

Acute and chronic cold exposure differentially affect cardiac control, but not cardiorespiratory function, in resting Atlantic salmon (*Salmo salar*)

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ABSTRACT

No studies have examined the effects of cold temperatures (~ 0 – 1 °C) on *in vivo* cardiac function and control, and metabolism, in salmonids. Thus, we examined: 1) how acclimation to 8 °C vs. acclimation (>3 weeks) or acute exposure (8–1 °C at 1 °C h⁻¹) to 1 °C influenced cardiorespiratory parameters in resting Atlantic salmon; and 2) if/how the control of cardiac function was affected. Oxygen consumption ($\dot{M}O_2$) and cardiac function [i.e., heart rate (f_H) and cardiac output (\dot{Q})] were 50% lower in the acutely cooled and 1°C-acclimated salmon as compared to 8 °C fish, whereas stroke volume (V_S) was unchanged. Intrinsic f_H was not affected by whether the fish were acutely exposed or acclimated to 1 °C (values ~ 51 , 24 and 21 beats min⁻¹ in 8 and 1 °C-acclimated fish, and 8–1 °C fish, respectively), and in all groups f_H was primarily under adrenergic control/tone (cholinergic tone 13–18%; adrenergic tone 37–70%). However, β -adrenergic blockade resulted in a 50% increase in V_S in the 1°C-acclimated group, and this was surprising as circulating catecholamine levels were ~ 1 –3 nM in all groups. Overall, the data suggest that this species has a limited capacity to acclimate to temperatures approaching 0 °C. However, we cannot exclude the possibility that cardiac and metabolic responses are evoked when salmon are cooled to ~ 0 –1 °C, and that this prevented further declines in these parameters (i.e., they ‘reset’ quickly). Our data also provide further evidence that V_S is temperature insensitive, and strongly suggest that changes in adrenoceptor mediated control of venous pressure/capacitance occur when salmon are acclimated to 1 °C.

1. Introduction

Climate change is predicted to increase average ocean temperatures by approximately 2–4 °C by the year 2100 (IPCC et al., 2021), and is increasing the severity and frequency of harmful conditions/events: including storms, marine heat waves, extreme reductions in water temperatures (i.e., ‘cold shock’ events) and hypoxia (Breitburg et al., 2018; Frölicher et al., 2018; Johnson et al., 2018; Oliver et al., 2018; Sampaio et al., 2021; Szekeres et al., 2016). Numerous biological functions in aquatic ectotherms, including fish, are affected by temperature as it is the ‘ecological master factor’ (Brett, 1971). Changes in physiological processes due to increased temperatures have been the main focus of climate change-related research in fish, and have involved measurements of upper thermal tolerance [critical thermal (CT_{Max}) and incremental temperature maxima (IT_{Max}): Gamperl et al., 2020; Gallant et al., 2017; Leeuwis et al., 2019; Zanuzzo et al., 2019]), and cardiorespiratory parameters. These parameters include oxygen consumption ($\dot{M}O_2$), cardiac output (the amount of blood pumped by the heart in 1

min; \dot{Q}), heart rate (f_H), stroke volume (the amount of blood pump in a heart beat; V_S), blood oxygen transport and tissue oxygen extraction (Anttila et al., 2014; Ekström et al., 2019; Gollock, 2006; Hvas et al., 2017; Leeuwis et al., 2021; Motyka et al., 2017; Penney et al., 2014) given their purported importance to thermal tolerance (Farrell et al., 2009; Jutfelt et al., 2018; Pörtner, 2010; Wang and Overgaard, 2007). However, with the exception of Antarctic species (Davison et al., 1997; Farrell and Steffensen, 2005), and a few other ‘polar’ species (Drost et al., 2016; Farrell et al., 2013; Franklin et al., 2013), there is very limited literature describing the effects of low (<5 °C) or cold (~ 0 –1 °C) temperatures on fish *in vivo* cardiovascular physiology. Costa et al. (2013) examined the effect of an acute temperature decrease from 8 to 1 °C, and acclimation to these temperatures, on *in vivo* cardiorespiratory parameters in the cunner (*Tautoglabrus adspersus*). However, this species is capable of metabolic depression at low temperatures (Costa et al., 2013; Gerber et al. in final prep), and thus, the results of this research are unlikely to be representative of most teleosts. Data on temperature dependent changes collected using *in situ* or *in vitro* preparations are limited (e.g., Aho and Vornanen, 2001; Gamperl and Syme, 2021;

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Abbreviations

AD	adrenaline
ADP	adenosine diphosphate
BSC	brain-sympathetic-chromaffin cell
CA	catecholamines
CT _{Max}	critical thermal maximum
CVP	central venous pressure
EDTA	ethylenediamine tetraacetic acid
f_H	heart rate
f_{Had}	adrenergic tonus
f_{Hch}	cholinergic tonus
f_{Hint}	intrinsic heart rate
Hct	haematocrit
Hb	haemoglobin
HPI axis	hypothalamus-pituitary-interrenal axis
IT _{Max}	incremental temperature maximum
K	condition factor
MCHC	mean cellular haemoglobin concentration

$\dot{M}O_2$	oxygen consumption
$\dot{M}O_2 / \dot{Q}$	tissue oxygen extraction
MS-222	tricaine methanesulfonate
NAD	noradrenaline
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
PO ₂	partial pressure of oxygen
PO _{max}	maximum power output
ppt	parts per thousand
\dot{Q}	cardiac output
Q ₁₀	fractional change in a rate over 10 °C
RMR	routine metabolic rate
ROS	reactive oxygen species
RVM	relative ventricular mass
SAN	sinoatrial node
SMR	standard metabolic rate
U _{crit}	critical swimming speed
V _S	stroke volume

Lurman et al., 2012), and may not accurately reflect temperature-dependent responses in *in vivo* cardiac performance. Finally, other literature on this topic is largely restricted to: the investigation of cardiac remodelling (including changes in relative ventricular mass; RVM) (Aho and Vornanen, 2001; Driedzic et al., 1996; Eliason and Anttila, 2017; Johnson et al., 2014; Keen et al., 2017; Klaiman et al., 2011); or to experiments where anaesthetized fish were given pharmacological agents (atropine and isoproterenol) and exposed to very rapid acute changes in temperature so that their ‘maximum’ heart rate and its Arrhenius breakpoint at high temperatures could be measured (e.g., see Gilbert et al., 2020; Gilbert and Farrell, 2021).

