: highlight the limitations of some lab-based methods of determining f_H parameters and thermal tolerance in fishes; and suggest that scope for f_H may be a more reliable and predictive measure of a fish's upper thermal tolerance than their peak f_H.

2.1 Introduction

Global sea surface temperatures are expected to rise by ~1.5°C in the next two decades (IPCC, 2022), and this will have a significant impact on many marine ectotherms, including fishes (Gamperl et al., 2020; Genin et al., 2020; Laufkötter et al., 2020; Pinsky et al., 2019; Reid et al., 2019; Viglione, 2021). In addition, an increase in the frequency and severity of storms and weather events (Bender et al., 2010) will increase temperature variability (Frölicher et al., 2018; Solomon, 2007; Szekeres et al., 2016; Viglione, 2021), and these temperature fluctuations present challenges for both wild and farmed fish.

Temperature has been described as the 'abiotic master factor' for fishes (Brett, 1971) as it controls / limits behaviour and physiological functions (Fry, 1947). Given that fish are ectotherms, and that their body temperature is largely dependent on that of the water they live in, changes in temperature impact many aspects of their biology, health, and welfare (Alfonso et al., 2021; Brett, 1971, 1979; Gamperl et al., 2020; Oppedal et al., 2011; Pörtner and Farrell, 2008; Tromp et al., 2018; Wade et al., 2019). Fish have a preferred thermal range at which growth and performance are maximum, and large deviations from this range have detrimental impacts including decreased appetite (and thereby growth), reproductive fitness, increased stress, and temperature-related mortality (Alfonso et al., 2021; Burke et al., 2020; Dahlke et al., 2020; Gamperl et al., 2020; Reid et al., 2019).

Limitations in performance beyond a species-specific thermal optimum (T_{opt}) have been linked to constraints in the capacity of aquatic organisms to meet their oxygen demands, as depicted in the widely recognized, but also controversial (Clark et al., 2013a, 2013b; Jutfelt et al., 2014; Lefevre, 2016; Norin et al., 2014), oxygen- and capacity-limited thermal tolerance (OCLTT) theory originally described by Pörtner (Pörtner, 2002, 2010; Pörtner et al., 2017) but based on results from Fry (1947).With regards to meeting the demands of increased temperature and determining a fish's upper thermal tolerance, an increase in heart rate (f_H) plays a key role in increasing oxygen delivery to the tissues (Farrell et al., 2009; Wang and Overgaard, 2007) as: increases in cardiac output ($\dot{Q}$; the amount of blood pumped by the heart per minute) with temperature are achieved solely by increases in f_H [i.e. stroke volume (S_V), the amount of blood pumped per heart beat, is largely unchanged; Clark et al., 2008; Farrell and Smith, 2017; Gollock et al., 2006; Mendonça and Gamperl, 2010; Steinhausen et al., 2008; Stevens et al., 1972)]; and recent studies suggest that increases in f_H are critical to the survival of fish at high temperatures when water oxygen levels are decreased (i.e. when experiencing hypoxia) (Gamperl et al., 2021; Leeuwis et al., 2021). Indeed, it has been suggested that cardiac (f_H) failure is functionally, and more ecologically, relevant than the temperature at which the fish loses equilibrium (i.e., reaches its critical thermal maximum, CT_{max}) (Sidhu et al., 2014).

Given concerns about the effects of increasing ocean temperatures and heat waves on fish populations (Lefevre et al., 2021; Little et al., 2020; Pörtner and Knust, 2007; Pörtner et al., 2017; Sinclair et al., 2016; van der Walt et al., 2021), the scientific community has been determining the upper thermal tolerance of numerous fish species, and examining how this parameter relates to oxygen consumption and cardiac function (Anttila et al., 2014; Casselman et al., 2012; Hansen et al., 2017; Leeuwis et al., 2019; Mignucci et al., 2021; Motyka et al., 2017; Muller et al., 2020). The most widely used method to determine the acute upper thermal tolerance of fishes is the CT_{max} test (Becker and Genoway, 1979). This test / protocol involves increasing water temperature at a constant rate (°C h^{-1}) from the fish's acclimation temperature until loss of equilibrium (LOE), defined as the inability of fish to maintain normal dorsal–

ventral orientation (Brauner and Richards, 2020). The rate of temperature increase varies greatly in the literature, with values ranging from 18°C h^{-1} (Anttila et al., 2013; Åsheim et al., 2020; Becker and Genoway, 1979; Gallant et al., 2017) to ~1–2°C h^{-1} (Blasco et al., 2020; Motyka et al., 2017; Leeuwis et al., 2019; Penney et al., 2014; Zanuzzo et al., 2019); the latter is the maximum that would be considered ecologically relevant under most conditions (Caissie et al., 2012; Desforges et al., 2023; Richards, 2011; Rodnick et al., 2004). Such tests have often been performed in respirometers so that oxygen consumption can be measured, and heart function can be monitored by implanting flow probes around the ventral aorta (Gamperl et al., 2011; Gollock et al., 2006; Keen and Gamperl, 2012; Mendonça and Gamperl, 2010). However, more recently, a 'rapid screening tool' originally developed by Casselman et al. (2012) has been increasingly used to determine the thermal tolerance of fishes. In this protocol, fish are anaesthetized and placed supine in a water bath, injected with atropine (to block cholinergic tone on the heart) and isoproterenol (to ensure maximum adrenergic cardiac stimulation), and f_H is measured using subdermal electrocardiogram (ECG) electrodes as water temperature is increased rapidly (at 10–18°C h^{-1}). However, there are concerns about how accurately f_H–temperature relationships and indices used to estimate a species' thermal tolerance (e.g., Arrhenius breakpoint temperature, ABT; temperature at peak heart rate, Tf_{Hpeak}; temperature of cardiac arrhythmias, T_{arr}) under these highly controlled / manipulated and unnatural conditions reflect those in free-swimming fishes (Motyka et al., 2017; also see references below).

Data storage tags (DSTs; also called data loggers) have recently been used to determine breakpoints in cardiac function (i.e., f_H) in anaesthetized Roman sea bream (*Chrysoblephus laticeps*; Skeeles et al., 2020) and white sea bream (*Diplodus capansis*; van der Walt et al.,

2021) following the protocol developed by Casselman et al. (2012) and acknowledge that there are limitations in using such manipulated conditions for estimating cardiac parameters. Further, Mignucci et al. (2021) recently compared the f_H of gilthead sea bream (*Sparus aurata*) that were implanted with DSTs while free-swimming in a tank versus recovered in a respirometer. These authors reported that fish in the respirometers had a higher resting f_H and suggested that biologging provides more reliable insights into the cardiac and behavioural responses of fish to environmental stressors. Clearly, more research needs to be conducted in this area so that we can understand the constraints of highly manipulated measurement conditions on fish cardiac function, and how they relate to measures of cardiac function (and thus thermal tolerance) in various species under 'real world' conditions. Such information is critical to ensuring that conservation and management policies implemented to protect fish species in the wild, or strategies to safeguard fish held in aquaculture operations, are based on accurate and reliable information about their thermal biology.

Therefore, the objective of this study was to compare measures of upper thermal (CT_{max}) and hypoxia (the oxygen level at which bradycardia is initiated) tolerance determined using f_H DSTs in free-swimming fish (after 4 weeks of post-surgical recovery) with that of fish: (i) exposed to the 'rapid screening tool' of Casselman et al. (2012); and (ii) fitted with blood flow probes in a respirometer. These experiments were performed on Atlantic salmon because it is a eurythermal fish whose environmental tolerances have been reported in several studies (e.g., Anttila et al., 2013, 2014; Gallant et al., 2017; Hvas et al., 2017; Leeuwis et al., 2019; Penney et al., 2014), global populations are declining (Dadswell et al., 2021; Chaput, 2012; Mills et al., 2013; Nicola et al., 2018), and it is an important aquaculture species that is experiencing elevated temperatures and hypoxic conditions at cage-sites in several regions

(Burke et al., 2020; Burt et al., 2012; Gamperl et al., 2021; Oldham et al., 2017; Oppedal et al., 2011; Stehfest et al., 2017).

2.2 Materials and Methods

This research was approved by the Institutional Animal Care Committee of Memorial University of Newfoundland and Labrador (protocol $^{\#}$21-05-KG) and performed in accordance with the Canadian Council on Animal Care Guidelines on the 'Care and Use of Fish in Research, Teaching and Testing' (https://ccac.ca/Documents/Standards/Guidelines/Fish.pdf).

2.2.1 Fish Husbandry

The Atlantic salmon (*Salmo salar*) used in these studies were age 1+, of Saint John River origin, and originally supplied by Cooke Aquaculture Inc. (Oromocto, NB, Canada) as pre-smolts. These fish were smolted at the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, Memorial University of Newfoundland and Labrador), and held there for ~9 months, prior to being moved to the Laboratory for Atlantic Salmon and Climate Change Research (LASCCR, Ocean Sciences Centre). At the LASCCR, the fish were held in 2.2 m^3 tanks supplied with seawater at 10°C and at ≥100% air saturation and exposed to a 14 h light:10 h dark photoperiod. During this period, they were fed 4 mm Signature Salmon Ration-PW (Northeast Nutrition Inc., Truro, NS, Canada) at a ration of 1% body mass (M_b) day^{-1}. In all experiments mass (g), fork length (cm) and ventricular mass (g) were recorded. The fish used in these experiments averaged 838.2 ± 13.4 g in mass, and 41.6 ± 0.3 cm in length (mean ± SEM).

2.2.2 Experimental Design

Group 1: Rapid screening using anaesthetized fish

Surgery

Prior to implantation, micro-HRT DSTs (8.3 mm×25.4 mm, 3.3 g; Star Oddi, Garðabær, Iceland) were inserted into Star Oddi's communication interface (COM-BOX), and Mercury (v.6.02) software running on a computer was used to program them. The start date and time, sampling intervals and frequencies were all set using this software. The DSTs were set to record f_H (at 100 Hz for 6 s) and temperature (5–45°C; ± 0.2°C) every 15 s for 4 recordings, followed by 1 recording where the ECG was saved, and this continued for the duration of the experiment. Raw ECGs were saved to validate the data collected (i.e., determine R–R intervals and f_H).

Each fish was netted from their tank and anaesthetized in oxygenated seawater containing tricaine methanesulfonate (Syncaine TMS; 0.2 g l^{-1}; Syndel Laboratories Canada, Nanaimo, BC, Canada) until ventilatory movements ceased. The fish were then placed supine on a wetted surgical sponge, and their gills were continuously supplied with ~10°C oxygenated seawater containing a maintenance dose of TMS (0.05 g l^{-1}). A small mid-ventral incision (1.1 ± 0.03 cm), at the posterior limit of the base of the pectoral fins, was made in the fish's body wall using a scalpel. A micro-HRT DST was then inserted into the abdominal cavity (blunt end first) towards the posterior of the fish, and then pulled forward using a suture (2-0) pre-tied at the middle of the DST to within 0.5 cm of the pericardium. A ½ circle, 28 mm, cutting edge needle (SE-MH 28, Mani Surgical Needles, Utsunomiya, Japan) was then used to pass the suture through the body wall and to start to close the incision. Finally, the incision was closed using 3-0 silk sutures: 2–4 interrupted stitches depending on incision length. Vaseline was applied to the incision to prevent water from entering the wound. DST implantation took approximately 5 min; thereafter, the fish was placed supine on a v-board and moved to a shallow water table (50 l) filled with 10°C oxygenated seawater containing a maintenance dose of TMS (0.05 g l^{-1}). The fish were completely submerged in the seawater, and aerated seawater from the bath was constantly pumped over the fish's gills. After f_H and temperature data had been recorded for 1 h, the fish were given sequential injections of atropine sulfate (1.2 mg kg^{-1}) and isoproterenol (4 µg kg^{-1}) (Sigma-Aldrich Canada Co., Oakville, ON, Canada) via the caudal blood vessels to block vagal tone and to maximally stimulate cardiac adrenergic β-adrenoreceptors, respectively (Casselman et al., 2012). Both drugs were dissolved in a 0.9% sodium chloride (NaCl) solution, and frozen at −80°C, prior to use.

Temperature challenge

Thirty minutes following the drug injections, water temperature was increased from the fish's acclimation temperature (10°C) to 28°C at 10°C h^{-1} (Casselman et al., 2012) using a circulating water bath connected to a titanium coil submerged in the seawater. Water temperature was recorded using a PT 100 probe (−30–150°C; resolution: 0.02°C, accuracy: ± 0.5°C) connected to a Firesting O_2 meter and a computer running Pyro Oxygen Logger software (PyroScience GmbH, Aachen, Germany). F_H and the fish's internal temperature were measured using the DSTs. In addition, in a subset of experiments (n=3), a calibrated thermocouple (Model: HHC201, Type K Thermocouple, −100–1372°C, accuracy: 0.1%; Omega Engineering Inc., Norwalk, CT, USA) was placed directly under the liver for the duration of the experiment to validate the DST's values of internal body temperature. Thereafter, the fish were euthanized in 0.4 g l^{-1} TMS. Following euthanasia, the heart was removed from the fish, and the bulbous and atrium were separated from the ventricle. The ventricle was then blotted dry on Kimwipes and weighed. Finally, the DST was retrieved, and the data downloaded.

Hypoxia challenge

No hypoxia challenge was performed on this group. Atropine sulfate blocks vagal tone and, thus, the fish are unable initiate bradycardia / slow their f_{H} when exposed to hypoxic water.

Group 2: Traditional respirometry

Surgery

Each fish was netted from their tank and anaesthetized in oxygenated seawater containing TMS (0.2 g l^{-1}) until ventilatory movements ceased. The fish were placed on a wet foam pad on a surgical table while their gills were irrigated continuously with oxygenated seawater containing a maintenance dose of TMS (0.1 g l^{-1}). Then, the salmon were placed on their right side, and umbilical tape was passed under the gill arches and secured to the surgical table to allow access to the opercular cavity. A small puncture was then made in the skin just below the junction of the second and third gill arches with a pair of sharp-pointed forceps, and the ventral aorta was carefully located by expanding the hole using blunt dissection. Once identified, the ventral aorta was freed from the surrounding tissue using a pair of curved forceps without damaging the pericardium, and a Doppler® flow probe (Model ES Cuff-type Transducer, 20 MHz, Iowa Doppler Products, Iowa City, IA, USA), 1.3 mm in diameter, was fitted around the ventral aorta. Finally, the flow probe lead was connected to a directional pulsed Doppler® flow meter (Model 545C-4; Bioengineering, University of Iowa, Ames, IA, USA) interfaced with a MP100 data acquisition system connected to a laptop computer running Acknowledge (v.3.8.2, BioPac Systems Inc., Goleta, CA, USA) to ensure that the signal was of high quality, and the probe lead was secured to the fish at three locations using 3-0 silk suture: one suture immediately ventral to the pectoral fin, one just below the lateral line and one in front of the dorsal fin.

Upon completion of surgery, individual fish were placed in a ~20 l cylindrical respirometer (20 cm diameter×54.6 cm length) submerged in a shallow (25 cm) water table containing fully aerated seawater at 10°C. The respirometer received a constant flow of water at a rate of 10 l min^{-1} from a submersible pump (model 1048; EHEIM GmbH & Co., Deizisau, Germany). Water in the experimental water table was supplied from a large (~300 l) reservoir whose temperature was controlled by a custom-designed heater / chiller (Technical Services, Memorial University of Newfoundland and Labrador). The fish were allowed to recover / acclimate inside the respirometers for ~20 h (i.e., until the first morning prior to the hypoxia challenge).

Hypoxia challenge

Approximately 20 h following surgery and acclimation to the respirometers, water oxygen levels were decreased step-wise every 60 min until the O_2 level of 30% air saturation was reached (Figure S1). Water air saturation was lowered by bubbling nitrogen gas into the seawater reservoir as controlled using a computer running WitroxCTRL software (Loligo Systems, Viborg, Denmark) that was interfaced with a fibre optic O_2 meter and an O_2 and temperature probe (Loligo Systems). This system controlled two solenoid valves which released air or nitrogen gas into the reservoir as required. Measurements of f_H were taken at every 5% decrease. Heart rate was recorded using the Doppler® flow probe and recording system, as previously described, and f_H (beats min^{-1}) was determined by averaging three sections of 15 consecutive systolic peaks.

After the measurements at 30% air saturation were taken, O_2 in the respirometers was increased to 100% over ~30 min. The fish were left undisturbed in the respirometers for ~36 h at their acclimation temperature (10°C) to recover.

Temperature challenge

An acute upper thermal challenge to the fish's CT_{max} was used to determine the salmon's upper thermal tolerance. This is a standard protocol in the Gamperl lab (see Ern et al., 2016; Leeuwis et al., 2019; Motyka et al., 2017; Norin et al., 2019; Zanuzzo et al., 2019), in which water temperature is increased by 2°C h^{-1} until the fish loses equilibrium (LOE) (Figure S1). F_H was recorded at each 1°C increment. The temperature at which LOE occurred was used as the CT_{max} value. Thereafter, the fish were euthanized with 0.4 g l^{-1} TMS, the heart was removed, and the bulbous arteriosus and atrium were separated from the ventricle. The ventricle was then blotted dry on Kimwipes and weighed. For the hypoxia experiment, f_{Hcrit} was determined as the point at which bradycardia occurred, and ABT, f_{HABT}, $Q_{10preABT}$, f_{Hscope} and f_{Hpeak} were determined as described below.

Group 3: Free-swimming fish with DSTs

Surgery

Prior to implantation, the DSTs (micro-HRT; Star Oddi) were set to record f_H (at 100 Hz for 6 s) and temperature (5–45°C; ± 0.2°C) every 2 h for 24 h prior to the hypoxia challenge and between the hypoxia challenge and thermal challenge, and every 10 min during the hypoxia and thermal challenges. Raw ECGs were saved with every recording to determine R–R intervals (f_H). The micro-HRT tags were implanted in the fish as described above. The fish were then placed into a 2.2 m^3 round tank with ~40 conspecifics (stocking density 18 kg m^{-3}), that was supplied with seawater at 10°C and with ≥100% air saturation in the LASCCR facility for 3 weeks. This duration of recovery was used as Zrini and Gamperl (2021) showed that this post-surgical period is required for resting f_H to return to stable levels. During this period, the fish were hand fed 1× daily at 1% M_b day^{-1} and maintained on a 14 h light:10 h dark photoperiod. Seven days prior to the hypoxia challenge, tagged fish (n=6 per experiment, see below) were moved into a 0.5 m^3 tank and remained in this tank for a week before the hypoxia and thermal challenges.

Hypoxia challenge

The oxygen level was decreased by bubbling nitrogen in the header tank which supplied the tank with 5 l min^{-1} of seawater. A stepwise decrease in oxygen every 60 min was achieved, with steps of 100%, 80%, 70%, 60%, 50%, 40% and 30% air saturation as described above (Figure S1). Temperature and oxygen were continuously monitored using a YSI 5500D MultiDO Optical Monitoring and Control Instrument (Yellow Springs Instruments, Yellow Springs, OH, USA), and a GoPro® camera mounted above the tank was used to monitor the fish in real-time and record behaviour throughout the experiment. Following the last step, oxygen was increased over ∼30 min to ∼100% air saturation, and the fish were then left undisturbed for 36 h.