Increased levels of stress biomarkers (i.e., plasma concentrations of cortisol, adrenaline (AD) and noradrenaline (NAD), and of tissue heat shock proteins) are strongly linked to stress exposure, including thermal stress (Alzaid et al., 2015; LeBlanc et al., 2012; Leeuwis et al., 2021; Sandblom and Axelsson, 2011). The release of catecholamines (primarily AD and NAD) from the chromaffin cells of the head kidney is a central component of the stress response (Reid et al., 1998), and modulates cardiac performance in fish (Farrell and Smith, 2017). However, there is also adrenergic nervous tone on the heart which varies between species, and is critical for supporting cardiac function when fish are exposed to stressors, including changes in temperature (Sandblom and Axelsson, 2011). Pharmacological blockade can be used to determine the contribution of parasympathetic and sympathetic influences (Altimiras et al., 1997; Axelsson, 1988; Butler et al., 1989; Farrell et al., 1986) on f_H and \dot{Q} , and to determine a fish’s intrinsic heart rate [i.e., the frequency of action potentials generated by the pacemaker cells in the sinoatrial node (SAN)] when other influences are removed (Aho and Vornanen, 2001; Ekström et al., 2016a; Mendonça and Gamperl, 2009; Sandblom and Axelsson, 2011)]. Such research is important in the context of climate change as the time course for the resetting of cardiac frequency (f_H) following acute (within hours) or prolonged/chronic (days to months) changes with temperature are not well understood, and the data are contradictory/variable. For example, the time required to reset the f_H in rainbow trout was described by Sutcliffe et al. (2020) to be just 1 h in fish acclimated to 4 °C in winter and exposed to 12 °C, and 8 h for fish acclimated to 12 °C and transferred to 4 °C. However, these authors did not see such an effect even after 10 weeks of acclimation to these temperatures in summer-acclimated fish. In addition, other studies have concluded that the resetting of f_H requires several weeks in fishes (Aho and Vornanen, 2001; Ekström et al., 2016a).

Cold temperatures are a significant challenge to the Atlantic salmon aquaculture industry and can impact fish welfare and production,

especially in countries such as Canada and Iceland. Temperatures normally range seasonally and with depth at sea-cages from ~2 to 20 °C (Björnsson et al., 2007a and b; Gamperl et al., 2021; Gollock, 2006). However, chronic exposure to cold (~0–1 °C) temperatures during the winter months, as well as acute daily drops in temperature (to < 2 °C) due to ‘turn over’ caused by rough conditions, are associated with large-scale mortalities at salmon sea-cages in these countries (Círfc, 2020; CBC News, 2020; Huffman, 2019; The Canadian Press, 2015). Our understanding of how warm (summer) temperatures affect cultured Atlantic salmon has improved greatly over the past few years (Anttila et al., 2014; Beemelmans et al., 2020, 2021; Gamperl et al., 2020; Gamperl and Syme, 2021; Hvas et al., 2017; Penney et al., 2014; Tromp et al., 2018). However, we have a very limited understanding of how the biology and physiology of this species is affected by short-term and long-term exposure to cold temperatures, and the results of this research can be contradictory (e.g., see Liu et al., 2020 vs. Sandnes et al., 1988). As part of a larger, comprehensive, research program on how cultured Atlantic salmon are impacted by cold (~0–1 °C) temperatures (including ionoregulation, cage-site behaviour and physiology, levels of stress biomarkers, tissue damage etc.), this study examined how acute exposure (i.e., a temperature drop over 8 h) and chronic acclimation to 1 °C: 1) influenced the salmon’s resting cardiorespiratory function (f_H , V_S , \dot{Q} , and $\dot{M}O_2$); 2) affected blood O₂-carrying capacity [haematocrit and haemoglobin] and circulating stress hormone levels; and 3) affected cholinergic and adrenergic tones on the heart, and intrinsic f_H , in this species.

2. Materials and methods

2.1. Fish husbandry and rearing conditions

Male Atlantic salmon were held in 0.8 m³ tanks in the Annex Tank Room of the Ocean Science Center at Memorial University. The tanks were initially supplied with seawater (~32 ppt) with temperature and oxygen levels maintained at 8 °C and >95% of air saturation, respectively, and with a 12 h light: 12 h dark photoperiod. There were two tanks each with 25 fish, and after approx. 2–3 weeks in the tanks, one tank remained at 8 °C, whereas the other tank had its temperature decreased from 8 °C to 1 °C at 1 °C per week (i.e., over 7 weeks). After all fish were acclimated to their respective temperatures for >3 weeks (range 3–8 weeks), the fish were used in experiments to examine the effect of acclimation to 1 °C, and an acute (over 7 h) drop in temperature from 8 °C to 1 °C, on Atlantic salmon cardiorespiratory function and

stress physiology (see below). A custom-built chiller made by Technical Services at Memorial University was used to provide water at 1 °C. This was the lowest temperature used as it was difficult to consistently achieve a temperature of 0 °C with this system/avoid temperatures going below 0 °C. All fish were fed a commercial salmon diet (5 mm, EWOS, Canada) by hand at ~1% body mass three times per week (i.e., a maintenance diet) and fish were not fed within 24 h of surgery. The weight [1.1 ± 0.7 kg; mean \pm standard error (s.e.m.)], fork length (42.3 ± 0.9 cm), condition factor (1.47 ± 0.01), and relative ventricular mass ($0.0745 \pm 0.003\%$) were similar for all fish used in the experiment (see Supplementary material, Table S1).

2.2. Surgical procedures and recovery

Fish were netted from their tank, and anaesthetized in oxygenated seawater containing tricaine methanesulfonate (MS-222, 0.2 g L⁻¹; Syndel Laboratories Ltd., Qualicum Beach, BC, Canada) until ventilatory movements ceased. The fish were then weighed and measured for fork length, and placed supine on a wetted foam pad upon a surgical table where their gills were irrigated continuously with cooled (~4 °C) and oxygenated seawater containing a maintenance dose of MS-222 (0.1 g L⁻¹). Each fish was fitted with a dorsal aortic cannula (PE 50, Clay-Adams; Becton Dickensen and Co., Sparks, MD, USA) as in Smith and Bell (1964) and Gamperl et al. (1994a) to allow for blood collection and for the injection of antagonists of cardiac control (see below). Following cannulation, the salmon were placed on their right side, and a Doppler® flow probe (Model ES cuff-type Transducer, 20 MHz, Iowa Doppler Products, Iowa City, IA, USA) ranging from 1.3 to 2 mm in diameter (depending on fish size) was fitted around the ventral aorta using the same procedures as described for rainbow trout in Gamperl et al. (1994a). Finally, the flow probe lead was connected to a directional pulsed Doppler® flow meter (Model 545C-4; Bioengineering, University of Iowa, Ames, IA, USA) to ensure that the signal was of high quality, and the probe lead was secured to the fish at 3 locations: just posterior to the pectoral fin, just below the lateral line, and just ventral to the dorsal fin.

After surgery was completed, each fish (N = 9 fish per group) was placed in a 20 L cylindrical respirometer (20 cm in diameter \times 54.6 cm long) to recover for ~18–24 h (i.e., until the first morning prior to experiments). The respirometers were submersed in a shallow (25 cm deep), insulated, experimental water table containing fully aerated seawater at either 8 °C or 1 °C (the fish's acclimation temperature) and received a constant flow of water at a rate of 10 L min⁻¹ from a submersible Eheim 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).

2.3. Acute temperature decrease

The morning following surgery (~18–24 h post-surgery), the flow probe leads were connected to the Doppler® flow meter and the fish were left undisturbed for another ~1 h before initial measurements were taken. Then, two groups remained at their respective acclimation temperatures (8 and 1 °C), while the third group was exposed to a decrease in temperature from 8 °C to 1 °C at 1 °C h⁻¹. Cardiac function and oxygen consumption measurements were subsequently made in all groups at times corresponding to every 1 °C decrease in temperature in the third group, during the second night (every 20 min), at 48 h after surgery (the second morning), and before and after the administration of antagonists of parasympathetic and sympathetic cardiac control. These pharmacological antagonists [atropine sulfate (1.2 mg kg⁻¹), bretylium tosylate (10 mg kg⁻¹) and propranolol hydrochloride (3 mg kg⁻¹)] were sequentially injected (separated by 45 min) through the cannula, followed by 0.4 mL of saline (0.9% NaCl) to clear/flush the cannula (Mendonça and Gamperl, 2009). Blood samples (1.5 mL) were

withdrawn from the dorsal aortic cannula and immediately replaced with saline at three time points: 1) 24 h post-surgery (i.e., prior to changes in temperature); 2) 1 h after the treatment fish reach 1 °C (and at the same time in the other groups); and 3) just prior to the injection of the pharmacological antagonists (i.e., approx. 48 h post-surgery). A schematic representation of the experimental design is provided in Fig. 1.

After the experiment was completed, all fish were euthanized using a lethal dose of 0.4 g L⁻¹ of MS-222, and an *in situ* post-mortem calibration of the flow probes was performed at physiologically relevant pressures using a peristaltic pump (MasterFlex Easyload®, Quebec, Canada) (Gamperl et al., 1994a) and a 'blood mimicking' solution (0.99% glycerol, 2.4% TritonX, 35% Orgasol in 200 mL of distilled water; Axelsson, pers. comm.). To accomplish this, after removal of the sinus venosus and atrium, the ventricle was bisected laterally, and a steel cannula attached to peristaltic pump tubing was tied into the ventricular lumen. Calibration was successful in approx. 80% of the fish. Finally, the two halves of the ventricle were weighed, and relative ventricular mass (RVM) was calculated as:

$$[(\text{ventricular mass} / \text{fish mass}) \times 100] \quad (1)$$

2.4. Cardiorespiratory function and neurohormonal control

Oxygen consumption ($\dot{M}O_2$; in mg O₂ kg⁻¹ h⁻¹) was measured using automated intermittent closed respirometry (Sandblom et al., 2014) and using methods consistent with recommendations for aquatic respirometry as detailed in Killen et al. (2021), Rodgers et al. (2016) and Svendsen et al. (2016). The flush pump and recirculation pump (both Eheim Model 1048; 10 L min⁻¹) were controlled by AutoResp® software (Version 2.1.0; LoligoSystems, Tjele, Denmark), and were intermittently turned on or off to either flush the respirometer with fresh seawater when on, or create a functionally sealed respirometry chamber when off. The duration of the 'flush' and/or 'recirculation' periods were adjusted throughout the experimental period to ensure an R² > 0.75 and to avoid a PO₂ inside the respirometers lower than 85% air saturation. This lower limit ensured that the water in the respirometer would return to 100% air saturation prior to the next measurement, and that the haemoglobin leaving the gills would still be near full O₂ saturation (Nikinmaa and Soivio, 1979). An R² > 0.75 was used due to the large signal to noise ratio exhibited by fish at colder temperatures (i.e., at 1 °C) when at rest or during measurements of SMR (see below), to ensure that $\dot{M}O_2$ was not overestimated (Chabot et al., 2020). However, the average R² was >0.9. $\dot{M}O_2$ recordings were made using an OXY-4 mini fiber-optic oxygen meter fitted with pre-calibrated dipping probes (PreSens Precision Sensing GmbH, Regensburg, Germany) that was interfaced with DAQ-4 and TEMP-4 modules (LoligoSystems, Tjele, Denmark). The signals from these modules were then fed into a computer running AutoResp® software. The rate of oxygen decline during the closed phase of the respirometry cycle (i.e., when the flush pump was off) was used by the AutoResp® software to calculate the $\dot{M}O_2$ of the fish after a 2 min 'wait' period at the beginning of the closed period (which varied between 5 and 15 min depending on temperature to ensure an acceptable R²). Given the limited number of $\dot{M}O_2$ measurements, standard metabolic rate (SMR) was determined during the 18h following day 2 (i.e., after the acute temperature decrease) by calculating the mean of the lowest 20% of the $\dot{M}O_2$ measurements after the removal of values with an R² < 0.75 (2/396, 66/392, and 33/332 for 8, 1 and 8 to 1 °C fish, respectively) (Chabot et al., 2016). To avoid external influences on fish cardiorespiratory parameters (Speers-Roesch et al., 2018), the lights remained on following surgery until the end of the experiments, and the water table was surrounded by 0.8 m high corrugated black plastic sheeting to prevent the fish from being disturbed by the presence of

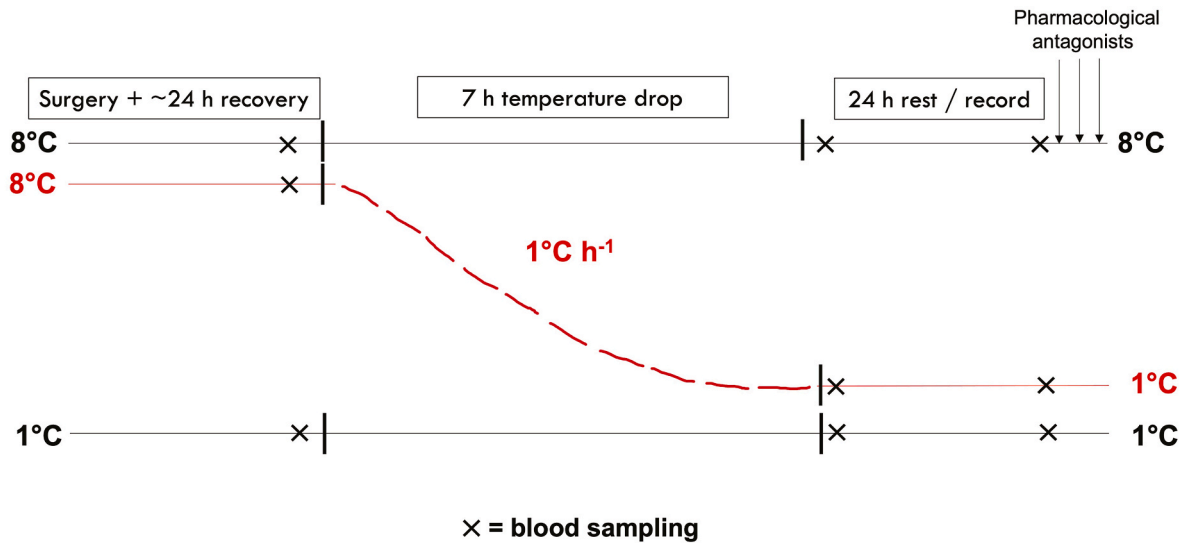


Fig. 1. Schematic diagram depicting the experimental design used to assess the effect of cold temperatures on the cardiorespiratory and stress physiology of the 3 groups of fish: acclimated and tested at 8 °C; acclimated and tested at 1 °C; acclimated to 8 °C and acutely exposed to a drop in temperature to 1 °C. The cardiorespiratory parameters (f_{H} , V_{S} , \dot{Q} and $\dot{M}\text{O}_2$) were recorded at the fish's acclimation temperature, at each 1 °C decrease in temperature, at rest on the second morning, and before each of the drug injections (atropine sulfate, bretylium tosylate and finally propranolol hydrochloride; separated by 45 min). Blood samples for various haematological parameters were taken (as indicated by an X) at the fish's acclimation temperature, an hour after these fish reached 1 °C, and on the morning of the 2nd day prior to drug injection (note: all groups were sampled at the same time points).

research personnel. Background measurements of $\dot{M}\text{O}_2$ were made in empty chambers at the end of the experiments, and these were negligible (<1%), indicating that no substantial microbial respiration was occurring (Rodgers et al., 2016; Svendsen et al., 2016).

Heart rate and cardiac output were recorded by connecting the flow probe leads to a pulsed Doppler® flow meter. Signals from the Doppler® flow meter were amplified and filtered using a data acquisition system (MP100A-CE; BIOPAC Systems, Inc., Santa Barbara, CA, USA) and a universal interface module (UIM100C; BIOPAC Systems, Inc.) connected to a laptop computer running AcqKnowledge® software (Version 3.8.2; BIOPAC Systems, Inc.). Heart rate (f_{H} ; beats min^{-1}) was determined by measuring the number of systolic peaks during two 30s intervals during the period when $\dot{M}\text{O}_2$ was being measured. The values for \dot{Q} were recorded in volts (V), and were converted to $\text{mL min}^{-1} \text{kg}^{-1}$ using the data from the calibrations described previously and the fish's weight (in kg). Stroke volume (V_{S}) was calculated as \dot{Q}/f_{H} . Oxygen extraction (the amount of O_2 consumed per mL of blood pumped) was estimated by dividing $\dot{M}\text{O}_2$ by \dot{Q} .