Temperature challenge

A CT_{max} test was used to determine the fish's upper thermal tolerance. Water temperature was increased by 2°C h^{-1} by increasing temperature in the header tank that supplied the tank with seawater, while maintaining water oxygen levels at ~100% air saturation (Figure S1). As in the hypoxia experiment, a YSI 5500D MultiDO Optical Monitoring and Control Instrument (Yellow Springs Instruments) and a GoPro® camera, were used to monitor the water and fish in real-time, respectively. The temperature at which LOE occurred was recorded as the CT_{max} value. Following LOE, the fish were euthanized with 0.4 g l^{-1} TMS, the heart was removed, and the ventricle was weighed. Then, the DSTs were recovered. Two experiments were conducted, and all fish were exposed to the hypoxia challenge prior to the temperature challenge (n=6 per experiment, n=12 fish total).

The data from the DSTs were downloaded using the COM-BOX and analysed using Mercury software running on a computer. For the first experiment, f_{Hcrit} was determined as the point at which bradycardia occurred, and ABT, f_{HABT}, $Q_{10preABT}$, f_{Hscope} and f_{Hpeak} were determined as described below.

2.2.3 Data Analysis

In these experiments, relative ventricular mass (RVM) was calculated as:

$$RVM = 100 \text{ x } [\frac{M_v}{M_b}] ,$$

where M_v is ventricle mass and M_b is body mass (both measured in grams). The oxygen at which bradycardia occurred (f_{Hcrit}) was determined by plotting f_{H} against air saturation (%) to find the change in slope for each individual. This was determined for each individual using the segmented package (v.1.4-1) in R, which uses a piecewise regression to determine the breakpoint in the relationship. However, f_{Hcrit} could not be determined for 2 fish tested in the respirometers (n=8). The normal exponential increase in f_{H} with temperature becomes discontinuous prior to arrhythmia (Casselman et al., 2012; Somero, 2011). The temperature at which this transition occurs is termed the ABT. ABT and the f_{H} at the ABT (f_{HABT}) were determined for each fish by plotting the natural logarithm of f_{H} against the inverse of temperature (in Kelvin) and finding the point of change in slope using the segmented package (v.1.4-1) in R (Casselman et al., 2012) (e.g., Figure S2). However, ABT could not be accurately determined for 3 fish tested in the respirometers and 5 fish free-swimming in the tank (n=7 for both groups). The Q_{10} for f_{H} was calculated for each individual across the temperature range from 10°C to a temperature just below the ABT ($Q_{10\mathrm{preABT}}$) using the formula:

$$Q_{10} = \left(\frac{f_{H2}}{f_{H1}}\right)^{10/(T_2-T_1)}$$

where f_{H1} and f_{H2} are the heart rate at the first (T_1) and second (T_2) temperatures, respectively. The scope for f_{H} (f_{Hscope}) was calculated as the difference between f_{H} at 10°C and the highest recorded f_{H} (f_{Hpeak}), regardless of temperature. Graphs were created using Prism 9 (www.graphpad.com) and statistical analyses was completed using R (http://www.R-project.org/). Assumptions of normality and homogeneity of variance were performed by visual inspection of Q–Q plots and histograms of residuals. Resting (or 'undisturbed') values for f_{H} were compared between groups using a one-way ANOVA using values at the beginning of sedation or resting measurements (at 10°C and 100% air saturation). Differences in morphometric measurements (RVM, mass, length), CT_{max}, ABT, f_{HABT}, $Q_{10preABT}$, f_{Hscope} and f_{Hpeak} were compared between the experimental groups using one-way ANOVA (Table S1). Unpaired t-tests were used to compare CT_{max}, f_{Hcrit} and f_H at 100% and 30% air saturation for fish tested in the respirometers and free-swimming in the tank (Table S1). Linear mixed-effects models (LME) with temperature / oxygen and treatment (i.e., group) as fixed effects, an interaction term for the two, and fish as a random factor, were used to assess changes in f_H. A linear regression was used to plot the relationship between f_{Hscope} and the temperature at f_{Hpeak} (T_{fHpeak}) for all groups. A linear regression was fitted to the f_H versus temperature relationship of free-swimming fish from 10 to 26°C during the CT_{max} test. The level of statistical significance used was $P<0.05$, and all values in the text, tables and figures are means ± SEM

2.3 Results

2.3.1 Body and Cardiac Morphometrics

There were no differences in fish size (mass or length) between the experiments ($P>0.05$) (Table 2-1). However, the RVM of fish tested using traditional respirometry was significantly higher than that of fish assessed using the 'rapid screening tool' (0.090 ± 0.004% versus 0.077 ± 0.003%, $P<0.05$) (Table 2-1). The RVM of free-swimming fish with implanted DSTs was not significantly different from that of the other treatments ($P>0.05$) (Table 2-1).

Table 2-1. Morphometric, *f*H, and tolerance metrics in the three treatment groups. Dissimilar letters indicate a significant difference ($P < 0.05$) between groups. All numbers are means ± SEM (n = 10 to 12).

	Group 1 'Rapid Screening Tool' n=11	**Group 2** Traditional Respirometry n=10	**Group 3** Free-Swimming with DSTs n=12
Mass (g)	851.7 ± 18.8^{a}	855.8 ± 36.3^{a}	811.2 ± 12.0^{a}
Fork Length (cm)	41.0 ± 0.4^{a}	42.1 ± 0.9^{a}	41.7 ± 0.3^{a}
RVM (%)	0.077 ± 0.003^{a}	0.090 ± 0.004^{b}	0.087 ± 0.003^{ab}
CT_{max}	-	25.9 ± 0.6^{a}	27.7 ± 0.1^{b}
ABT (°C)	14.6 ± 0.2^{a}	18.1 ± 1.6^{ab}	18.4 ± 1.4^{b}
Resting f_H @10°C	69.9 ± 1.1^{a}	68.7 ± 1.7^{a}	48.9 ± 1.9^{b}
f_{Hpeak}	126.0 ± 4.5^{a}	138.4 ± 4.7^{ab}	153.1 ± 4.7^{b}
T_{fHpeak}	20.15 ± 0.4^{a}	23.20 ± 0.7^{b}	26.49 ± 0.4^{c}
f_{Hscope}	57.8 ± 3.8^{a}	70.8 ± 4.0^{a}	104.2 ± 4.6^{b}
$Q_{10preABT}$	2.21 ± 0.05^{a}	1.64 ± 0.20^{b}	1.94 ± 0.10^{ab}
f_H at 100% air sat	-	73.8 ± 17.2^{a}	45.6 ± 2.4^{b}
f_H at 30% air sat	-	36.9 ± 11.2^{a}	25.2 ± 2.2^{b}
O_2 level at Bradycardia	-	52.60 ± 2.62^{a}	62.12 ± 2.40^{b}

RVM, relative ventricular mass; CT_{max}, critical thermal maximum; ABT, Arrhenius breakpoint temperature; f_H, heart rate; f_{Hpeak}, peak heart rate; Tf_{Hpeak}, temperature at peak heart rate; f_{Hscope}, heart rate range from 10°C to peak; $Q_{10preABT}$, temperature coefficient from 10°C to the Arrhenius breakpoint temperature.

2.3.2 Resting f_H

Resting f_H values at 10°C were significantly lower in the free-swimming fish with DSTs (48.9 ± 1.9 beats min^{-1}, $P<0.05$; Table 2-1, Figure 2-1) than in fish assessed using the 'rapid screening tool' and traditional respirometry (69.9 ± 1.1 and 68.7 ± 1.7 beats min^{-1}, respectively; Table 2-1, Figure 2-1).

2.3.3 Acute Hypoxia Challenge

The f_H values for free-swimming fish with DSTs and fish tested in the respirometers remained consistent with values at 100% air saturation until the initiation of bradycardia. The initiation of bradycardia (f_{Hcrit}) was significantly different between groups and occurred at 62.1 ± 2.4% air saturation in free-swimming fish and 52.6 ± 2.6% air saturation in fish tested in the respirometers ($P<0.05$) (Table 2-1, Figure 2-2). The mean f_H in both groups was significantly lower by 45% air saturation than the initial f_H (at 100% air saturation). F_H for fish in the respirometers continued to decline linearly from the initiation of bradycardia until 30% air saturation whereas the f_H of free-swimming fish reached a plateau at ~45% air saturation and remained between 24 and 28 beats min^{-1}, ~40% below initial values. Reduced swimming / movement of free-swimming fish was observed as O_2 level was decreased and many (~50% of fish) struggled to maintain equilibrium at 30% air saturation, whereas there were no observable changes in behaviour for fish tested in respirometers. At 30% air saturation, f_H was significantly lower in free-swimming fish (25.2 ± 2.2 beats min^{-1}) than in fish in the respirometers (36.9 ± 11.2 beats min^{-1}) (Table 2-1, Figure 2-2). The average f_H decreased by ~40% in the free-swimming fish and ~50% for fish in respirometers (by ~20 and 37 beats min^{-1}, respectively) at 30% air saturation as compared with normoxic values.

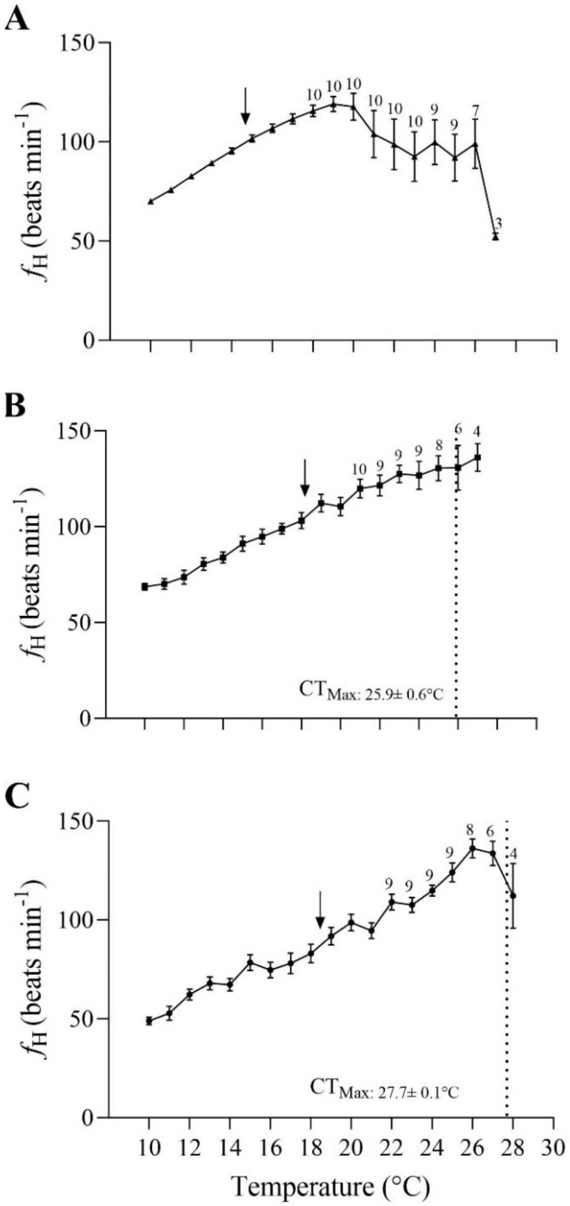

Figure 2-1. Changes in the heart rate (f_H) of Atlantic salmon during a temperature challenge. F_H is shown for (**A**) fish implanted with DSTs and given a 'rapid screening tool' (n=11), (**B**) fish tested using traditional respirometry and implanted with Doppler® flow probes (n=10) and (**C**) free-swimming fish held with conspecifics and implanted with DSTs (n=12). Numbers above the symbols indicate decreased sample size at a specific temperature. The average critical thermal maximum (CT$_{max}$) value (mean ± SEM) is shown using a dotted line in panels B and C. Arrows indicate the Arrhenius breakpoint temperature (ABT). Temperature was increased at 10°C h^{-1} in **A** and at 2°C h^{-1} in **B** and **C**.

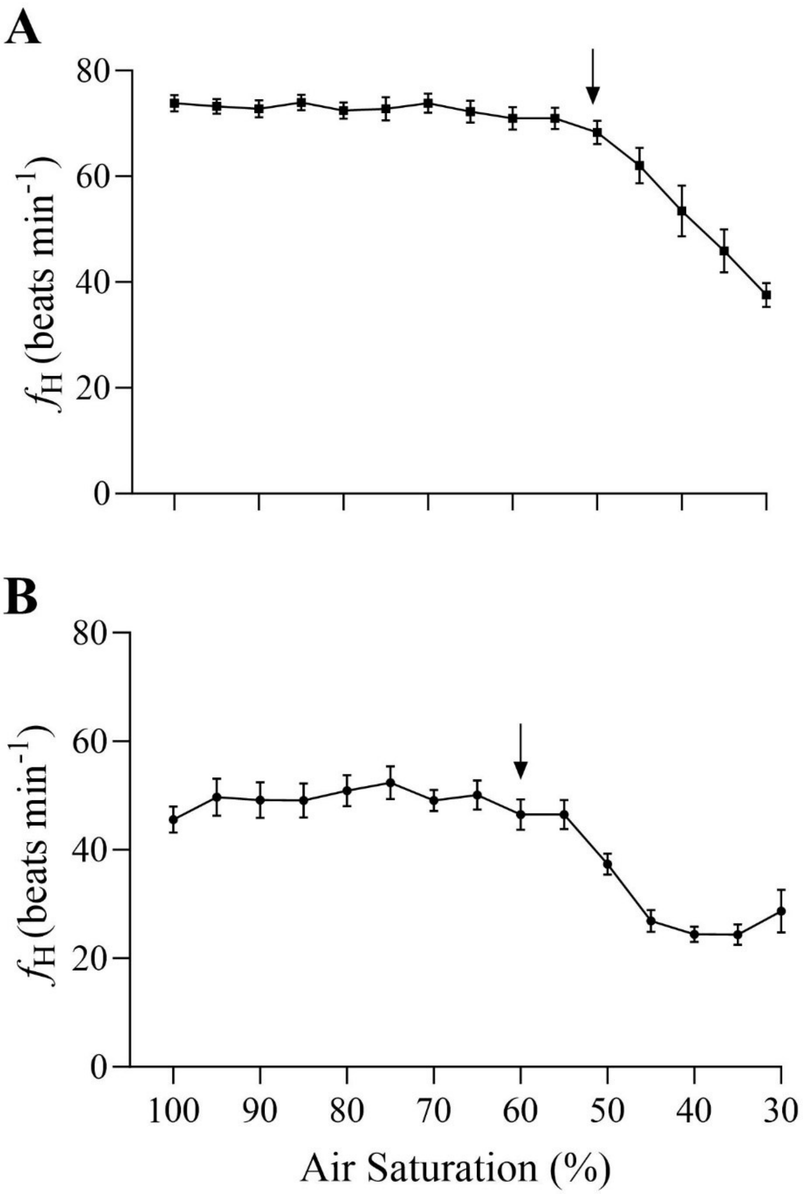

Figure 2-2. Heart rate (f_H) responses to the lowering of water oxygen level from 100% to 30% air saturation. Average f_H values at each 5% decrease in air saturation are plotted for (**A**) fish fitted with Doppler® flow probes and tested using traditional respirometry (n=10) and (**B**) free-swimming fish in a tank with conspecifics and implanted with DSTs (n=12). The arrow indicates the average ABT as determined using individual fish.

2.3.4 Temperature Challenge

The ABT was significantly lower (14.6 ± 0.2°C) using the ‘rapid screening tool’ than when fish were tested in the respirometers and in free-swimming fish (18.1 ± 1.6 and 18.4 ± 1.4°C, respectively) (Table 2-1). The f_{Hpeak} was also significantly lower using the ‘rapid screening tool’ compared with that of free-swimming fish (126.0 ± 4.5 beats min^{-1} versus 153.1 ± 4.7 beats min^{-1}, $P<0.05$) (Table 2-1). In contrast, the temperature at which f_{Hpeak} was reached was significantly different between all groups, with the lowest temperature measured in the ‘rapid screening tool’ (20.2 ± 0.4°C) followed by the fish in respirometers (23.2 ± 0.7°C) and, finally, free-swimming fish (26.5 ± 0.4°C) (Table 2-1). The free-swimming fish also had a significantly higher f_{Hscope} (104.2 ± 4.6 beats min^{-1}) as compared with the fish in respirometers and fish assessed using the ‘rapid screening tool’ (70.8 ± 4.0 and 57.8 ± 3.8 beats min^{-1}, respectively) (Table 2-1). The $\mathrm{CT}_{\mathrm{max}}$ of free-swimming fish was 27.7 ± 0.4°C, and this value was significantly higher than that of fish tested in the respirometers (25.9 ± 0.6°C) (Table 2-1, Figure 2-1). Both fish in respirometers and free-swimming fish in the tank displayed an increase in activity / movement near their $\mathrm{CT}_{\mathrm{max}}$.

The internal (core) body temperature recorded by the DST and the thermocouple was consistently lower than the water temperature during the ‘rapid screening tool’ (temperature increase: 10°C h^{-1}), and this difference was 2.5°C at 28°C (Figure 2-3). In contrast, DST temperature in the free-swimming fish was always consistent with that measured in the water (Figure 2-3).

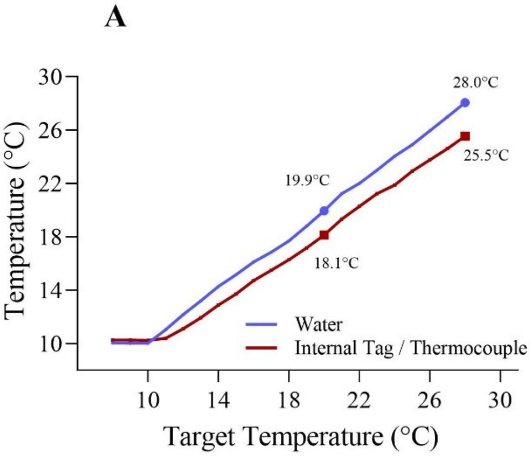

Figure 2-3. Water temperature and internal tag (DST) temperature during the temperature challenge for anesthetized and free-swimming Atlantic salmon. The rate of temperature increase was (**A**) 10°C h^{-1} for anesthetized fish versus (**B**) 2°C h^{-1} for free-swimming fish. In three anesthetized fish, core body temperature was also measured with a calibrated thermocouple. This gave identical data to that recorded by the DST.

2.4 Discussion

To predict (and implement conservation and management strategies to mitigate) the potential impacts of climate change-related environmental challenges on marine fish populations, it is critical that we have accurate data on their sub-lethal and lethal tolerances. It has been suggested that f_{Hpeak} and its associated rate transition temperature can be used to determine the thermal tolerance of fishes, and that rapid measurements of f_{Hpeak} offer functional and ecological insights into the acute upper thermal limits of this taxon (e.g., their CT_{max}) (Ferreira et al., 2014; van der Walt et al., 2021). Thus, the goal of this study was to compare two commonly used methods of determining a fish's f_{H} response to acute warming and their CT_{max} with that measured in free-swimming individuals. Further, this study examined the salmon's f_{H} response to hypoxia and the oxygen level at which bradycardia was initiated in free-swimming fish versus those post-surgery in a respirometer. Overall, the data reveal that the 'rapid screening tool' and measuring f_{H} responses in a respirometer do not provide values for these important parameters that are quantitatively similar / comparable to those in free-swimming fishes. For example: the ABT as determined by the 'rapid screening tool' was ~4°C lower in Atlantic salmon than that determined in a respirometer and in free-swimming fish; the temperature at f_{Hpeak} differed considerably between the three methods (~20, ~23 and ~26.5°C, respectively); the CT_{max} of free-swimming fish was ~1.8°C higher than that measured for fish in a respirometer fitted with a Doppler® flow probe; and the f_{Hcrit} and f_{H} at 30% air saturation were very different in these two groups. These differences are likely due, in part, to the much higher starting f_{H} in the 'rapid screening tool' and respirometer studies, and the reduced scope for f_{H} that this affords. However, the rate of heating / warming, and potential differences between water temperature and the fish's core temperature, must also be considered as potential sources of error / variation.