Q_{10} values (i.e., the fractional change in a rate over a 10 °C range) were calculated as an index of the effect of chronic (acclimation) and acute temperatures (T) changes (8 to 1 °C) on $\dot{M}\text{O}_2$, f_{H} and \dot{Q} and $\dot{M}\text{O}_2/\dot{Q}$ (R) using the following equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{(T_2-T_1)}\right)} \quad (2)$$

The 'intrinsic' f_{H} (f_{Hint} ; beats min^{-1}) after the administration of all drugs, cholinergic tonus (% f_{Hch} ; %) and adrenergic tonus (% f_{Had} ; %) were calculated for the salmon's heart rate as described by Axelsson (1988):

$$\% f_{\text{Hch}} = [(f_{\text{H}} \text{ after atropine} - f_{\text{H}} \text{ before atropine}) / f_{\text{Hint}}] \times 100 \quad (3)$$

$$\% f_{\text{Had}} = [(f_{\text{H}} \text{ after atropine} - f_{\text{Hint}}) / f_{\text{Hint}}] \times 100 \quad (4)$$

After the study was completed, we discovered that 45 min [as was used in Mendonça and Gamperl, 2009 and on which our methods were based] was likely not long enough to ensure that bretylium completely prevented the release of catecholamines from sympathetic nerve

terminals (e.g., see Smith et al., 1985). Thus, the % contribution of nervous vs. humoral influences on the adrenergic tonus were not calculated, and the data on the effects of bretylium tosylate on cardiac function are only presented in the supplement (Fig. S2). These effects were relatively minor, and would not have influenced the effects of propranolol on cardiac function as this latter drug would block both neural and humoral β_1 - and β_2 -adrenergic effects on cardiac function.

2.5. Haematological parameters

Blood samples (1.5 mL) were collected (see previously provided sampling details) and immediately aliquoted for the analysis of various haematological parameters. First, blood was drawn into micro-haematocrit tubes and centrifuged at 10,000×g for 2 min to determine haematocrit (Hct; %). An aliquot of 50 μL of whole blood was then collected for the measurement of blood haemoglobin (Hb) concentration using the cyanomethaemoglobin method (Drabkins reagent, D5941; Sigma Aldrich, Oakville, Canada) with absorbance read at 540 nm using a plate reader (SpectraMax 5, Molecular Devices, San Jose, USA). Hb concentrations were calculated from standard curves generated using bovine Hb (Sigma, H2500). Mean cellular Hb concentration (MCHC, in mg mL^{-1}) was calculated as Hb concentration/Hct \times 100. The remaining blood sample was centrifuged for 1 min at 10,000×g in a mini-centrifuge (05-090-128, Fisher Scientific). Three hundred μL of plasma was then pipetted into a 1.5 mL, opaque, Eppendorf® tube containing 15 μL of 0.2 M EDTA and 15 μL of 0.15 M glutathione (which served as antioxidants) for later measurement of circulating catecholamine levels. The remaining plasma was pipetted into 50 μL aliquots for the measurement of plasma cortisol and lactate. All samples were immediately frozen in liquid N_2 and stored at -80°C .

2.6. Measurement of plasma lactate, cortisol and catecholamines

ELISA kits were used to measure plasma levels of cortisol (Neogen Life Sciences, 402710, Lexington, KY, USA) and adrenaline and noradrenaline (Abnova KA1877, Taipei, Taiwan), following the manufacturer's instructions. Plasma samples for catecholamine measurement were analyzed within 2 months of storage at -80°C . Plasma lactate was first deproteinized with 6% (v/v) perchloric acid, then measured

spectrophotometrically at 340 nm using the production of NADH/NADPH by lactate dehydrogenase (Sigma L2500), and [lactate] was calculated in reference to standard curves (Sigma L6402).

2.7. Statistical analyses

All statistical analyses were performed using Rstudio v. 1.4.1717 with R v. 4.1.0. A Rosner's Test for outliers ($\alpha = 0.05$; which was selected as the best test for the identification of outliers by R) was used on all datasets prior to statistical analysis. This analysis revealed that there were very few outliers: these include the majority of cardiorespiratory measurements for one fish in the 8°C-acclimated group, and lactate data at one sampling point for one of the acutely cooled fish. A general linear model (lm function) and ANOVAs were used to assess the main factor of 'group' on morphometric variables [mean weight (g), fork length (cm), condition factor (K) and RVM], SMR, intrinsic heart rate ($f_{H_{int}}$), and the various values of 'tone' on f_H between the three groups. If there was a significant effect ($p < 0.05$), a Tukey's HSD *post-hoc* test examined where the differences occurred. A general linear mixed model (lmer function) was used to analyze all cardiorespiratory, pharmacologically-induced and haematological data. This model included 'fish' as a random factor, and 'group' and 'time' of measurement and their interaction as fixed effects. Main effects were analyzed using ANOVAs (anova function) with type III sums of squares, and if the model indicated a significant fixed effect ($p < 0.05$), a Bonferroni (fdr method) *post-hoc* test identified statistical differences. Data for plasma lactate were transformed ($x^{-0.333}$) prior to statistical analysis as this parameter failed normality testing (Shapiro-Wilks). All data shown are means \pm s.e.m, and $p < 0.05$ was used as the threshold for determining statistical significance.

3. Results

3.1. Haematological response to cold exposure

There were no differences in Hct or [Hb] between the groups.