2.4.1 Resting f_H

Most commonly, measurements to assess environmental tolerances of fishes have been performed within a laboratory setting because of the practicality of manipulating conditions in a controlled manner (e.g., changing temperature or dissolved oxygen) and the requirement of being in close proximity to a data acquisition system and computer to monitor physiological variables. More recently, advancements in bio-logging (DSTs) have enabled the continuous monitoring of several parameters in free-swimming fish, with many users recommending a minimum of 1–2 weeks of post-surgical recovery before starting measurements (Bjarnason et al., 2019; Brijs et al., 2018, 2019; Ekström et al., 2018; Hvas et al., 2020). More specifically, studies have noted that while initial stabilization of f_H takes a minimum of 2–4 days post-surgery, there is a further and more gradual decline in f_H that lasts for up to 3 weeks (Føre et al., 2021; Hvas et al., 2020; Yousaf et al., 2022; Zrini and Gamperl, 2021). Given the short recovery period of fish fitted with Doppler® flow probes, it is not surprising that the f_H for salmon at rest was elevated (by ~20 beats min^{-1}, by 38%) as compared with fish free-swimming in tanks after 4 weeks of recovery. Assessing the contribution of confinement versus surgery to the reported elevation in the f_H of fish in the respirometers was beyond the scope of this study. However, Altimiras and Larsen (2000) compared the resting f_H of rainbow trout (*Oncorhynchus mykiss*) measured in a swim tunnel using biopotentials in the water (i.e., a non-invasive technique) with that of previous studies that used surgical techniques to record this parameter and reported that f_H was much lower using the former method. This latter study suggests that stress associated with short post-surgical recovery (18–66 h) was a major contributor to the much higher f_H in the respirometer-confined salmon in this study. Indeed, Porter et al. (2022) recently measured the plasma cortisol levels of salmon at 24 h post-surgery in a respirometer at 8°C, and these values (mean 47.9 ng ml^{-1}) were much greater than those measured in the same population of fish when quickly sampled from their holding tanks (5–10 ng ml^{-1}; Vadboncoeur et al., 2023b). However, the effects of confinement itself cannot be overlooked. For example, Mignucci et al. (2021) recently reported that gilthead seabream (*Sparus aurata*) implanted with DSTs had a higher f_H than that of free-swimming fish (~75 versus 105 beats min^{-1}, respectively) independent of time post-surgery. Clearly, confined and instrumented fish have substantially elevated f_H and, thus, are questionable to use in estimating resting f_H in free-swimming fishes in the wild or in aquaculture.

2.4.2 Hypoxia Response

The slowing of f_H in response to decreased / low dissolved oxygen levels, known as hypoxic bradycardia, is an important physiological response and potentially aids in the survival of fish that encounter low levels of oxygen (Farrell, 2007; Stecyk, 2017). This reflex response occurs as a result of increased cholinergic nervous tone within the cardiac branch of the vagus nerve of fishes as described by Stecyk (2017). While the significance of hypoxic bradycardia is not fully understood, several cardiac benefits resulting from this reflex have been proposed, including increased time for diffusion of oxygen into the myocardium, reduced cardiac oxygen demands, increased coronary blood flow during the prolonged diastolic period, and improved cardiac contractility (Farrell, 2007; Joyce et al., 2016; McKenzie et al., 2009).

This response of f_H to decreasing dissolved oxygen levels has been studied in other salmonids at similar temperatures (10–12°C; e.g., *Oncorhynchus mykiss*; Holeton and Randall, 1967; Marvin and Heath, 1968; Randall and Smith, 1967), and the onset of bradycardia for this species (~50–65% air saturation) is very similar to what I report for Atlantic salmon in respirometers post-surgery and when free-swimming. Also consistent with this literature is the linear decrease in f_H reported for salmon in the respirometers. The f_H for fish in the respirometers decreased by 50% (30 beats min^{-1}) from the initiation of bradycardia at 52.6 ± 2.6% to 30% air saturation. The response to hypoxia in free-swimming salmon with DSTs differed in two ways as compared with these fish. First, the f_{Hcrit} in the latter group occurred at 62.1 ± 2.4% air saturation, at ~10% higher air saturation than for fish in the respirometers. While there are limited data with which to compare this finding, Mignucci et al. (2021) reported similar results in gilthead seabream (*Sparus aurata*). Mignucci et al. (2021) did not mathematically calculate the breakpoint in the oxygen–f_H response (f_{Hcrit}); however, based on a visual interpretation of Figure 2 in their paper, it appears that there was also an earlier breakpoint (i.e., decrease in f_H) in free-swimming fish as compared with those in respirometers. Second, the decrease in f_H between the oxygen level at the initiation of bradycardia (f_{Hcrit}) and 30% air saturation was not linear in the free-swimming salmon. It decreased by ~24 beats min^{-1} (50%) from 55% to 45% air saturation but did not decrease further. This is unlikely to be a result of the activity level of the free-swimming fish. Although their activity was not quantified in this study, it decreased with the seawater oxygen level, and fish were inactive and just maintaining their position in the water column at the lowest oxygen levels used in this study. This decrease in activity is not a novel finding. For example, Schurmann and Steffensen

(1994) reported that Atlantic cod (*Gadus morhua*) decreased their swimming activity threefold between ~60% and 20% air saturation.

Instead, I propose that this different f_H response between the two groups may be related to the extent of cholinergic tone on the heart of resting fish in respirometers versus free-swimming fish. Cholinergic tone is a major determinant of f_H in fish, initiates bradycardia and has been shown to be affected by several factors (Sandblom and Axelsson, 2011; Wood and Shelton, 1980; Wood et al., 1979). In instrumented Atlantic salmon (confined to a respirometer) at 8°C, cholinergic tone is only 12.4%, and this low tone likely contributed to their higher resting f_H values (59.8 ± 2.6 beats min^{-1}; Porter et al., 2022). It is quite possible that the low f_H in the free-swimming fish in this experiment was mediated, at least partially, by higher cholinergic tone on the heart and, thus, these fish likely had limited scope to decrease f_H as the oxygen level in the water decreased. The hypothesis that the reduction in f_H induced by bradycardia is dependent on resting f_H / cholinergic tone is clearly worth further investigation.

2.4.3 Acute Warming Response

While f_H increased linearly with temperature in all fish, the ABT was substantially lower for fish exposed to the 'rapid screening tool' (14.6 ± 0.2°C) in comparison to the ~18°C measured in both free-swimming fish and those in respirometers. This is a large (and important) difference, and would have been even greater if, as for most previous studies using the Casselman et al. (2012) protocol, I had only measured water temperature. In this study data loggers simultaneously recorded f_H and internal (core) temperature during the 'rapid screening tool', and this temperature differed from that of the water (Figure 2-3), whereas this difference was not seen in the free-swimming fish at a warming rate of 2°C h^{-1}. While the size of the salmon exposed to the ‘rapid screening tool’ (∼800 g) likely contributed to the large difference in core versus water temperature that I report, this overall result / finding is not surprising. A difference of 1–2°C has been reported for small Atlantic cod (*Gadus morhua*; ∼100 g) and a 0.2°C discrepancy has even been reported in zebrafish (*Danio rerio*) (Jutfelt et al., 2019; Morgan et al., 2019). In addition, Sandblom et al. (2016) identified this issue in preliminary studies with perch (*Perca flaviatilis L.*) and reduced the rate of heating in their ‘rapid screening tool’ to 3°C h^{-1} to avoid this issue. These above issues question the use of the ‘rapid screening tool’ (especially at high rates of heating) for determining the ABT of f_H and, thus, its accuracy when estimating the T_{opt} of fishes.

With respect to estimating / determining maximum cardiac values related to upper thermal tolerance, the ‘rapid screening tool’ also gives very different values when compared with those of free-swimming fish implanted with DSTs and given a long (appropriate) period of surgical recovery. In fact, $T_{f\text{Hpeak}}$ was > 6°C lower with the ‘rapid screening tool’ (20.15 ± 0.4 versus 26.49 ± 0.4°C), and this value was ∼7.5°C lower than the CT_{max} of the free-swimming fish implanted with DSTs. This is the first study to directly compare these methods for determining thermal tolerance-related parameters in fishes and raises significant concerns about the use of this protocol for determining peak f_H values (and thus upper temperature tolerance) in fishes, and the suitability of using values derived from this method for predicting the impact of marine heatwaves on the distribution and survival of fishes. This is particularly true given the confidence that I have in the temperature-related data obtained in the free-swimming fish. The temperature–f_H relationship obtained for these fish is extremely similar to that reported by Gamperl et al. (2021) for salmon implanted with DSTs in a commercial sea-cage during a marine heat wave (see Figure 2-4). In comparison to the ‘rapid screening tool’ and the free-swimming fish with DSTs, the data for fish in the respirometers was intermediate. The ABT and f_{H} were comparable to values for the free-swimming fish, whereas $T_{f\mathrm{Hpeak}}$ was ~3.5°C lower.

With regard to what is potentially mediating the difference in f_{Hpeak} (and thus the estimates of upper thermal tolerance that would be derived from them) between these experiments, the data are quite revealing. In the 'rapid screening tool', resting f_{H} following injections of atropine and isoproterenol was artificially elevated (69.9 ± 1.1 beats min^{-1}), and this in addition to a lower f_{Hpeak} (126 ± 4.5 beats min^{-1}) limited the fish's f_{Hscope} (57.8 ± 3.8 beats min^{-1}) as compared with free-swimming conspecifics with DSTs (104.2 ± 4.6 beats min^{-1}). Indeed, if you plot the $T_{f\mathrm{Hpeak}}$ for all three groups versus their f_{Hscope} (Figure 2-5), there is a very strong positive relationship (r^2=0.713) between these two parameters. Collectively, these data highlight the importance of the available scope for f_{H} in determining a fish's upper thermal tolerance, and of eliminating or reducing stress (and therefore resting f_{H}) in fish used in protocols designed to determine their upper thermal tolerance. Further, they emphasize how stressors in the natural environment or in aquaculture operations could result in these animals being more susceptible to acute increases in temperature.

We were not able to measure $\mathrm{CT}_{\mathrm{max}}$ for fish given the 'rapid screening tool' as the salmon were anaesthetized throughout, or confidently determine their T_{arr} (the temperature at which arrhythmias first occur) from the ECG recordings in fish with DSTs as the measurement period of the tags used was only 6 s. However, I did obtain values for $T_{f\mathrm{Hpeak}}$ and $\mathrm{CT}_{\mathrm{max}}$ of the free-swimming salmon and those tested in respirometers. Despite showing no significant difference in f_{Hpeak}, the free-swimming fish had values for $T_{f\mathrm{Hpeak}}$ and $\mathrm{CT}_{\mathrm{max}}$ that were ~3.3 and 1.8°C higher, respectively, than those of fish in the respirometers. That the $\mathrm{CT}_{\mathrm{max}}$ of these two groups were quite similar / comparable is not surprising. Based on a number of studies that have compared CT_{max} between different fish populations / families (Anttila et al., 2013; Bartlett et al., 2022; Ignatz et al., 2023), those acclimated to temperature differences of <10°C (Anttila et al., 2015; Beitinger et al., 2000; Morgan et al., 2019), or exposed to suboptimal conditions (e.g. hypoxia; Anttila et al., 2015; Ern et al., 2016), it appears that CT_{max} displays limited plasticity / variability (~1.5–3.0°C) when values are compared at the same rate of heating. This is likely due the specific mechanisms that determine when a fish loses equilibrium (this parameter defining when a fish has reached its CT_{max}) (Ern et al., 2023).

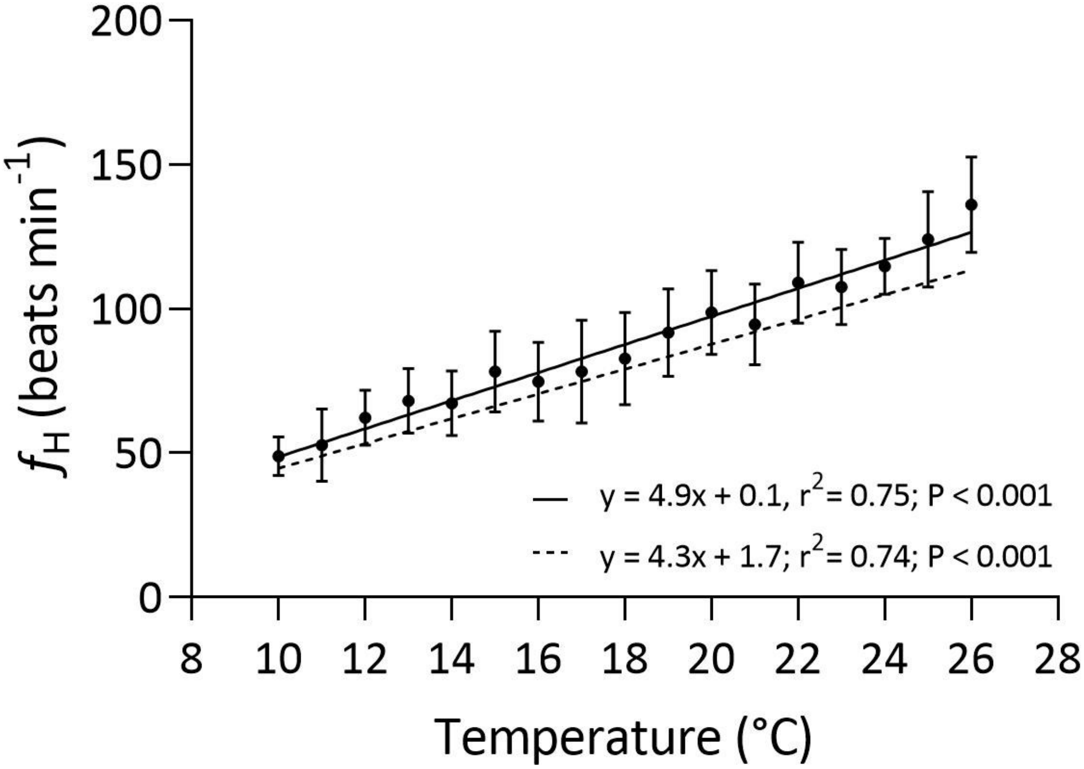

Figure 2-4. The average f_H of free-swimming Atlantic salmon implanted with DSTs during a temperature challenge at 2°C h^{-1} (n=12). The solid line represents the linear regression for this relationship in fish (n=12) in this study (with symbols indicating mean ± SEM). The dashed line is the day-time linear regression reported in Gamperl et al. (2021) for free-swimming fish (n=5) in a commercial sea-cage implanted with f_H DSTs during a summer heat wave in Newfoundland in 2019.

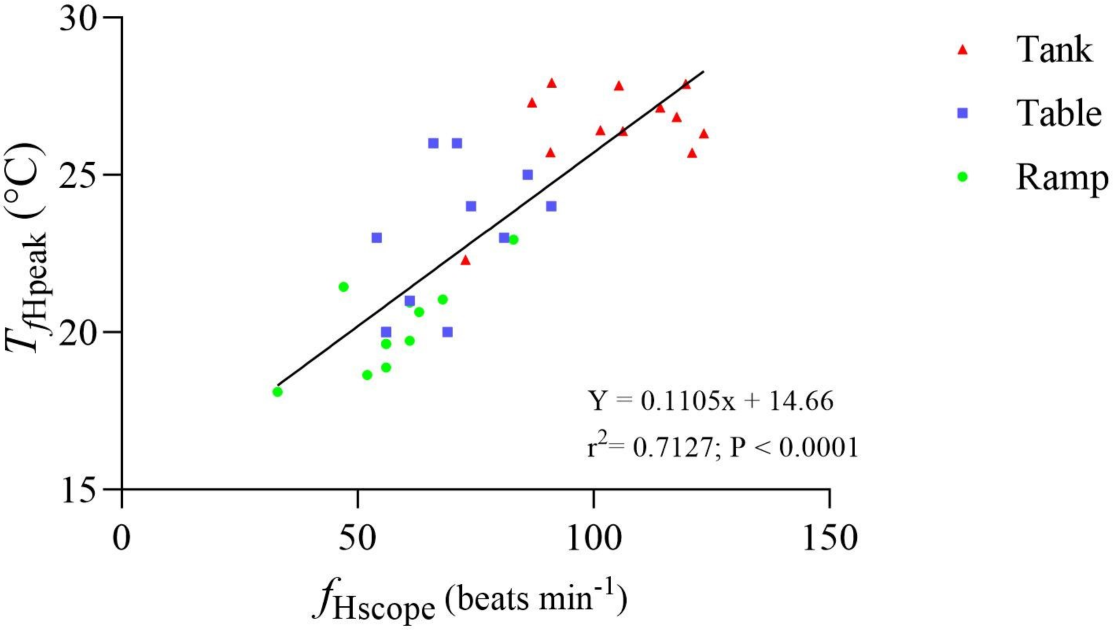

Figure 2-5. Relationship between the scope for heart rate (f_{Hscope}) and the temperature of peak f_{H} ($T_{f\mathrm{Hpeak}}$). The symbols represent each treatment group: circles, fish anesthetized and implanted with DSTs (n=11), squares, fish implanted with Doppler® flow probes and tested using traditional respirometry (n=10), and triangles, free-swimming fish held with conspecifics and implanted with DSTs (n=12).

2.5 Conclusions and Perspectives

There is accumulating evidence that the geographical distribution of aquatic ectotherms can be predicted based on their thermal limits (Payne et al., 2021; Stuart-Smith et al., 2017; Sunday et al., 2012), and that increases in the frequency and severity of temperature extremes (i.e. heat waves and ‘cold shocks’ / ‘cold stress’) will be important determinants of the survival and population strength (biomass) of fish species in the future (Cheung and Frölicher, 2020; Cheung et al., 2021; Genin et al., 2020; Perry et al., 2005; Reid et al., 2022; Szekeres et al., 2016). Thus, obtaining meaningful and accurate values for the thermal limits of various species-specific biological processes will be key to implementing conservation and management strategies (Bates and Morley, 2020; Desforges et al., 2023; McKenzie et al., 2016; Ørsted et al., 2022). This may be particularly true with regard to the impacts of heat waves as recent analyses suggest that the heat failure (mortality) rate of ectothermic species, including fishes, doubles (on average) for every 1°C increase within the stressful temperature range (Jørgensen et al., 2022; Ørsted et al., 2022). While CT_{max} (loss of equilibrium) has been used by a number of authors to estimate the upper thermal tolerance of fishes, the rates of heating used are often faster than ecologically relevant (i.e. > 5°C h^{-1}; Caissie et al., 2012; Desforges et al., 2023; Gilbert and Farrell, 2021; Richards, 2011; Rodnick et al., 2004), and others have suggested that CT_{max} is not the most ecologically relevant measure of a fish’s thermal tolerance (Bartlett et al., 2022; Ignatz et al., 2023). For example, high rates of heating do not allow for the balance in damaging and regenerative (or ‘plastic’) responses that ultimately define critical boundary temperatures (Ørsted et al., 2022). Thus, other protocols and methodologies need to be developed, evaluated and validated, with regard to determining the thermal tolerance of fish to short-term (acute) warming events.

In this first of its kind study, I compared the temperature dependent f_H responses of Atlantic salmon when exposed to temperatures approaching their upper thermal limit using three different protocols / experimental approaches. This study clearly shows that there are major differences in temperature-dependent f_H parameters obtained using the three methods, and that the 'rapid screening tool' greatly underestimated the thermal limits / tolerance of free-swimming Atlantic salmon. This finding is troubling given the number of researchers using this protocol to assess the upper thermal tolerance of fishes. Further, it highlights the rapid screening tool's limitations with respect to determining the upper thermal tolerance of fish in their natural environment and suggests that this protocol should not be used to predict the vulnerability of wild fishes to heat waves (e.g., van der Walt et al., 2021). However, it is not surprising as this protocol (Casselman et al., 2012) was originally designed to examine whether ABT could be used to estimate a species' T_{opt} for aerobic scope, not a species' upper thermal tolerance. Also, the injection of atropine and isoproterenol artificially elevates the fish's initial (resting) f_H and, thus, reduces the available scope to increase f_H – a parameter which our results suggest may be a key factor in determining a fish's environmental tolerances / limits (e.g., see Figure 2-5).

Instead, our results support the conclusion of Mignucci et al. (2021) that biologging on free-swimming fishes provides more ecologically relevant insights into the cardiac and behavioural responses of fish to environmental stressors and suggest that the $T_{f\text{Hpeak}}$ of free-swimming fishes is a good / accurate predictor of the upper thermal tolerance of this taxon. This is based on the extremely similar relationship between f_H and temperatures from 10 to 20°C in this lab-based study and that determined for Atlantic salmon in a large sea-cage recorded during a heat wave in Newfoundland (Gamperl et al., 2021; Figure 2-4), and that the

$T_{f\mathrm{Hpeak}}$ and CT_{max} of salmon in this study were within 1.8°C. In addition, there are several features of DSTs that make them a valuable tool for assessing fish thermal biology going forward. First, the cost of heart rate DSTs is decreasing, and there are DSTs that are capable of simultaneously recording f_H and activity (3D acceleration). These aspects will allow DSTs to be used by a broader range of scientists, and to account for changes in behaviour (e.g., swimming speed: Warren-Myers et al., 2023; Zrini and Gamperl, 2021) when using them to examine aspects of the thermal biology and bioenergetics of fishes. There are also DSTs now available that are capable of recording ECGs for intervals up to 15 s at 100 Hz (as opposed to the 6 s recording limit in the DSTs used in this study) which will make the accurate determination of T_{arr} (although I believe $T_{f\mathrm{Hpeak}}$ is a more ecologically relevant measure of a fish's thermal tolerance) possible. Finally, the environmental tolerances of several individuals can be measured simultaneously. The latter point is important as this allows for data on a large number of fish to be collected in a relatively short period of time, and this was one of the arguments for developing the 'rapid screening tool'.

This, however, is not to say that there are no drawbacks to using this experimental approach. First, DSTs have a limited battery life, they cannot presently be implanted into very small fish (i.e., the mass of a tag should not exceed 2% of fish's body mass (Makiguchi and Kojima, 2017; Wright et al., 2019)) and measuring f_H in free-swimming fishes requires that you have an appropriate place to hold the fish post-implantation. Second, measuring f_H in free-swimming fishes does not allow one to examine the mechanistic basis/es for differences in environment-dependent f_H responses and tolerances between species, populations or as impacted by specific conditions. In this regard, our results suggest that protocols used to measure heart function in fishes, and that allow for the measurement of other physiological parameters (e.g., oxygen consumption) and blood sampling / agent administration, could be modified so that they provide more accurate measures of thermal tolerance. The salmon implanted with Doppler® flow probes had equivalent values for the ABT for f_H, a value for f_{Hpeak} that was not significantly different from that of free-swimming fishes, and a CT_{max} that was only 1.8°C lower as compared with the latter group. It is possible that longer surgical recovery times, and approaches to reduce confinement stress (e.g., Altimiras and Larsen, 2000), could lower the fish's resting f_H and, thus, allow the fish to have a more realistic f_{Hscope} available to meet environmental and other challenges.

Finally, our results highlight the potential interaction between the degree of stress experienced by fishes, and how this influences their capacity to respond to other environmental challenges. With regards to f_H, it has already been shown in sablefish (*Anoplopoma fimbria*) (Leeuwis et al., 2021) and Atlantic salmon (Gamperl et al., in preparation) that the cholinergic-mediated bradycardia induced by hypoxia prevents fish from being able to increase f_H during an acute thermal challenge and reduces their thermal tolerance (CT_{max}). In addition, in this study, I suggest that the low f_H in truly resting (unstressed) fish limits their capacity to reduce f_H when exposed to oxygen-limited conditions (i.e., due to an already high cholinergic tone on the heart), and that stress-induced increases in f_H limit the f_{Hscope} available to deal with rising temperatures and, thus, result in a reduction in upper thermal tolerance. Both of these hypotheses require validation via additional experiments, but potentially add greatly to our understanding of fish cardiorespiratory physiology and the mechanistic basis of environmental tolerances.

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CHAPTER 3: Understanding How Winter Conditions in the North Atlantic Affect the Physiology and Behaviour of Atlantic Salmon in Sea-Cages

This chapter will be submitted to the journal *Aquaculture* as: Rebeccah M. Sandrelli, Andrew K. Swanson and A. Kurt Gamperl. Understanding how winter conditions in the North Atlantic affect the physiology and behaviour of Atlantic salmon in sea-cages.

Abstract

In recent years there have been a number of instances where declines in water temperature are suspected to have contributed to winter mortalities (i.e., 'winter chill') at salmon cage-sites in Atlantic Canada. In addition, it is expected that temperature variability due to more intense and frequent winter storms will increase with climate change and may further threaten Atlantic salmon health and welfare in sea-cages. To better understand cage-site conditions and aspects of salmon behaviour and physiology during the winter, I fitted sea-caged Atlantic salmon in Newfoundland (Canada) in two consecutive years with internal and external data storage tags (DSTs) that recorded depth, temperature, heart rate (f_H) and 3D-acceleration (swimming activity). Tags were surgically attached / implanted in late October / early November when water temperatures were 8-10°C, and the DSTs were collected at the time of harvest. Although, tag retrieval was limited due to some early mortality and problems with fish identification during harvest, the data collected for both years were consistent.

Water temperature decreased at a rate of 0.35 – 0.57°C week^{-1} from October / November to March, and the coolest average weekly temperature experienced by the fish was 1.10°C in early March of both years. Average f_H decreased with temperature starting in the fall, and the fish primarily occupied the upper 5 m of the 30 m deep cage for the duration of this study, despite the fact that temperatures were generally homogeneous with depth. Nonetheless, vertical migrations to deeper waters were observed during some periods (e.g., at night during the coldest months), and thus, the average depth of the fish was greater during these periods.

These data reveal that temperatures in sea-cages in Newfoundland remain below 5°C for ~5 months and are below 2°C for ~1.5 months. Thus, understanding how cold temperatures affect

various aspects of salmon biology, and monitoring these fish during this period, is critical to the sustainability and future of this industry.

3.1 Introduction

With the stagnation of global fisheries landings, the aquaculture industry currently provides approximately half of the world's seafood (FAO, 2022). Further, the global demand for seafood is rapidly rising, and the aquaculture sector will need to increase production levels considerably to meet consumer needs (Cai and Leung, 2017). The Atlantic salmon (*Salmo salar*) is amongst the top five most economically important aquaculture finfish species worldwide (FAO, 2022), and is the main species produced by the Canadian aquaculture industry with production primarily in the provinces of British Columbia, New Brunswick and Newfoundland and Labrador.

In Newfoundland (Canada), production was anticipated to more than double from ~20,000 metric tonnes (MT) to 50,000 MT by 2030 ("Support Growth of the Aquaculture Industry to 50,000 MT Annually for Salmon and 10,750 MT Annually for Mussels – The Way Forward", 2018) by expanding the existing industry (Northern Harvest Sea Farms / MOWI Canada East and Cold Ocean Salmon / Cooke Aquaculture Inc.), and the arrival of Grieg Seafood NL Ltd. in the province. Despite this, production has declined since its peak in 2013 (Government of Newfoundland, 2022) due to a number of challenges including sea lice, viral and bacterial pathogens, and unfavourable environmental conditions such as extreme seasonal temperatures (Barange et al., 2018; Burke et al., 2020; Calado et al., 2021; Falconer et al., 2020, 2022; Gagné and Leblanc, 2018; Islam et al., 2022; Reid et al., 2019).

There has been significant attention devoted to understanding the consequences of climate change on salmon aquaculture and assessing strategies to mitigate these impacts, with the primary foci being rising average sea temperatures, hypoxia (reductions in water oxygen levels), and marine heat waves (Antilla et al., 2015; Beemelmanns et al., 2021a, 2021b; Gamperl et al., 2020, 2021; Ignatz et al., 2023; Stehfast et al., 2017). Surprisingly, however, there has been little effort to understand the impacts of cold temperatures (< 5°C) on salmon physiology until recently (Liu et al., 2020; Porter et al., 2022: Porter and Gamperl, 2023; Tang et al., 2022; Vadboncoeur et al., 2023a, 2023b). This is despite the fact that winter storms are predicted to increase in frequency and intensity (IPCC, 2022), which can lead to episodes where deeper waters mix with cold surface waters resulting in rapid reductions in temperature (Johnson et al., 2018; Szekeres et al., 2016; U.S. Global Change Research Program, 2017). Further, Atlantic Canada's salmon aquaculture industry has experienced several recent 'winter chill' mortality events (i.e., in 2014, 2015, 2019 and 2020 (CBC News, 2020; Huffman, 2019; Pennel, 2014; The Canadian Press*,* 2015)). It has been presumed that 'winter chill' is associated with temperatures below 0°C which cause blood and tissues to freeze, however, other factors such as disease, stress and crowding may also be contributing factors (Círíc, 2020; Vadboncoeur, 2023a, 2023b).

Monitoring fish health, welfare and behaviour at aquaculture sites, particularly in winter, can be challenging given the number of fish in a cage, sea ice cover, rough seas and winter storm events. However, valuable insights can be gained from the long-term, continuous, monitoring of animals that are exposed to natural and aquaculture-related stressors. Recent advancements in the development of biologgers [also known as data storage tags (DSTs)] have allowed for the monitoring of a variety of environmental and physiological parameters in aquaculture-reared salmonids including depth, temperature, heart rate and acceleration / activity (Føre et al., 2018; Gamperl et al., 2021). Further, their use has provided valuable information on the stress / physiological responses induced by various aquaculture procedures (e.g., crowding, brailing, transport, euthanasia; Brijs et al., 2018, 2019; Føre et al., 2021; Hvas et al., 2020) and their behavioural and physiological responses to extremes in summer water temperatures (Gamperl et al., 2021). However, to date, there have been no investigations on how winter conditions in the North Atlantic affect the physiology and behaviour of salmon reared in sea-cages. This research has recently been made more feasible by the development of DSTs that can record ECGs for longer durations (e.g., at 100 Hz for 15 seconds; https://www.star-oddi.com; Garðabær, Iceland), and thus, are capable of accurately determining heart rate (f_H) at cold temperatures (i.e., at low heart rates).

Understanding the distribution, physiology, and behaviour of farmed fish during periods of cold exposure could provide the industry with valuable information regarding how to mitigate the impacts of these events on fish health, welfare and production. I implanted salmon with a Star-Oddi HRT-ACT (that measures heart rate, 3-D acceleration and temperature) DST and externally attached a milli-TD (temperature and depth) DST to record various physiological and behavioural parameters in the fall of 2020 and 2021, through to the spring of the following year.

3.2 Methods

This study was approved by the Animal Care Committee of Memorial University of Newfoundland and Labrador (protocol $^{\#}$20-03-KG), and experimental procedures were performed in accordance with the Canadian Council on Animal Care Guidelines on the ‘Care and Use of Fish in Research, Teaching and Testing’ (https://ccac.ca/Documents/Standards/Guidelines/Fish.pdf).

3.2.1 Location and Fish Husbandry

Farmed Atlantic salmon (*Salmo salar*), originally of St. John River Stock (New Brunswick), were reared in commercial sea-cages ~2 km apart on either side of a fjord in the Bay d'Espoir (Site $^{\#}1$: 2020-2021 and Site $^{\#}2$: 2021-2022; Figure S3) on the south coast of Newfoundland, Canada. Fish were reared using typical aquaculture protocols and stocking densities in circular sea-cages [150 m circumference, 20 m depth (with extended conical bottom; to 30 m)] and fed to satiation daily by an automated feeding system. Mortality dives were conducted weekly by the company. This research was conducted in collaboration with Cold Ocean Salmon, a subsidiary of Cooke Aquaculture Inc. (CAI).

3.2.2 Data Storage Tags (DSTs)

DST Specifications

The externally attached milli-TD DSTs (weight in air, 12 g; diameter, 13 mm; length, 39.4 mm) record temperature (-3 to 40°C; ± 0.1°C accuracy) and depth (0 to 100 m; 0.6% accuracy). The internally implanted milli-HRT ACT DSTs (weight in air, 12 g; diameter, 13 mm; length, 39.5 mm) record ECGs (15 s) and f_{H}, tri-axial acceleration (accurate to ± 32 m-g) and temperature (0 to 45°C; accurate to 0.2°C). Custom ordered milli-HRT ACT DSTs were used at Site $^{\#}1$. These DSTs had centi-HRT housings (larger casing: weight in air, 19 g; diameter, 15 mm; length, 50 mm) and battery, and this increased the battery life of the DST. Time recorded from both tags is accurate to ± 1-minute month^{-1}. All tags were produced by Star-Oddi (Garðabær, Iceland), and combined, did not exceed 2.2% of the salmon's body weight.

DST Programming and Preparation

Prior to implantation, the tags were inserted into Star-Oddi's communication interface (COM-BOX) and connected to a computer. Sea Star (v. 8.55) and Mercury (v. 5.59) (Star-Oddi, Garðabær, Iceland) software were then used to program the milli-TD and milli-HRT ACT DSTs, respectively. The start date, sampling time intervals and frequencies were set using this software. Mercury (v. 5.63) was used to calculate sampling intervals for the milli-HRT ACT-custom DSTs using centi-HRT ACT G2 as the selected recorder type. The DSTs were set to begin recording on October 27th, 2020 (Site $^{\#}$1) and November 1st, 2021 (Site $^{\#}$2) at 12:00:00 AM Newfoundland Standard Time (UTC-3:30). The milli-TD tags were set to record depth and temperature at 5-minute intervals. The milli-HRT ACT custom tags (used at Site $^{\#}$1) were set to record heart rate (f_H) (at 100 Hz for 15 seconds: saving the raw ECG every 6 hours) and temperature and acceleration (at 10 Hz for 600 seconds, saving raw acceleration data every 42 hours) every 1 hour, whereas the milli-HRT ACT tags (used at Site $^{\#}$2) were set to record heart rate (f_H) (at 100 Hz for 15 seconds: saving the raw ECG) every 6 hours, and temperature and acceleration (at 1 Hz for 60 seconds, saving raw acceleration data every 42 hours) every 2 hours.

The milli-HRT ACT DSTs were prepared for implantation by tying two 30 cm pieces of non-sterile 2-0 silk suture around the tag, with the knots secured and stationed directly over the front sensor and directly behind the back sensor so that the loose ends were approximately the same length. The milli-TD DSTs were prepared using the tag holder kit provided by Star-Oddi which includes two plastic molds and two silicone pads. The milli-TD DSTs were prepared for attachment by: looping two pre-sterilized pieces of 30 cm stainless steel (1/8 hard T316L, 0.02” diameter, Malin Co. Brookpark, OH) wire over the tag, and passing the ends of the wire through one of the kit’s silicone pads and the pre-drilled holes in the kit’s plastic mold; and by attaching the DST to one of the plastic molds (which has a cut-out for the tag) using stainless steel wire (see Zrini and Gamperl, 2021). All DSTs were sterilized with 70% ethanol, and the milli-HRT ACT DSTs with attached silk suture were soaked in sterile seawater prior to implantation.

Surgical Implantation / Attachment

On October 26th, 2020, twenty-three pre-adult Atlantic salmon in two sea-cages at Site #1 [average mass of 1.68 ± 0.07 kg (range 1.15 to 2.36 kg) and a fork length of 52.35 ± 0.53 cm (range 47.4 to 57.2 cm)] were implanted with an external DST (n=5) or internal and external DSTs (n=18). On November 1st, 2021, twelve Atlantic salmon in one sea-cage at Site #2 [average mass of 4.66 ± 0.26 kg (range 3.2 to 6.04 kg) and a fork length of 69.63 ± 2.01 cm (range 50.5 to 76 cm)] were implanted with an external DST (n=2) or internal and external DSTs (n=10). Tagging occurred when surface temperatures at the cage-site were ~ 8-10°C.

A box seine (~1.5 m long x 3.5 m wide x 1.5 m deep) attached to the inside border of the sea-cage was used to aid in the netting of fish for tagging, and for holding tagged fish for up to 24 hours post-surgery. Fish were individually netted from the box seine into a fish tote (~1.5 m long x 1.5 wide m x 1.5 m deep) supplied with a continuous flow of seawater. Individual fish were then netted and anesthetized in seawater containing 0.2 g L^{-1} tricaine methanesulfonate (Syncaine TMS, Syndel Laboratories Canada, Nanaimo, British Columbia, Canada), had their mass and fork length recorded after loss of equilibrium, and were immediately transferred to a surgery table where they were placed in a supine (ventral side up) position on a wetted sponge. Throughout the surgery, the salmon's gills were irrigated with seawater containing 0.1 g L^{-1} TMS delivered from an oxygenated reservoir. To begin surgery, lidocaine hydrochloride (Hospira Healthcare Corp. Kirkland, Quebec, Canada) was administered subcutaneously (1 mg kg^{-1}) along the location of the intended incision (dose divided between 3-4 injections). A small mid-ventral incision (~2-3 cm), at the posterior limit of the base of the pectoral fins, was made in the fish's body wall using a scalpel and cotton swabs were used to stop any bleeding at the incision site. A milli-HRT ACT DST was then inserted, blunt end first, towards the posterior of the fish, and then pulled using the attached sutures to within 0.5 cm of the pericardium. A ½ circle, 28 mm, cutting edge needle (SE-MH 28, Mani Surgical Needles, Japan) was then used to pass the silk sutures through the body wall at the front and back of the incision to secure the tag and to start to close the incision. Finally, the incision was closed using 3-0 Prolene® suture (with 24 mm cutting needle) (8684G, J&J Ethicon Suture, San Lorenzo, Puerto Rico) and 4-7 interrupted stitches depending on incision length. Vaseline® was applied to the incision to prevent water from entering the wound during initial recovery / healing.

Immediately following implantation of the milli-HRT ACT, the fish was turned over, and four pre-sterilized stainless steel hypodermic needles (15 gauge, 3.5" long) were passed through the skin and muscle below the dorsal fin to allow the stainless-steel wire of the prepared milli-TD DST to be guided through. Then, the hypodermic needles were removed and the 4 wires exiting the muscle were passed through the kit's other silicone pad and plastic mold. These wires were then twisted together to secure the DST to the fish (see Zrini and Gamperl, 2021).