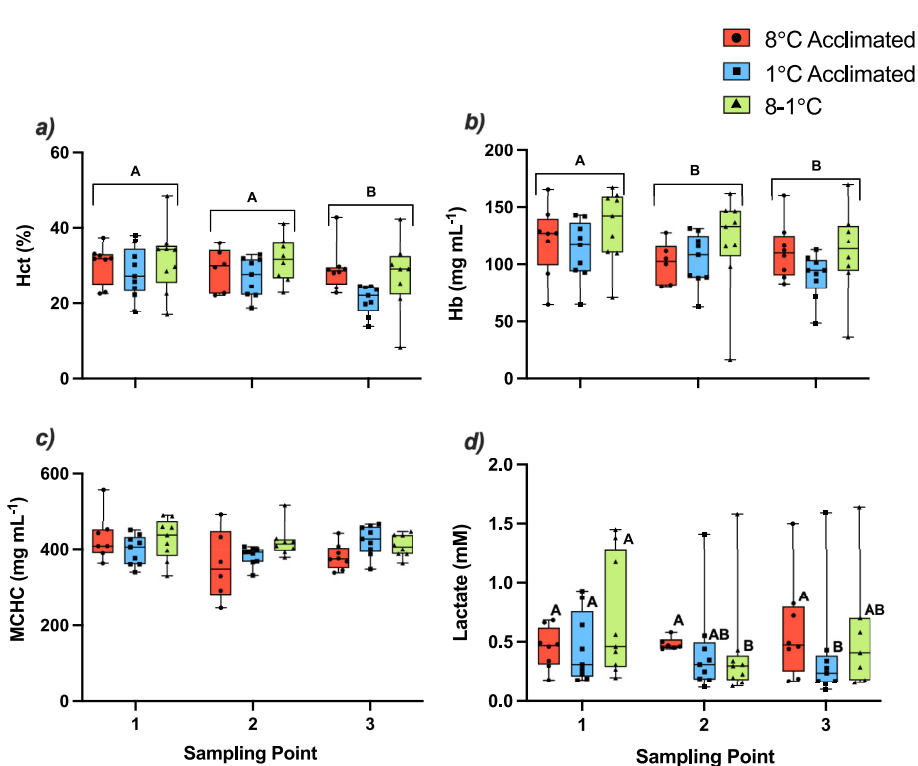


Fig. 2. Haematological parameters in Atlantic salmon acclimated to 8 (red, ●) and 1 °C (blue, ■), and acutely cooled from 8 to 1 °C (green, ▲). Shown are graphs of (a) haematocrit (Hct), (b) haemoglobin (Hb), (c) mean cellular Hb concentration (MCHC), and (d) plasma lactate. Symbols without an uppercase letter in common are significantly different ($p < 0.05$) between the sampling points within a group. There were no significant differences between groups at a particular sampling point. Values are means \pm s.e.m. with $n = 6-9$ per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

However, on average, all groups had slightly lower Hct (%) values during the third sampling as compared to the first sampling (values \sim 28–30%), in addition to significantly lower [Hb] during the second and third samplings (Fig. 2a and b) compared to the first sampling (108.9 ± 6.0 mg mL⁻¹ and 103.4 ± 5.6 mg mL⁻¹ vs 122.2 ± 5.7 mg mL⁻¹, respectively). No significant differences in MCHC were observed during this experiment, with values approx. 350–450 mg mL⁻¹ (Fig. 2c). Lactate decreased significantly in fish exposed to the gradual decrease in temperature from the first to second sampling (an hour after they had reached 1 °C); i.e., from 0.69 ± 0.17 mM to 0.41 ± 0.15 mM, respectively (Fig. 3d), and the following morning it was 0.57 ± 0.17 mM. However, these values were not significantly different than those measured in the other two groups.

Acclimation and sampling point also had an interactive effect ($p < 0.001$) on cortisol concentrations in these instrumented and confined fish (Fig. 3a). Acclimation to 1 °C resulted in significantly higher cortisol concentrations (82.6 ± 8.2 ng mL⁻¹) at the first sampling post-surgery than measured in both the 8 °C acclimated and 8 °C fish that were to be acutely cooled (47.9 ± 5.0 ng mL⁻¹, $p < 0.01$ and 41.2 ± 4.9 ng mL⁻¹, $p < 0.001$, respectively). Cortisol levels increased (although not significantly) 1 h after the 8 – 1 °C fish reached 1 °C (to 54.4 ± 8.4 ng mL⁻¹), and again to 66.2 ± 9.6 ng mL⁻¹ by the third sampling point ($p < 0.05$ between the first and third samplings). Adrenaline concentrations were, on average, lower during the second sampling in all groups, but then increased slightly in the fish acutely exposed to 8-1 °C (1.7 ± 0.3 nM to 2.3 ± 0.5 nM, respectively, from the second to third sampling). However, [AD] were extremely low and the changes did not reach the significance level of $p < 0.05$. (Fig. 3b). Noradrenaline levels were significantly higher at the first sampling (approx. 18 h post-surgery) in the fish that were to be acutely cooled as compared to the 1 °C acclimated fish (Fig. 3c), and this trend was consistent throughout the experiment. However, it is important to note that levels of AD and NAD were very low ([mean] < 2.5 nM over the entire experiment).