Following tag insertion and attachment, all fish received an intraperitoneal injection of the antibiotic enrofloxacin (Baytril®, Bayer; 10 mg kg^{-1}), and were recovered in a fish tote with aerated flowing seawater. This antibiotic was administered prophylactically to prevent bacterial infections at the site of the incision. The surgeries and associated procedures took ~15 minutes per fish, and they were closely monitored until they began to swim freely and were then returned to the box seine inside the sea-cage. After 24 hours, the box seine was carefully lowered so that the fish could enter the sea-cage. There were no mortalities in the box seine (i.e., within 24 hours post-surgery).

Tag Retrieval and Analysis

Weekly mortality dives were conducted by cage-site staff. Cages at Site $^{\#}$1 were harvested in June and October of 2021, and Site $^{\#}$2 was harvested in March of 2022. The external DSTs were used to identify experimental animals during harvest and the mortality dives.

Data from the milli-HRT ACT and milli-TD tags were downloaded by putting them into a COM-BOX and using a computer with associated Star-Oddi software. Manual calculations of f_H and heart rate variability (HRV) were performed using the saved ECGs. To calculate f_H, the time between R wave peaks was measured (in seconds), averaged, and then 60 was divided by the average to obtain the fish's f_H in beats minute^{-1} (bpm). HRV was calculated as the standard deviation of the time between successive R wave peaks (in ms). The milli-HRT ACT tag records when the sensor is measuring acceleration above standard gravity and the software calculates external acceleration (EA) as a vectoral sum dynamic of body acceleration, or VeDBA, (measured in m-g at 1 Hz for 60 seconds, where g is the acceleration of gravity or 9.8 m s^{-2}), which is then averaged over 1 minute. In addition, the software performs a 360-degree static calibration on each DST for each of the axes. The Mercury software reports minimum, maximum and average EA values recorded over the 1-minute sampling period. VAR is the variance in EA calculated as the standard deviation squared over a set sampling period (measured in m-g^2 at 1 Hz for 60 seconds). Values of VAR greater than 222 m-g^2 were used to estimate when the fish were not swimming constantly [i.e., they were engaged in burst-coast swimming (Zrini and Gamperl, 2021) or potentially struggling etc.]

Graphing and statistical analyses were performed using Prism 10 (GraphPad Software Inc., San Diego, CA, USA). The depth and temperature data from the milli-TD were both graphed for each individual fish as were the means for each site. Weekly temperatures were calculated based on the milli-TD recordings at each site, and a linear regression between week and temperature was fitted until the lowest weekly temperature for each site was recorded. Daily temperature recordings at depths of 1, 5, 10 and 15 m were provided by Cold Ocean Salmon (Cooke Aquaculture Inc.), and these data were plotted following the completion of each study. These daily recordings were also used to calculate weekly temperatures from the week of tagging until the lowest recorded temperatures, and linear regressions were fitted for each depth. Average values of temperature, heart rate, HRV, EA and the percentage of non-steady swimming were calculated for each day and night. At each site, relationships between heart rate, HRV and EA versus temperature were determined using linear regression analysis. Relationships between heart rate and external acceleration were also calculated at each using the linear regressions.

3.3 Results

3.3.1 Fish Recovery and Tag Retrieval

At Site $^{\#}1$, a mortality dive conducted 10 days post-tagging recovered 3 fish with both milli-HRT ACT and milli-TD tags, and 15 days post-tagging an additional 2 fish were retrieved. However, the milli-HRT ACT tags were missing (Table S2). Fish / tags were recovered by commercial divers so limited information on incision and fish condition were available. Additional fish were recovered ~1 month (n=2) and ~2 months (n=1) post-tagging with both tags recovered (Table S2). The data for these fish were analyzed, and the f_H, activity, distribution and temperatures occupied did not differ from that obtained for the surviving fish for much of the time they were in the sea-cage. However, large vertical migrations, followed by a major drop in f_H, were observed in the days prior to the last viable f_H (Figure S4). No mortalities (fish or tags) were recovered during weekly dives at Site $^{\#}2$ (Table S2).

Harvest occurred in June and October 2021 at Site $^{\#}1$ and 3 fish (1 milli-HRT ACT and 3 milli-TD tags) and 1 fish (1 milli-TD and milli-HRT ACT tag missing) were recovered, respectively (Table S2). Harvest at Site $^{\#}2$ occurred in March 2022 and 2 milli-HRT ACT and 1 milli-TD tags from 3 fish were recovered (1 tag was missing for each fish, Table S2).

3.3.2 Data Analysis and Quality

Data for harvested fish was analyzed from tag start time (October 27th, 2020, at 12:00 am NST) to April 1st, 2021 for Site $^{\#}1$ (156 days), and from November 1st, 2021 to March 24th, 2022 for Site $^{\#}2$ (142.5 days). During these periods 3744 and 1711 f_H measurements, and 534 and 497 associated ECG tracings, were recorded for Site $^{\#}1$ and Site $^{\#}2$, respectively. The quality of the f_H measurements were $QI_0 = 71.4 \pm 7.3\%$, $QI_1 = 27.7 \pm 7.6\%$, $QI_2 = 0.13 \pm 0.08\%$; $QI_3 = 0.84 \pm 0.4\%$. All saved ECGs had identifiable PQRS intervals, and therefore, could be used to manually calculate and validate f_H values determined by the software. Less than 1.5% of f_H values were deemed outliers and removed (i.e., values < 10 beats min^{-1} or > 200 beats min^{-1} or with a QI_2 or QI_3 indicating that the PQRS complex could not be identified by the software).

3.3.3 Temperature and Distribution

Temperature and depth were recorded by the externally attached milli-TD tags every 5 minutes for the duration of the study and totaled 44928 and 41040 measurements per fish at Site $^{\#}1$ (n=4) and Site $^{\#}2$ (n=1), respectively (Figure 3-1A and 3-1B, Figure S5). Water temperatures at tagging were ~8-10°C but decreased steadily following tagging. This temperature decrease averaged 0.35 and 0.57°C $week^{-1}$ for Sites $^{\#}1$ and $^{\#}2$, respectively, until the lowest weekly temperature of 1.10°C occurred in early March for both years (Figure 3-2). Analysis of the data provided by Cold Ocean Salmon revealed a very similar weekly decrease in temperature of 0.29-0.35 and 0.55-0.57°C (depending on depth) for Site#1 and Site #2, respectively. On average, the temperature difference between 5 and 15 m was 0.36 ± 0.03°C and 0.40 ± 0.03°C at the two sites, respectively, while the difference between the temperature at these depths and at the surface (i.e., at 1 m) for Site #1 and Site #2 was 0.66 ± 0.05°C and 1.08 ± 0.08°C, respectively. The maximum daily difference recorded between depths was ~3.6°C, with the temperature at the surface being colder (Figure 3-3). Following the coolest temperature recorded in early March to the end of the analyzed period (April 1st / March 24th), temperatures increased slightly, however they averaged ~1.7°C (Figures 3-2 and 3-3, Figure S5). Collectively, these data reveal that temperatures at any depth within the sea-cages studied remained below 2°C for ~1.5 months and below 5°C for ~5 months (December to May; see Figure S5 for data on sea-cage temperatures).

Although temperature steadily decreased over much of the experimental period, the salmon generally remained in the upper ~5 metres of the sea-cage (Figure 3-1, Figure 3-4, Figure S5) at both Site #1 and Site #2. While, on average, the fish remained in the upper part of the sea-cage, they continued to frequent all depths throughout the study (Figures 3-1 and 3-4, Figure S5). For several of the fish this appeared to be at particular intervals / times [i.e., in November / December and when cage temperatures were ~ 2°C (March – April)]. However, for the one fish that data were obtained for at Site #2, it was evident that it made excursions to the bottom of the cage throughout the study period (Figure 3-1B). The salmon's overall depth preference had a diurnal pattern, with the tagged fish occupying deeper depths during the night and shallower depths during the day. This pattern was particularly evident in early March, when temperatures were the lowest, with the mean depth of the fish being ~5 m deeper during the night (Figure 3-4).

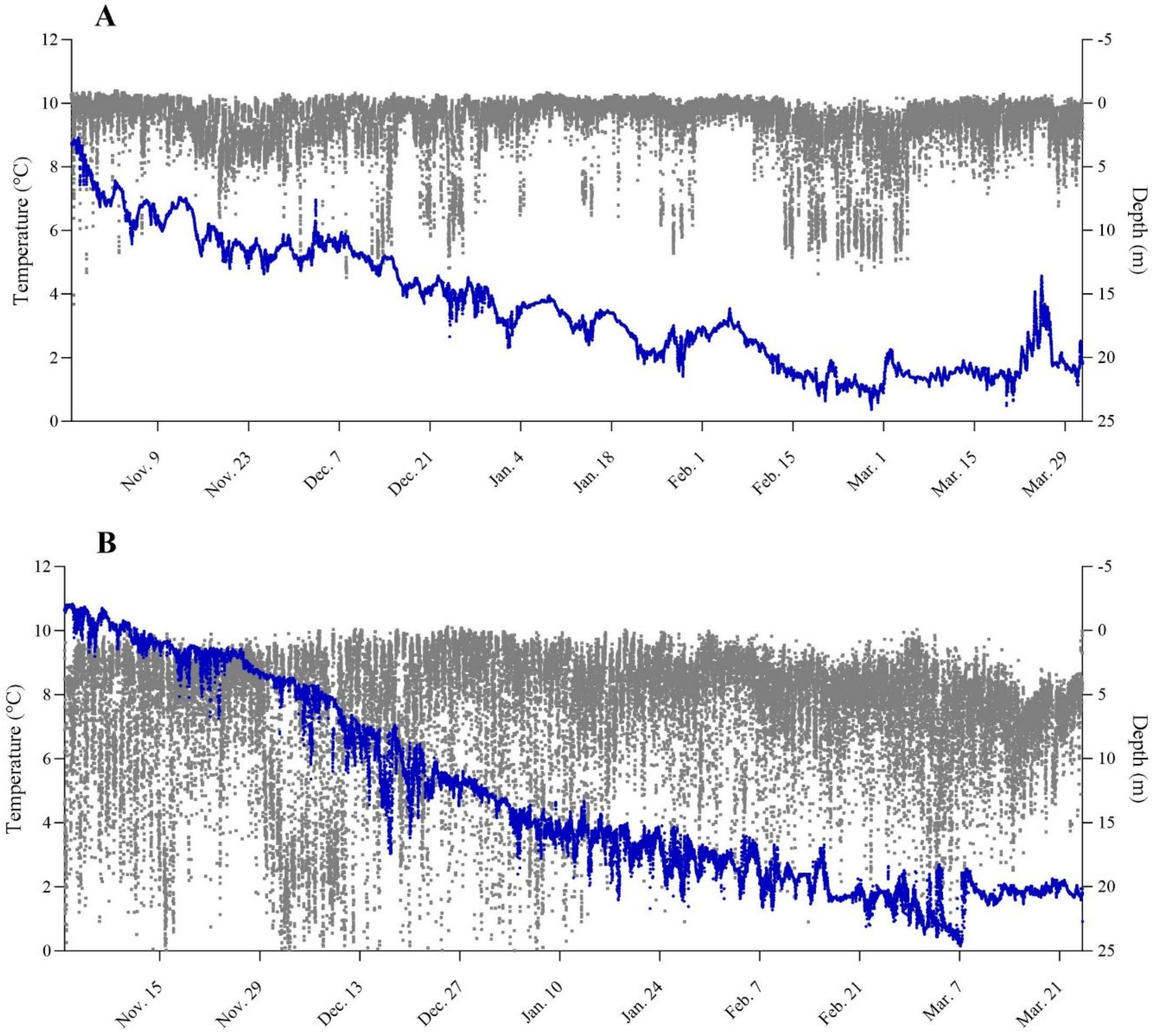

Figure 3-1. Mean depth (gray) and temperature (blue) measured every 5 min in four fish (A) at Site #1 and one fish (B) at Site #2 fitted with milli-TD tags over the duration of the study.

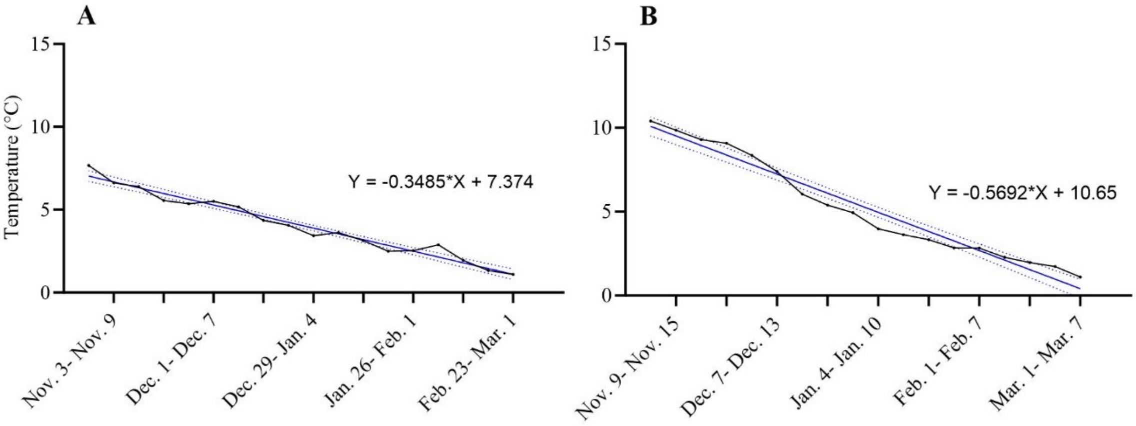

Figure 3-2. Mean weekly cage-site temperatures recorded by milli-TD tags attached to (A) four fish at Site #1 and (B) one fish at Site #2 until the lowest weekly temperature. Linear regressions were calculated based on these data to determine the weekly decline in temperature.

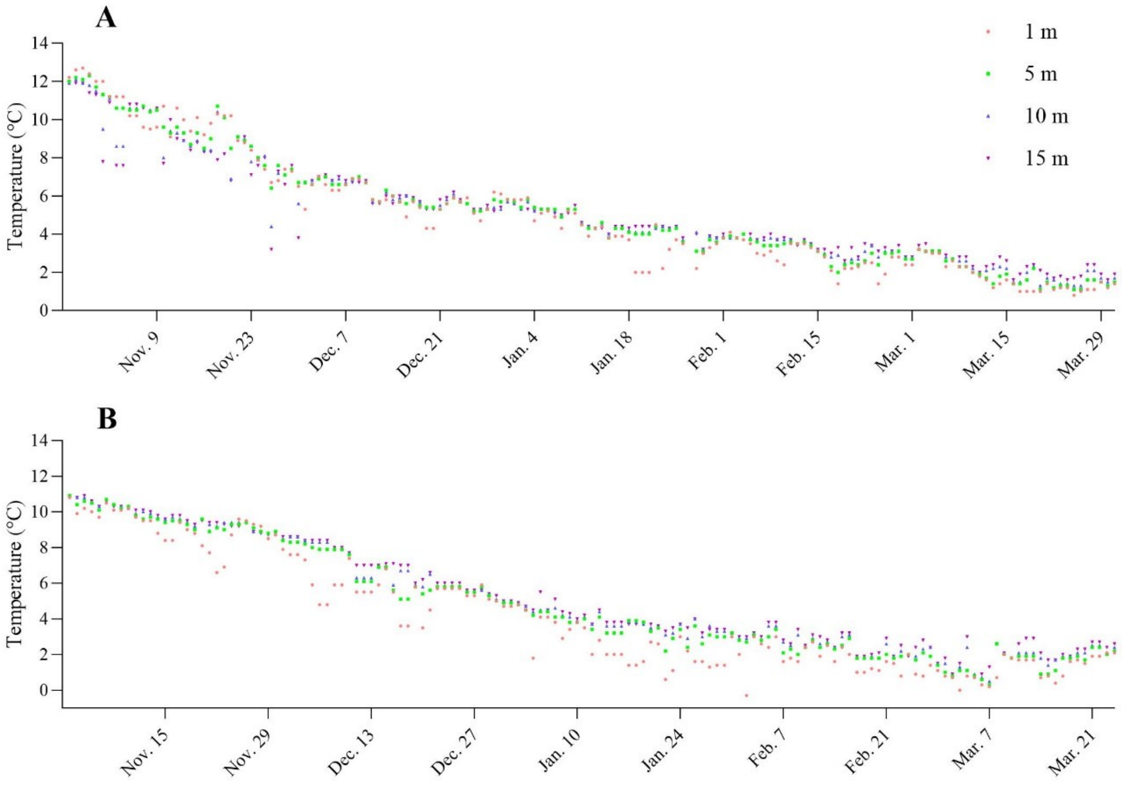

Figure 3-3. Daily temperatures at 1, 5, 10, and 15 m depth at (A) Site $^{\#}$1 (2020/2021) and (B) Site $^{\#}$2 (2021/2022), as recorded by our industrial partner (Cold Ocean Salmon / Cooke Aquaculture Inc.)

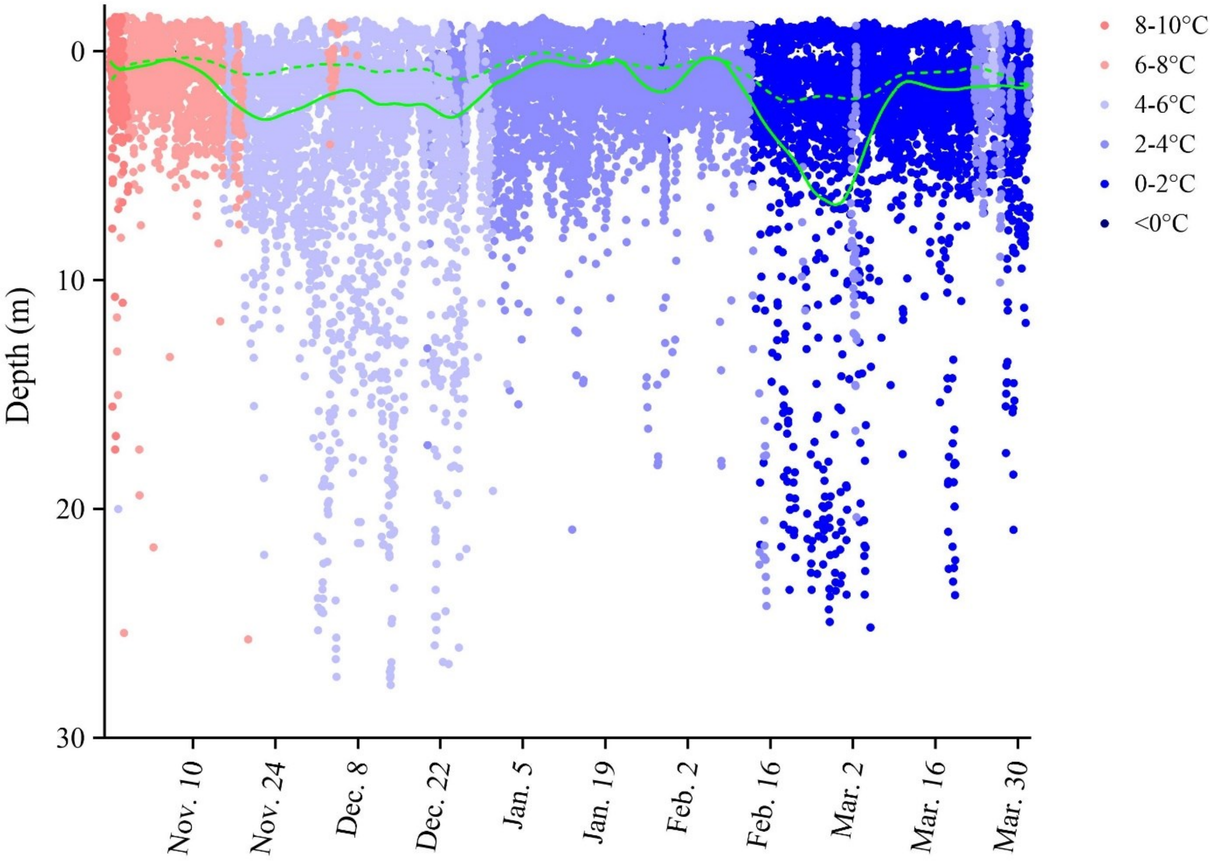

Figure 3-4. Cage-site temperature at various depths over the duration of the study. The data were obtained from milli-TD tags that were attached to four Atlantic salmon at Site $^{\#}1$ (2020/2021). The Lowess curves (green) show the mean depth occupied by the salmon during the day (broken line) and night (solid line).

3.3.4 Heart Rate and Activity

At the beginning of this study, water temperatures were ~8 and 10°C at Site $^{\#}1$ and Site $^{\#}2$, respectively, and f_H was ~50 beats minute^{-1}. Thereafter, the average heart rate closely followed the decrease in temperature. Heart rate was ~40 beats minute^{-1} when temperatures were ~5°C and ~20 beats minute^{-1} at ~1-2°C (Figure 3-5A, Figure 3-5B and Figure 3-5E, Figure 3-5F, respectively). Indeed, the f_H and temperature relationships were highly significant when the data for all retrieved tags were combined (Figure 3-6A, Figure 3-6E). While statistical analysis did not identify a diurnal pattern in f_H over the duration of this study for two of the three fish, overall, heart rates were higher during the day-time in comparison with night-time values (Figure 3-5A, Figure 3-5E, Table S3). Although higher heart rate variability values (HRV) were observed at the beginning of the study, an overall relationship between HRV and temperature was not clearly identified (Figure 3-6B, Figure 3-6F). External acceleration remained constant, between ~10 and 15 m-g, for the majority of the study. Finally, while a clear relationship was not identified between non-steady swimming and temperature, it appears that the fish regularly engaged in 'burst-coast swimming' and that this was more prevalent during the day-time (Figure 3-5D, Figure 3-5H).

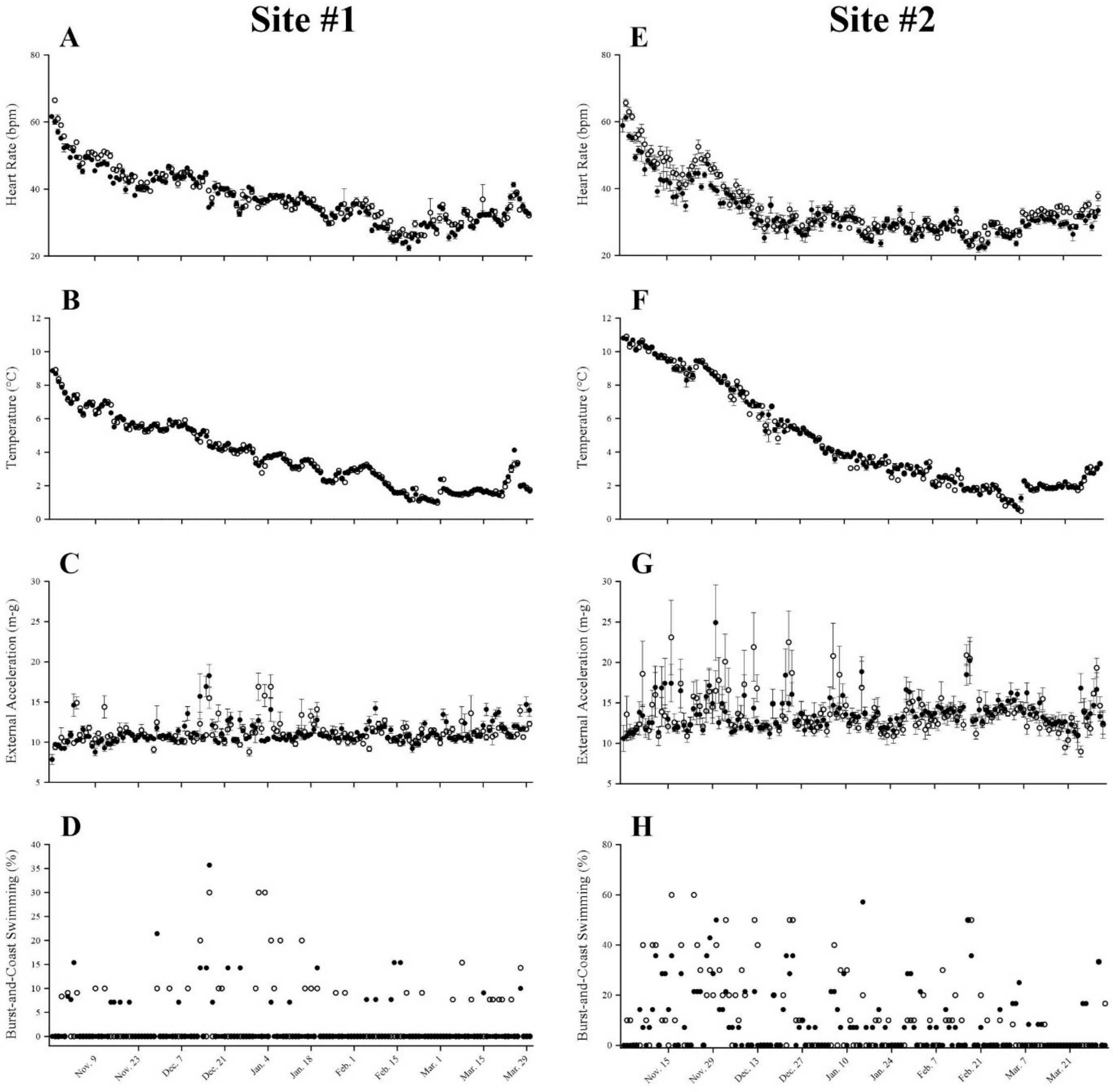

Figure 3-5. Average day-time (open circles) and night-time (closed circles) values for heart rate (A, E), temperature (B, F), external acceleration (C, G) and the percentage of non-steady swimming (D, H) measured for one fish at Site $^{\#}1$ and two fish at Site $^{\#}2$.

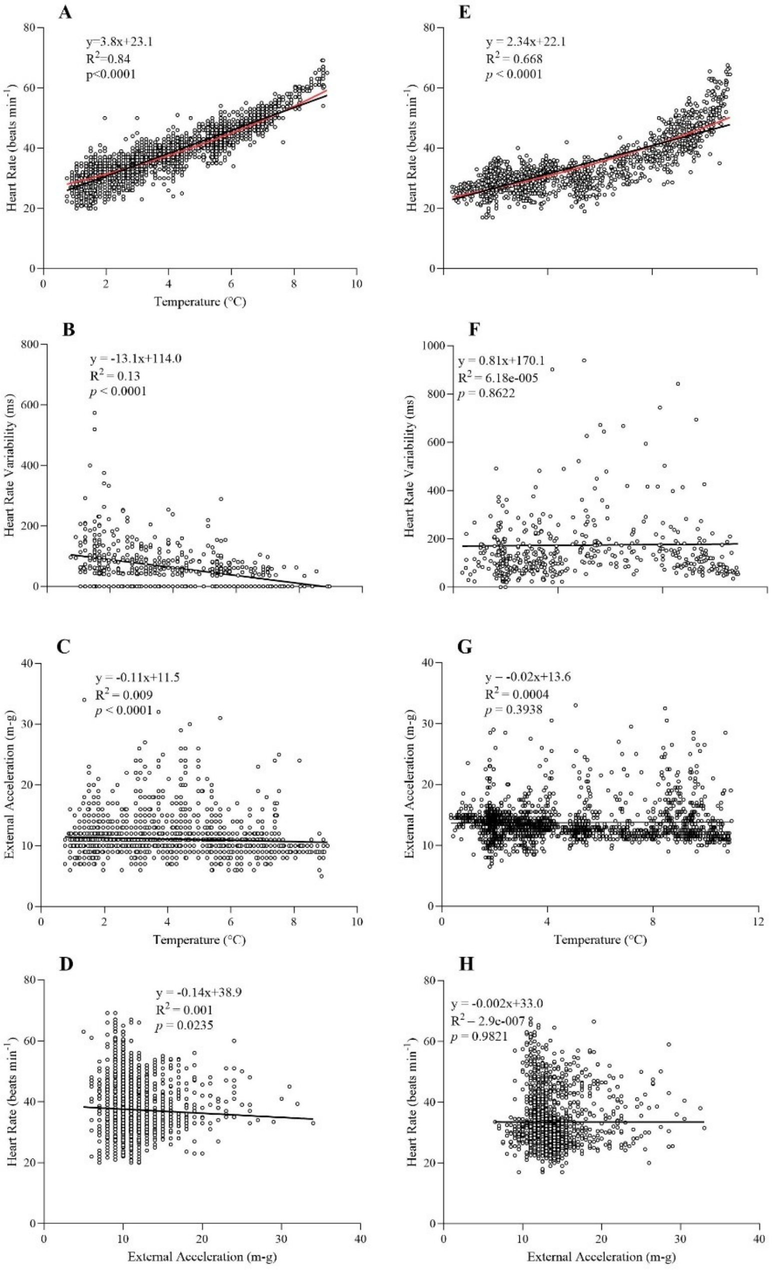

Figure 3-6. Relationships between recorded temperature and heart rate (A, E), heart rate variability (B, F), and external acceleration (C, G), and between heart rate and acceleration (D, H). Panels A-D show data retrieved from one fish from Site $^{\#}$1 and E-H is the average data from two fish at Site $^{\#}$2. Linear regression line (black line) and equations are shown in each panel. Note: an exponential equation (red line) was also fit for heart rate (A, E); R^2=0.835 and 0.701, respectively.

3.4 Discussion

The finfish aquaculture industry will continue to be impacted by climate change given the predicted increase in average seawater temperatures, and in the frequency and severity of storms and weather events. Year-round production in sea-cages in the North Atlantic means that temperature-related challenges are not confined to the warm summer waters, but also occur in the winter. Thus, additional knowledge and understanding of cage-site conditions during the winter is critical if the industry is to implement management strategies to mitigate issues with decreased production, and fish health and welfare. The goal of this study was to use DSTs to simultaneously record the heart rate, acceleration (swimming activity), temperature and depth of sea-caged Atlantic salmon from November to April, and thereby, better understand their physiology, distribution and behaviour when they are exposed to the coldest temperatures ($< 5^{\circ}C$) of the year.

3.4.1 Mortality and Tag Retrieval

In the immediate two weeks following surgery, a 22% mortality rate based on retrieved fish and associated tags was observed at Site $^{\#}1$ (Table S2), with an overall mortality of 35% over the study. This may be attributed to several factors. First, we had purchased ‘centi-sized’ HRT-ACT tags to take advantage of the additional battery capacity, and they were intended to be implanted into fish that were ~2.3 kg and larger. However, the fish available for this study were considerably smaller (average 1.68 ± 0.07 kg). I believe the size of the tags, combined with poor healing (i.e., due to the cool temperatures) and the limited recovery period (24 hours), resulted in the loss of a number of internal tags and associated mortality. Further, tag attachment / surgery is associated with significant stress (Hvas et al., 2020; Macaulay et al., 2021; Wright et al., 2019), and elevated heart rates in free-swimming fish can be observed for up to 3 weeks post-surgery / implantation of DSTs (Føre et al., 2021; Hvas et al., 2020; Yousaf et al., 2022; Zrini and Gamperl, 2021).

No mortalities were retrieved at Site $^{\#}$2, yet tags from only 3 out of the 12 tagged fish were retrieved at harvest. Similarly, there were 11 fish with tags not retrieved at Site $^{\#}$1. The missing fish / tags may not have been retrieved during the harvest (processing of the cages) as a result of misidentification of fish and / or expelled tags. Also, there were health / environmental issues at both sites that resulted in significant mortalities, limiting the opportunity for inspection of fish for DSTs. Finally, the tags were intended to be deployed for approximately 4-5 months, instead it took 238 and 363 days to completely harvest the cages at Site $^{\#}$1, and 142 days at Site $^{\#}$2. This extended period of deployment could have led to the loss of the external (milli-TD) tags, and thus, precluded the identification of tagged fish at harvest. This hypothesis would fit with the findings of Macauley et al. (2021), who reported that while the mortality of tagged fish in sea-cages can be quite variable, their model predicted that mortality of tagged fish would be approximately 36% at 100 days post-surgery. Collectively, the above highlights the difficulties that can arise from working in sea-cages in the winter and deploying tags for extended periods of time. Further, it provides several insights that can be used to improve tag retrieval in the future.

3.4.2 Temperature

The tagging at Sites $^{\#}$1 and $^{\#}$2 occurred almost exactly one year apart. Temperatures at the time of tag implantation were comparable (8°C vs. 10°C), and a similar decline in weekly mean temperatures occurred between November and March. These values were 0.35 and 0.57°C week^{-1} based on the data obtained from milli-TD DSTs and was similar to that obtained based on the aquaculture company's temperature logs. Overall, this study reveled that sea-cage temperatures are less than 5°C for nearly half of a year's production cycle, and below 2°C for approximately 1.5 months. Thus, in Newfoundland, these cool / cold water temperatures can have significant impacts on production and the time that the salmon must remain in the cages prior to harvest.

Overall, temperatures throughout the sea-cage were relatively homogeneous over the study period; although there were times that temperatures at the surface (1 m) were 2-4°C lower than in the rest of the water column. This limited temperature variability is in stark contrast to what has been observed during the warmest summer months at salmon sea-cages. For example, Stehfast et al. (2017) reported a major crowding event that resulted from water temperature stratification and a subsequent hypoxic squeeze at a sea-cage in Tasmania, Australia. Further, temperatures at the surface of sea-cages during the Newfoundland heat wave of 2019 were often approximately 10 – 12°C above those measured at 15 – 20 meters depth (Burke et al., 2020; Gamperl et al., 2021). While the latter studies have led to the implementation of management practices / protocols (i.e., deeper nets, submersible cages, and additional aeration / oxygenation etc.) to ultimately mitigate the challenge of summer temperatures, it does not appear that similar efforts would be of benefit to salmon in the winter.

3.4.3 Cage Use and Fish Distribution

While there was some individual variation in the data recorded regarding cage use by the fish, overall, the fish displayed remarkably similar movement patterns. In the days immediately following tagging, the fish predominately occupied the upper 5 meters of the cage with occasional migrations to deeper depths (e.g., see Figure S5). This pattern in depth distribution continued for much of the study, however, there were periods where they would use the deeper depths of the cage quite regularly. For example, many of the fish made more frequent migrations to the deeper parts of the cage in November / December, and again when temperatures reached ~2°C particularly during the night-time in this latter period. Interestingly, this is the opposite to what has been observed during warm temperatures in summer (Gamperl et al., 2021; Stehfast et al., 2017), where fish avoided the warm surface waters during the day and returned to shallower depths at night. As temperatures within the sea-cage during this study remained relatively homogenous with depth, it is unlikely that temperature explains the fish's diurnal change in depth distribution. Instead, this behaviour is probably related to feeding which only occurs between daylight hours. For example, while the salmon continued to be fed to apparent satiation throughout the winter months (until ~1°C), feeding behaviour and overall feed consumption decrease as metabolic demands are reduced (Ibarz et al., 2007; Porter et al., 2023). For example, a recent study by Vadboncoeur et al. (2023b) reported a substantial decrease in salmon feed consumption (and behaviour) during a seasonal decline (~1°C week^{-1}) in temperature. Feeding / appetite began to decrease at 6°C, with the complete loss of appetite by 1-2°C. Similarly, Liu et al. (2020) reported that Atlantic salmon stopped feeding below 2°C. Thus, it is likely that the tagged fish within this study also decreased and / or stopped feeding at these low temperatures, and this was reflected in their cage-site depth distribution.

3.4.4 Heart Rate and Acceleration

Heart rates at the beginning of this study, at temperatures between 8-10°C, were ~50 beats min^{-1}. This is very similar to values reported by other studies (Gamperl et al., 2021; Sandrelli and Gamperl, 2023; Warren-Myers et al., 2021; Yousaf et al., 2022). Given that temperatures began to decline shortly after the fish were returned to the cage, a recovery period characterized by stable f_H values (i.e., no further decrease in f_H) was not seen. Instead, f_H was observed to closely follow the seasonal decline in temperatures (Figures 3-5 and 3-6), with the lowest average heart rates (20-25 beats min^{-1}) recorded at temperatures below 2°C. A diurnal effect on f_H was not found to be statistically significant, likely due to the small sample size. This, however, may also be due to the fact temperatures were <5°C for a large proportion of this study and were relatively homogeneous from the surface to the bottom of the cage. For example, while diurnal changes in f_H of ~5 beats min^{-1} have been reported at water temperatures of ~5°C (Føre et al., 2021; Svendsen et al., 2021), these are much lower than the ~25 beats min^{-1} that Brijs et al. (2018) found at 15°C.

External acceleration was more variable from October to January compared to January to March / April (i.e., at temperatures >4°C) with values often ranging between 15 and 25 m-g. However, this pattern of activity ended as temperature fell further, and little variation in activity (external acceleration) was observed over the rest of the winter (Figure 5). While it is difficult to draw firm conclusions from these data given the small sample size, they are consistent with lab-based studies on the effects of cold temperatures on salmon swimming performance. Lab based studies on fish acclimated to control (8-11°C) vs. cool / cold (1-4°C) temperatures report decreases of 20-35% in the critical swimming speed of salmon due to factors independent of metabolic capacity (Porter and Gamperl, 2023; Riseth et al., 2020; Taylor et al. 1996).

3.5 Conclusions and Perspectives

Farmed aquatic animals are an important, and arguably, a critical source of protein and food for the growing human population. As with wild fish populations, the aquaculture industry faces a number of climate change-related challenges (Cheung and Frölicher, 2020; Cheung et al., 2021; Falconer et al., 2020; Genin et al., 2020; Islam et al., 2022; Perry et al., 2005; Reid et al., 2019; Szerkeres et al., 2016). While the main focus has been on addressing the effects of high temperatures, it is clear based on recent losses of fish in the winter that research needs to be conducted on the effects of temperatures as low as 0°C on fish health and welfare (Reid et al., 2022; Szekeres et al., 2016).

I fitted Atlantic salmon with DSTs that could record temperature, heart rate, acceleration and depth to further understand how temperatures in sea-cages in Newfoundland change seasonally, and how they might affect fish activity, physiology and distribution. Although aquaculture companies monitor water temperature and oxygen, and feeding behaviour, quite closely, their observations can be limited particularly during the winter months as sea ice cover, limited day-light hours and storms can hinder monitoring. Although, there are now several publications on how temperatures down to 1°C affect the physiology (i.e., stress hormone and heat shock protein levels, ion regulation, and cardiac function) and swimming performance of Atlantic salmon (Porter at al., 2022; Porter and Gamperl, 2023; Reid et al., 2022; Vadboncoeur, 2023a; 2023b), these data have all been collected in laboratory-based settings where the fish are highly manipulated and confined. Further, the literature provides several caveats with regard to directly comparing lab-based studies to those on free-swimming fishes (Mignucci et al., 2021; Sandrelli and Gamperl, 2023).