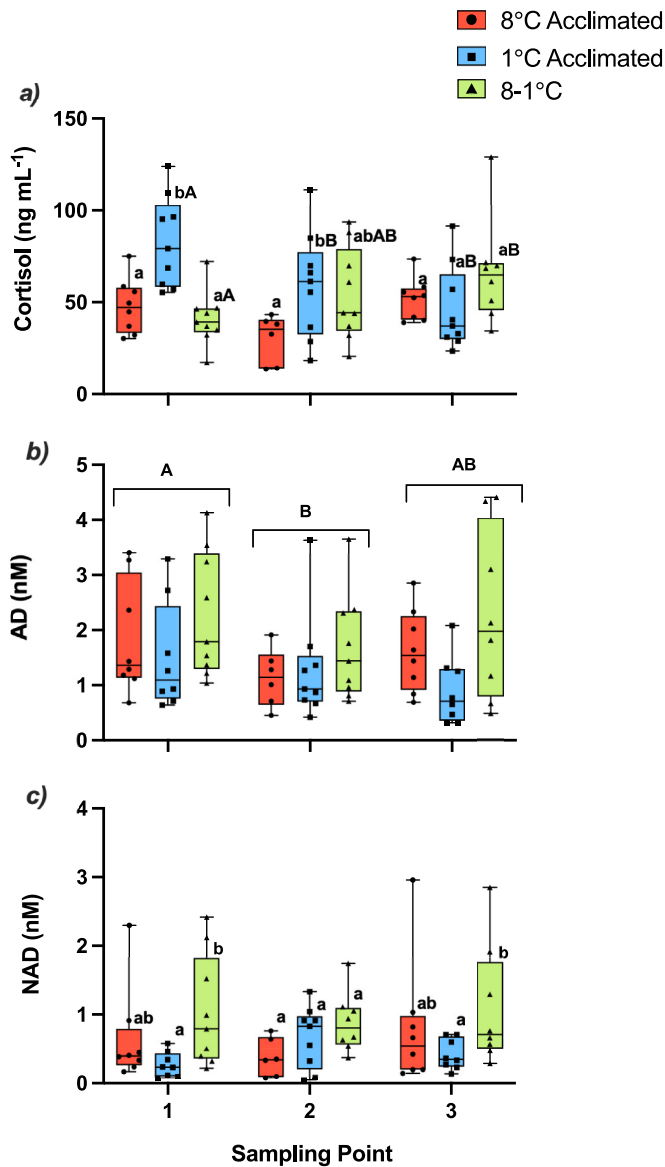


Fig. 3. Changes in stress hormone levels in Atlantic salmon acclimated to 8 °C (red, ●) and 1 °C (blue, ■), and acutely cooled from 8 to 1 °C (green, ▲). Shown are graphs of (a) cortisol, (b) adrenaline (AD) and (c) noradrenaline (NAD). Symbols without a letter in common are significantly different ($p < 0.05$) between groups at a particular sampling point (lowercase) and between sampling points (uppercase). Values are means \pm s.e.m. with $n = 6-9$ per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Cardiorespiratory response to cold exposure

Atlantic salmon acclimated to 1 °C had a significantly lower initial f_H than both the 8 °C acclimated group ($p < 0.0001$) and the 8 °C fish that were to be acutely cooled ($p < 0.0001$) (31.2 ± 1.4 beats min^{-1} vs 59.8 ± 2.6 beats min^{-1} and 62.3 ± 1.5 beats min^{-1} , respectively) (Fig. 4a). However, there was a significant interaction between acclimation temperature and measurement time ($p < 0.0001$) on f_H (see Supplementary Table S2). The fish acutely exposed to decreasing temperature had a significantly lower f_H when compared to 8 °C acclimated fish starting at 6 °C (52.7 ± 1.5 vs. 62.6 ± 1.4 beats min^{-1} , respectively, $p < 0.0001$), but higher values than those fish already acclimated to 1 °C until 2 °C. At 2 and 1 °C (i.e., after 6 and 7h) acutely cooled fish had f_H values of 35.7 ± 1.0 and 32.1 ± 0.8 beats min^{-1} , respectively, vs. 33.1 ± 0.5 and 32.5

± 0.8 beats min^{-1} for 1 °C acclimated fish, and these values were not significantly different.

Stroke volume (V_S) remained relatively constant in all groups throughout the experiment (~ 0.25 mL kg^{-1}) (Fig. 4b). Thus, V_S did not compensate for the decrease in f_H as the 8 °C fish were acutely cooled to 1 °C. This resulted in a significant decrease in \dot{Q} in these fish after they reached 3 °C (from 11.5 ± 0.8 mL $\text{min}^{-1} \text{kg}^{-1}$ at 8 °C to 9.0 ± 1.2 mL $\text{min}^{-1} \text{kg}^{-1}$ at 3 °C, $p < 0.05$; Fig. 4c). This was the same temperature at which these fish had similar \dot{Q} values to 1 °C acclimated fish (7.2 ± 0.9 mL $\text{min}^{-1} \text{kg}^{-1}$, $p = 0.254$). By 1 °C, fish exposed to the gradual decrease in temperature had a \dot{Q} of 7.1 ± 1.3 mL $\text{min}^{-1} \text{kg}^{-1}$, and this was very similar to that recorded for the 1 °C acclimated salmon at this time point (6.1 ± 0.3 mL $\text{min}^{-1} \text{kg}^{-1}$).

Resting values of $\dot{M}O_2$ during the first measurement were significantly lower ($p < 0.01$) in the 1 °C acclimated group than both the 8 °C acclimated group and the fish to be acutely cooled (27.1 ± 1.8 mg O_2 $\text{kg}^{-1} \text{hr}^{-1}$ vs 53.6 ± 5.4 mg O_2 $\text{kg}^{-1} \text{hr}^{-1}$ and 61.5 ± 6.3 mg O_2 $\text{kg}^{-1} \text{hr}^{-1}$, respectively) (Fig. 4d). $\dot{M}O_2$ in the fish acutely exposed to lower temperatures decreased by 2-fold between 8 and 1 °C, and was not significantly different as compared to the 1 °C acclimated fish at this temperature (33.7 ± 5.2 vs. 24.1 ± 1.7 mg O_2 $\text{kg}^{-1} \text{hr}^{-1}$, respectively). $\dot{M}O_2/\dot{Q}$ was not significantly affected by either the chronic or acute changes in temperature. Under all of these conditions it was approximately 0.07 ± 0.004 mg O_2 mL blood $^{-1}$ (Fig. 4e).