In this study, I was able to successfully record f_H, temperature, depth and swimming activity in Atlantic salmon in sea-cages in the fall / winter in Newfoundland. In addition, I was able to show that: 1) water temperatures fell by 0.35 and 0.57°C week^{-1} until early March when temperatures were ~1°C, and heart rate closely followed temperature; 2) temperatures remain below 5°C for five months, and while often lower at the surface, they were generally homogenous from 5-20 m; and 3) while fish mainly occupied the upper 5 meters of the cage, there were some excursions to deeper depths at night and during the periods of lowest temperature. However, I also learned several valuable lessons with regard to the implantation / use of these tags for long-term studies at cold temperatures. First, although the DSTs used at Site $^{\#}1$ had larger batteries, which made frequent and long-term recordings possible, these tags were considerably larger and likely more invasive to the fish. This, combined with the effects of cold temperatures on wound healing, probably led to the loss of a number of tags / mortalities. Second, retrieving DSTs from tagged fish placed in commercial cages (150 m circumference, with 50,000 – 60,000 other fish) for periods in excess of 6 months, and having tagged fish identified on a processing line, is not easy and leads to a poor rate of tag recovery. Finally, the recent increase in maximum recording time for ECGs (from 6 to 15 seconds at 100 Hz) allows for very accurate measurements of f_H at low water temperatures. For example, at temperatures < 2°C, I had < 10 f_H values that were QI=3 (i.e., the PQRS interval could not be identified) of the hundreds that were recorded. It is this technological advancement, and successful testing of acoustic HRT DSTs in Newfoundland under cage-site conditions, that should allow such technology to be incorporated into real-time aquaculture monitoring systems. Real-time monitoring of f_H, activity and fish depth could be an important addition, and enable the industry to remotely identify, and respond quickly, when issues threaten fish health and welfare.

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CHAPTER 4: Summary, Research Limitations and Perspectives

4.1 General Discussion

As fish are ectotherms, their body temperature is dependent on water temperature, and thus, changes in this environmental parameter impact nearly all aspects of their biology, health and welfare (Alfonso et al., 2021; Brett, 1971; Gamperl et al., 2020; Oppedal et al., 2011; Pörtner and Farrell, 2008; Tromp et al., 2018; Vadboncoeur et al., 2023b; Wade et al., 2019). This is of particular concern in the era of climate change as temperatures are expected to continue to rise, and more frequent storms and weather events will increase variability (IPCC, 2018, 2022). This temperature variability will not only increase the occurrence of marine heat waves, but also of winter storms that can result in dangerously cold temperatures and potentially 'winter chill'. Thus, the goal of this thesis was to better understand the impacts of both high and low temperature extremes using the economically important Atlantic salmon as a model species.

There has been significant research into the impacts of warming temperatures on fishes, including the Atlantic salmon, with many studies assessing upper thermal limits (Ignatz et al., 2023; Lefevre et al., 2021; Little et al., 2020; Pörtner et al., 2017; Sinclair et al., 2016; van der Walt et al., 2021). However, the methods vary considerably with regard to heating rate (ranging from 1-2°C h^{-1} to 10-18°C h^{-1}), usually involve short post-handling / surgery recovery periods and often confinement, and the impact of the latter two factors on a fish's thermal tolerance are not well understood. In addition, it is difficult to predict how these measures compare to those that would be obtained in free-swimming fish in the wild and in culture. In contrast, our understanding of the effects of cold temperatures on fish physiology and biology is extremely limited, despite several winter chill mortality events that have been recently observed in both Canadian and Icelandic aquaculture (Círíc, 2020; *Harsh weather linked to die-off at Newfoundland salmon farm*, 2020; Huffman, 2019; The Canadian Press, 2015).

The overall goal of this thesis was to provide information that could be used to make accurate, and more ecologically-relevant, predictions regarding climate change-related impacts on the Atlantic salmon, and by extension other fishes. By using heart rate DSTs implanted into free-swimming fish, I was able to: 1) compare several commonly used methods (and metrics) of assessing upper thermal and hypoxia tolerance and determine the limitations of these measures; and 2) better understand the behaviour, distribution and physiology of sea-caged Atlantic salmon during the fall / winter when the lowest temperatures are experienced. This information will be beneficial to many key stakeholders and decision makers (including scientists, conservation biologists, aquaculture companies and fisheries managers), and allow them to better predict and plan for the impacts of increasing water temperatures and low dissolved oxygen levels, as well as the effects of low winter water temperatures and associated 'winter chill' events.

4.2 Research Findings and Novel Insights

4.2.1 Methods Used to Assess Environmental Tolerances

While it is difficult to accurately predict the impacts of climate change on fishes, determining environmental tolerances can both establish species-specific baseline values and help guide mitigation strategies. Most studies have examined temperature- and hypoxia-related impacts on fishes using controlled laboratory experiments (Anttila et al., 2014; Casselman et al., 2012; Hansen et al., 2017; Leeuwis et al., 2019; Mignucci et al., 2021; Motyka et al., 2017; Muller et al., 2020). This setting allows researchers to precisely regulate environmental conditions (i.e., isolate the experimental parameter of interest), but also to make measurements that require access to computers and data acquisition systems (e.g., cardiac function, oxygen consumption), or to sample blood for the analysis of various haematological parameters. The novel research conducted in this thesis revealed that there were major differences in the f_H-temperature relationships between the protocols tested, and in particular, raise concerns about the use of the ‘rapid screening tool’ to investigate fish thermal biology. For example, this method severely underestimated the Atlantic salmon’s thermal limits, with the values for ABT and Tf_{Hpeak} ~3.8 and 6.3°C lower, respectively, than measured in free-swimming salmon. This is not surprising as this method was originally developed to determine a species’ thermal optimum for aerobic scope, and not upper thermal tolerance. Further, the fish are anesthetized to remove the influence of activity, and injections of atropine and isoproterenol are used to ensure that heart rate is maximized at all temperatures during the assessment of acute thermal tolerance. As a consequence, the available scope to increase f_H during the protocol is limited, and this is a major concern as my research suggests that scope for f_H may be a key (critical) determinant of a species’ upper thermal tolerance.

In Chapter 2, it was also not surprising that free-swimming fish implanted with DSTs and given a four-week post-surgical recovery period, had a lower resting f_H (average 48.9 ± 1.9 beats min^{-1}) and a greater value for f_H scope (104.2 ± 4.6) as compared to the salmon tested in the respirometers and using the ‘rapid screening tool’ (70.8 ± 4.0 and 57.8 ± 3.8, respectively). Firstly, the stress associated with anaesthesia and surgery is acknowledged by many users of DSTs, and a minimum recovery period of 1-2 weeks is recommended before taking measurements (Bjarnason et al., 2019; Brijs et al., 2018, 2019; Ekström et al., 2018; Hvas et al., 2020; Zrini and Gamperl, 2021). However, this period of recovery is not possible in respirometry studies. Fish cannot be deprived of food for extended periods of time as this would be a confounding influence, and it is ethically and experimentally questionable as to whether fish can / should be confined for such lengthy timeframes. Importantly, the elevation in f_H and decrease in scope for f_H in fish confined to the respirometers post-surgery resulted in lower values for Tf_{Hpeak}, ABT and CT_{max} by 0.3, 3.3 and 1.8°C, respectively. These data clearly show that acute temperature tolerance tests on fish implanted with Doppler® flow probes, and recovered in respirometers, may underestimate a species' acute thermal tolerance by approximately 2 – 3°C.

Overall, the results in Chapter 2 indicate that evaluating thermal and hypoxia limits using free-swimming fish implanted with DSTs, and given a four-week recovery period, provide much more relevant insights into the thermal biology and tolerances of both wild and cultured fish. This is an extremely important finding, as predictions about how climate change will impact various fishes need to be accurate to allow management and / or conservation efforts to have the desired effects.

4.2.2 Sea-caged Atlantic Salmon in the Fall / Winter in the North Atlantic

Aquaculture production in sea-cages occurs year-round, yet it remains unclear what specific temperature conditions the salmon are exposed to during the fall / winter in Atlantic Canada, and how these temperatures influence their behaviour, distribution and physiology. This aspect of their culture is of increasing interest, and concern, given mortality events that have occurred in the region in recent years (*Harsh weather linked to die-off at Newfoundland salmon farm*, 2020; Huffman, 2019; The Canadian Press, 2015). The continuous monitoring of sea-caged Atlantic salmon with implanted / attached DSTs in Chapter 3 allowed for several important insights. First, the fish experienced a decline in temperature of between 0.35 and 0.57°C week^{-1} from mid November until reaching ~ 1°C in early March, and temperatures remained at the latter value for a period of approximately 5-6 weeks. Further, this temperature profile closely matched the company's daily temperature recordings at various depths (1, 5, 10, and 15 m). Second, temperatures within the sea-cages were below 5°C for five months, and while cooler temperatures were sometimes observed at the surface, they were generally homogeneous between 5 – 20 meters. Third, f_H closely followed the decline in temperature, and this was not surprising given the strong heart rate-temperature relationships that have previously been described for this species at warmer temperatures (10-20°C) (Gamperl et al., 2021). Finally, although the salmon mainly occupied the upper 5 meters of the cage, deeper excursions were observed throughout the study. These were particularly evident at night during the coolest temperatures.

While the data recovered were consistent between all individuals in the two years, the low number of tag retrievals in this study is an obvious limitation. Nonetheless, valuable lessons were learned regarding the implantation / use of these DSTs for long-term studies at cold temperatures. While the DSTs used at Site $^{\#}$1 had larger batteries, which made frequent and long-term recordings possible, these tags were considerably larger and likely more invasive for the fish. Additionally, the tagging occurred when temperatures were ~8-10°C and began decreasing almost immediately following implantation / attachment. These cold temperatures likely impacted wound healing, and probably contributed to the loss of a number of tags / mortalities. Second, retrieving DSTs from tagged fish placed in large commercial cages (150 m in circumference, with 50,000 – 60,000 other fish) for periods in excess of 6 months, and having tagged fish identified on a processing line, is difficult and leads to the recovery of a limited number of tags.

4.3 Future Research

4.3.1 Stress and Climate Change

It is difficult to predict climate change-related impacts as stressors such as temperature and low oxygen are often coincident with others such as crowding and disease (Combe et al., 2023; Gamperl et al., 2020; Godwin et al., 2020; Stehfast et al., 2017; Vadboncoeur et al., 2023a, 2023b). Therefore, it is not surprising that my thesis highlights the potential interaction between the degree of stress experienced by fishes, and how this influences their capacity to respond to other environmental challenges. Specifically, in Chapter 2, I found that: the low f_H in undisturbed (unstressed) fish limited their capacity to reduce f_H when exposed to oxygen-limited conditions; and that stress-induced increases in resting f_H limit the f_{Hscope} available to deal with rising temperatures, and that this results in a reduction in upper thermal tolerance. With regard to the first finding, I hypothesize that this was due to an already high cholinergic tone on the heart, as it has been recently shown that cholinergic regulation of f_H has important implications for a fish's capacity to respond to other environmental challenges. Specifically, in both sablefish (*Anoplopoma fimbria*) (Leeuwis et al., 2021) and Atlantic salmon (Gamperl et al., in preparation) cholinergic-mediated bradycardia induced by hypoxia prevents fish from being able to increase f_H during an acute thermal challenge, and this reduces their upper thermal tolerance (CT_{max}).

4.3.2 Acoustic Transmission of f_H DSTs

The low tag retrieval in Chapter 3 highlights the challenges associated with tagging free-swimming fishes within a commercial sea-cage during the winter. Although recovering fish within a facility prior to release into the cage or tagging when water temperatures are >10°C, may improve overall tagging success (i.e., reduce post-surgical mortalities), this may or may not improve tag retrieval at harvest. One solution to this issue, would be the use of acoustic tags that are capable of transmitting the data (e.g., f_H, temperature, depth etc.) in real-time to hydrophones / acoustic receivers in the cage, as this would allow for the continuous monitoring of tagged fish, and thus, eliminate the need to recover the fish / DST. Further, the use of such tags would allow the transmitted information to be integrated into existing real-time monitoring systems at commercial sea-cages, and for cage-site staff to be alerted if values were outside a set range. For example, given the tight relationship between water temperature (0 – 20°C: Chapter 3; Gamperl et al., 2021) and f_H, monitoring this latter parameter would allow the industry to determine / identify when fish in the cages are experiencing significant stress and to make management decisions to mitigate the situation and avoid negative consequences on production, and potentially the loss of fish.

In August 2022, in collaboration with the manufacturer (Star Oddi), I implanted five f_H tags (similar to the tags used within Chapter 2 and 3 of this thesis) that were capable of the acoustic transmission of f_H and temperature. These tags transmitted data every 30 minutes to hydrophones (receivers) located at various depths (4, 8 and 12 m) in the center of the cage and attached to the cage's periphery (see Figure S6). The purpose of this study was to evaluate how the transmission power of the tags affected data collection and battery life, and to determine the best location to place the hydrophones. This research and development project was very successful. Tag design is currently being finalized, and the commercial release of this product is expected in the next few months.

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Appendix

Table S1. Summary of statistical values generated from the ANOVAs, unpaired t-tests and linear regressions. (I) Fish anesthetized and implanted with DSTs (n=11), (II) fish tested using traditional respirometry and implanted with Doppler® flow probes (n=10) and (III) free-swimming fish held with conspecifics and implanted with DSTs (n=12). Bolded *P*-values are statistically significant ($P > 0.05$).

		F	R^2	*P*	N1	N2
ANOVA and Tukey's Post-Hoc Summary						
Mass vs. Group		1.180	0.07	0.3212		
Length vs. Group		1.033	0.06	0.3683		
RVM vs. Group		4.244	0.2205	**0.0238**		
	I vs. II			**0.0251**	11	10
	I vs. III			0.0941	11	12
	II vs. III			0.7554	10	12
ABT vs. Group		4.641	0.2967	**0.0208**		
	I vs. II			0.0592	11	7
	I vs. III			**0.0384**	11	7
	II vs. III			0.9807	7	7
f_{Hrest} vs. Group		53.56	0.7812	**<0.0001**		
	I vs. II			0.8508	11	10
	I vs. III			**<0.0001**	11	12
	II vs. III			**<0.0001**	10	12
f_{Hpeak} vs. Group		8.867	0.3715	**0.0009**		
	I vs. II			0.1753	11	10
	I vs. III			**0.0006**	11	12
	II vs. III			0.0837	10	12

Tf_{Hpeak} vs. Group		37.86	0.7162	**<0.0001**		
	I vs. II			**0.0011**	11	10
	I vs. III			**<0.0001**	11	12
	II vs. III			**0.0004**	10	12
f_{Hscope} vs. Group		34.52	0.6971	**<0.0001**		
	I vs. II			0.0940	11	10
	I vs. III			**<0.0001**	11	12
	II vs. III			**<0.0001**	11	12
$Q_{10\mathrm{PreABT}}$ vs. Group		7.744	0.4131	**0.0028**		
	I vs. II			**0.0020**	11	7
	I vs. III			0.1792	11	7
	II vs. III			0.1685	7	7
Unpaired T-Tests						
CT_{Max}	II vs. III	29.76	0.3349	**0.048**	10	12
f_{Hcrit}	II vs. III	1.436	0.2926	**0.0113**	9	12
f_{H} 100%	II vs. III	2.855	0.8162	**<0.0001**	10	12
f_{H} 30%	II vs. III	1.058	0.4389	**0.0011**	10	11
Linear Regressions						
f_{Hscope} vs Tf_{Hpeak}		79.38	0.7127	**<0.0001**	34	
f_{H} vs Temperature	III	609.2	0.7510	**<0.0001**	204	

Table S2. Information on fish tagging and DST retrieval. Fish highlighted in orange were collected as mortalities, whereas fish highlighted in green indicate those retrieved at harvest. Bolded tag numbers indicate retrieved tags. Fish that are not highlighted indicate that their tags were never recovered.

Cage-Site	Cage #	Fish #	milli-HRT-ACT	milli-TD	Weight (kg)	Length (cm)
Site #1	7	1	x	**B3099**	1.46	53
		2	MAL0219	B2653	1.6	50.4
		3	MAL0221	B2661	1.49	51.2
		4	x	B2676	1.38	50
		5	MAL0216	B2659	1.49	51.8
		6	MAL0218	B2672	1.43	50.2
		7	x	**B2663**	1.15	47.4
		8	MAL0234	B2662	1.88	57.2
		9	**MAL0233**	**B3097**	1.45	52.8
		10	MAL0230	B3095	1.5	50
		11	**MAL0235**	**B3098**	1.42	48.5
	1	12	MAL0228	B3093	1.91	52.8
		13	MAL0214	**B2667**	2.34	56.4
		14	MAL0227	B3101	2.36	55.2
		15	MAL0226	**B3092**	2.2	55.2
		16	x	B3094	1.5	
		17	**MAL0222**	**B3096**	1.51	50.2
		18	MAL0224	B2673	2.25	55
		19	**MAL0229**	**B3088**	1.7	53.8
		20	x	**B2655**	1.58	52.7
		21	**MAL0231**	**B2651**	1.9	52.8
		22	**MAL0232**	**B3102**	1.5	52
		23	**MAL0223**	**B3091**	1.54	53.1
Site #2	2	1	MAL0351	B3088	4.86	70.5
		2	MAL0352	B3099	5.92	75
		3	**MAL0354**	B2667	6.04	75.5
		4	MAL0353	B2660	3.98	69.5
		5	MAL0355	B2669	4.7	72
		6	MAL0356	B2663	3.51	63.8
		7	**MAL0357**	B2657	4.75	72.5
		8	MAL0358	B3097	4.02	67
		9	MAL0359	B3091	4.08	71
		10	MAL0360	B3096	3.2	50.5
		11	-	**B3098**	5.68	76
		12	-	B3102	5.22	72.3

Table S3. Summary of statistical values generated from the unpaired t-tests for diurnal heart rate of three fish with milli-HRT ACT tags.

		F	R^2	*P*	N1	N2
Unpaired T-Tests						
Fish 11 – Site #1	night vs. day	1.027	0.0017	0.4721	156	157
Fish 3 – Site #2	night vs. day	1.104	0.0240	**0.0088**	142	143
Fish 7 – Site #2	night vs. day	1.368	0.0061	0.1754	141	142

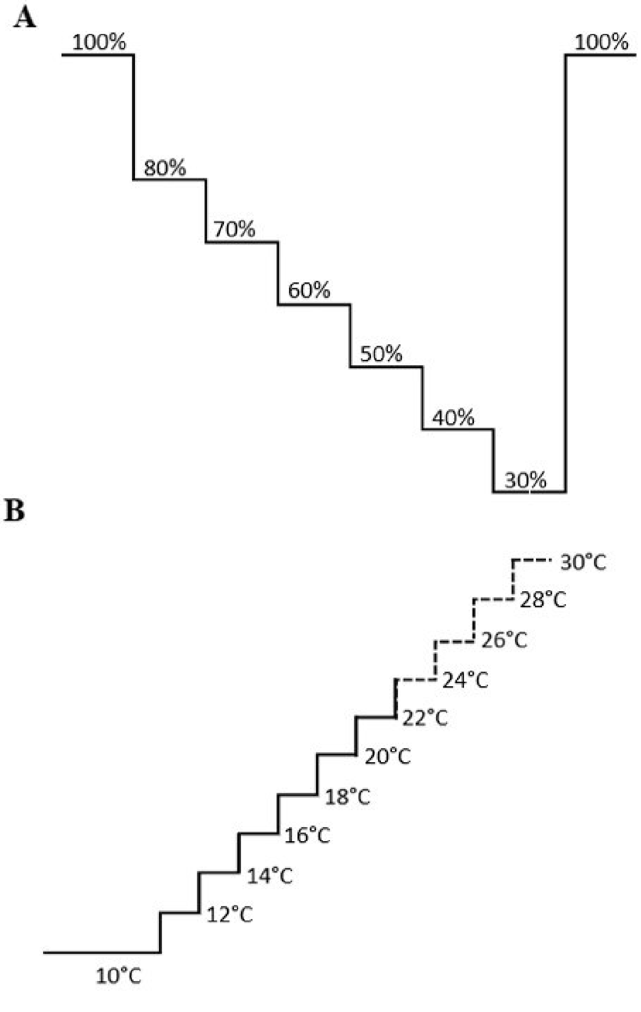

Figure S1. Schematic representation of changes in water oxygen level (% saturation) and temperature (°C) during the hypoxia (A) and temperature (B) challenges, respectively. These protocols were used for both fish tested using traditional respirometry and for free-swimming fish. Each step represents a 1-hour time increment. Fish tested using the ‘rapid screening tool’ underwent a similar temperature increase at 10°C h $^{-1}$.

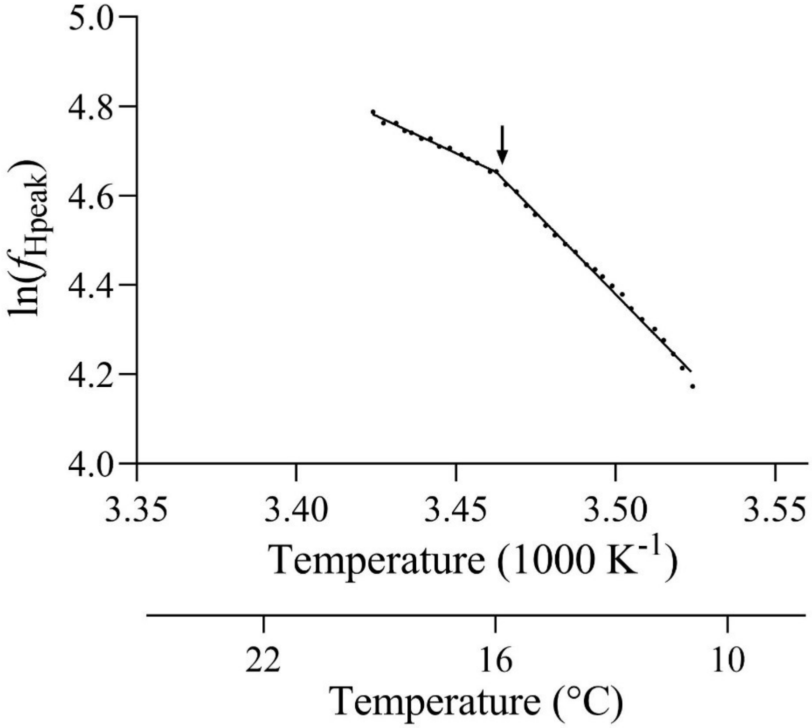

Figure S2. Arrhenius breakpoint plot of the natural log of maximum heart rate (ln f_{Hpeak}) against body temperature, plotted as the inverse of temperature in Kelvin (K). Secondary x-axis notes temperature in Celsius (°C) for reference. The point at which the change in slope occurs is the Arrhenius breakpoint temperature (ABT) indicated by (↓).

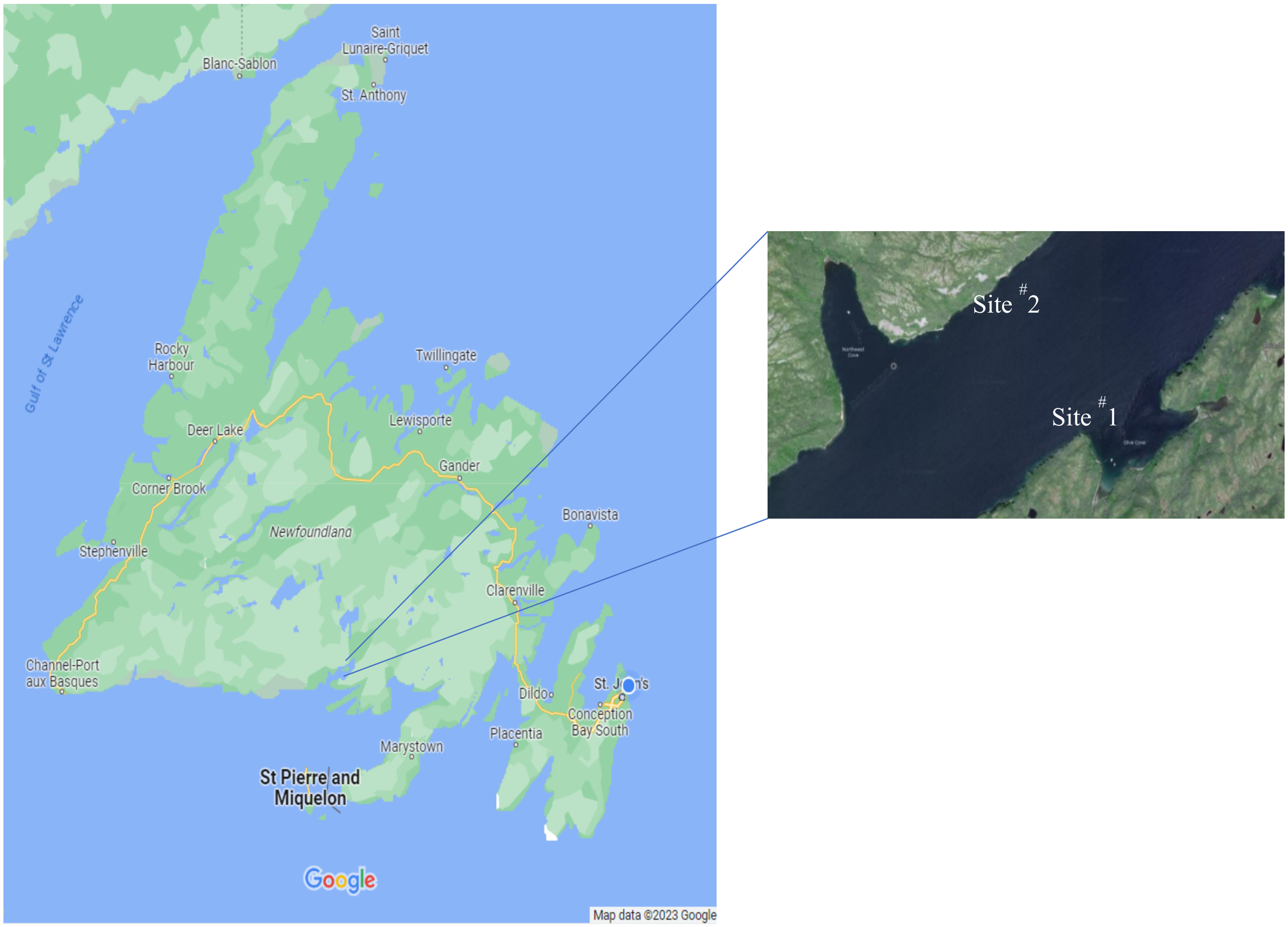

Figure S3. Cage-site locations on the south coast of Newfoundland, Canada based on Google Maps©.

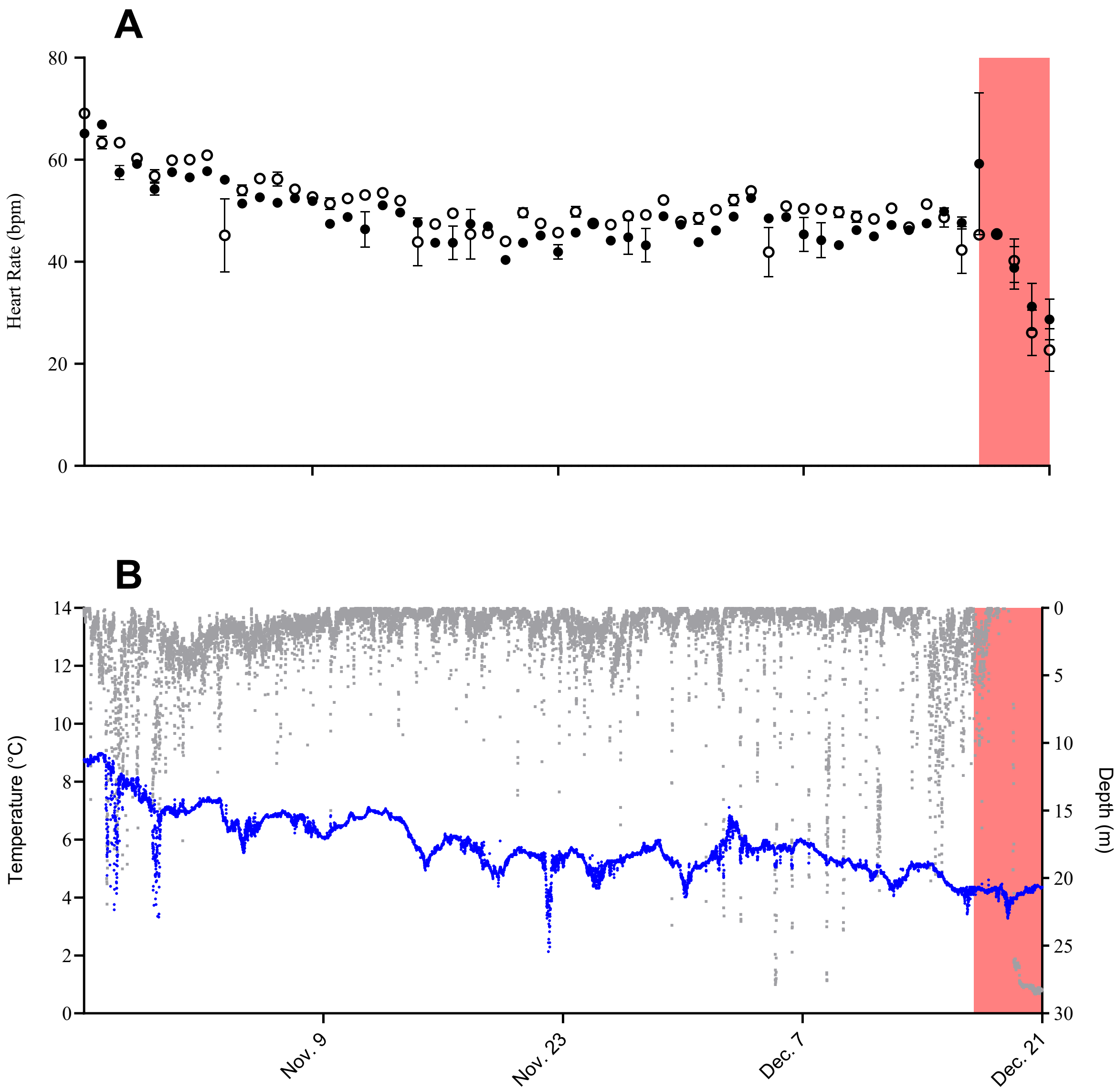

Figure S4. Heart rate (A) and temperature / depth (B) profiles for one fish retrieved ~2 months post-surgery at Site $^{\#}$1. (A) Average day-time (open circles) and night-time (closed circles) f_{H} based on recordings every 1 hour by a milli-HRT ACT tag (mean ± SEM). (B) depth (gray) and temperature (blue) data based on recordings made every 5 minutes by a milli-TD tag. The red shaded area indicates where f_{H} and depth decrease and increase, respectively, just before the last viable f_{H} is detected.

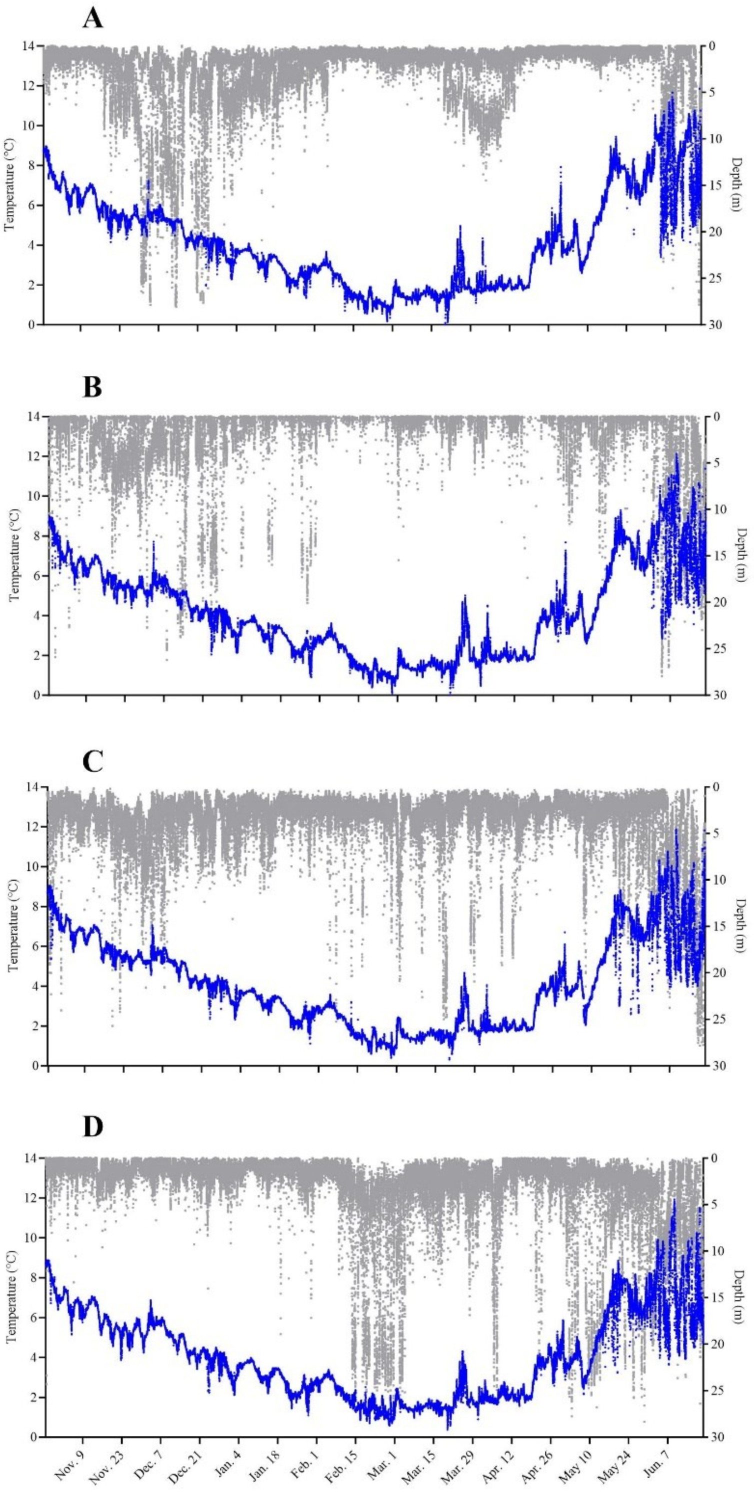

Figure S5. Depth (gray) and temperature (blue) data for 4 individual salmon based on recordings made every 5 minutes by milli-TD tags at Site #1 from tagging (October, 2020) until harvest (June, 2021). A-Fish 1, B-Fish 7, C-Fish 11, D-Fish 15 from Table S2.

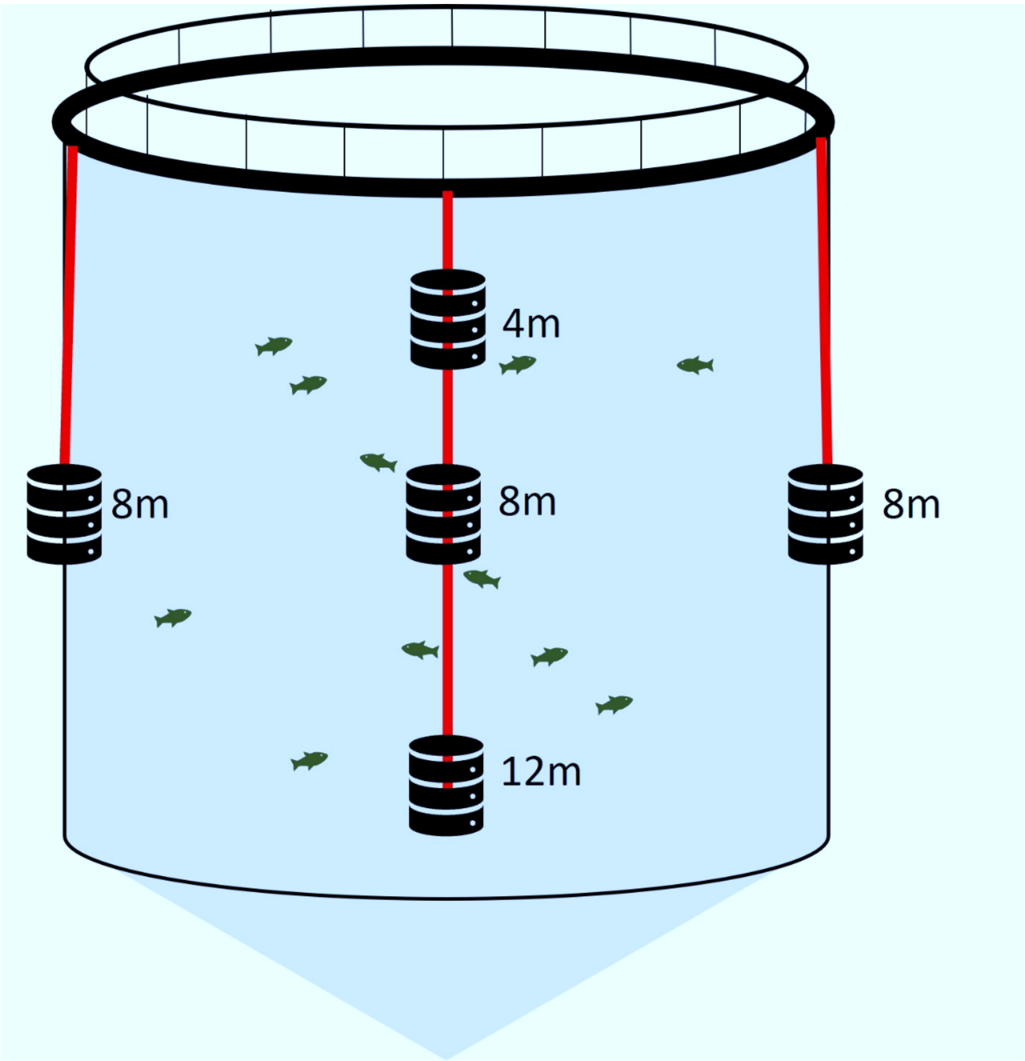

Figure S6. Schematic of acoustic receivers located in a commercial sea cage at depths of 4, 8 and 12 m.