Changes in cardiac and metabolic parameters occurred rapidly in Atlantic salmon when acutely exposed to cold temperatures (~ 1 °C). For example, fish that were chronically acclimated to 1 °C had only slightly lower cardiorespiratory Q_{10} values for \dot{Q} and f_H as compared to those acutely exposed to this temperature change (Table 1). Further, the Q_{10} values were all above 2.5, and this suggests that there was limited thermal compensation when the salmon were acclimated to cold temperatures as compared to acutely exposed. The overnight measurements of standard metabolic rate (SMR) following the temperature change were significantly lower in both the 1 and 8 to 1 °C fish as compared to the 8 °C acclimated fish (Table 2). The SMR values at 8 and 1 °C (~ 45 and 20 mL O_2 $\text{kg}^{-1} \text{hr}^{-1}$; Table 2) were approximately 67 and 85% of routine $\dot{M}O_2$ values just prior to the injection of the pharmacological antagonists (66.5 ± 6.2 ; and 21.6 ± 1.5 and 28.5 ± 3.1 mL O_2 $\text{kg}^{-1} \text{hr}^{-1}$, respectively).

3.3. Neurohormonal control on the heart

Heart rate increased significantly in all groups (by ~ 4.1 beats min^{-1} , 10%) after atropine injection (the blockade of cardiac muscarinic receptors; $p < 0.01$), whereas propranolol (β_1 - and β_2 adrenoreceptor blockade) decreased f_H by approximately 7.0 beats min^{-1} or 21% ($p < 0.0001$) (Fig. 5a). The calculated cholinergic and adrenergic, 'tones' on the heart were not different between the 8 °C and 1 °C acclimated fish, with values ranging from ~ 12 to 15% to 36–53%, respectively (Table 2). However, the 8-1 °C group had a significantly higher adrenergic tone ($69.8 \pm 9.5\%$) than the 8 °C acclimated fish ($p = 0.006$; Table 2). Intrinsic f_H was not significantly different between the 1 °C and 8-1 °C groups (23.6 ± 0.9 beats min^{-1} and 20.9 ± 1.1 beats min^{-1} , respectively), but these values were less than 50% of that measured in the 8 °C acclimated group (50.5 ± 1.3 beats min^{-1}) (Q_{10} value ~ 3).

Atropine injection had no effect on \dot{Q} or V_S (Fig. 5b and c). Propranolol injection also had few effects on these parameters in the three groups. However, there was a significant increase in V_S after propranolol injection in the 1 °C acclimated group (by 55%), and this resulted in this parameter being significantly greater in this group than in the 8-1 °C group at the end of the experiment (Fig. 5). This result/effect was even more apparent when the raw (in volts beat $^{-1}$) and relative (in % change from initial values) data for V_S are shown [i.e.; so that fish where the calibration was not successful could be included in the analysis

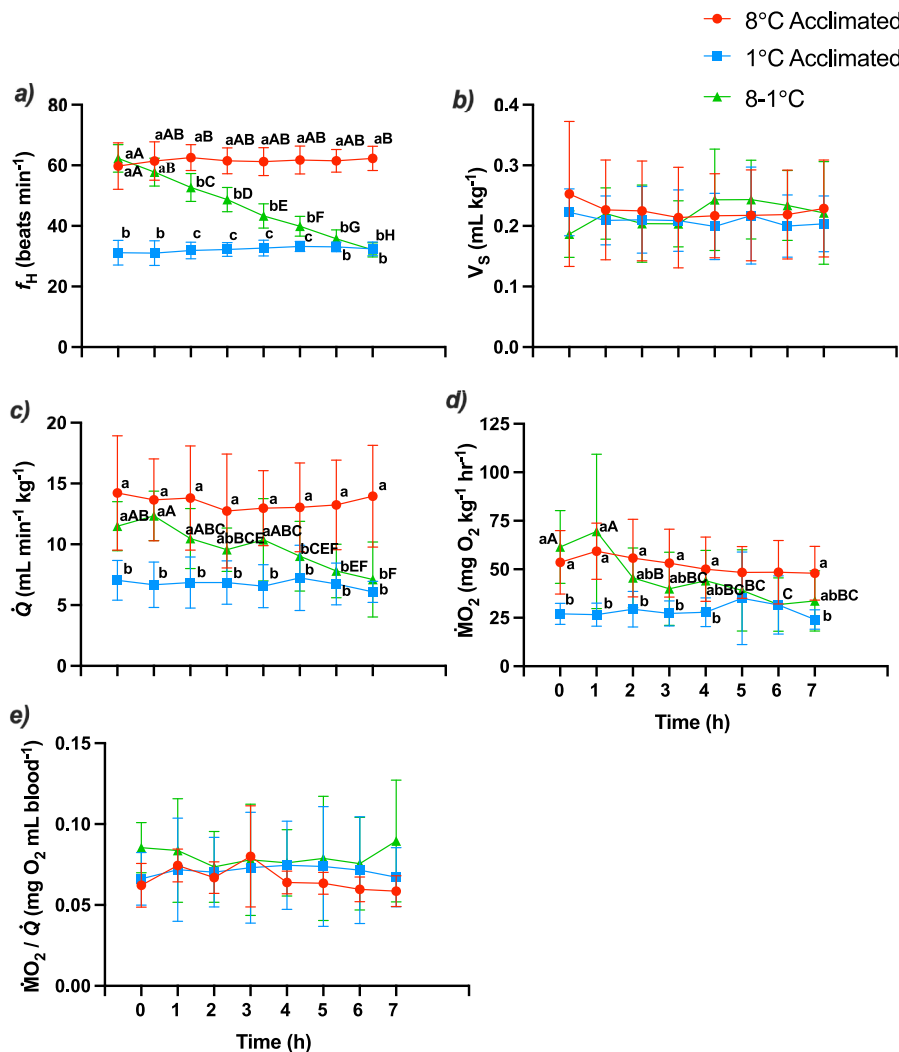


Fig. 4. Cardiorespiratory responses of Atlantic salmon acclimated to 8 (red, ●) and 1 °C (blue, ■), and acutely cooled from 8 to 1 °C (green, ▲). Shown are (a) heart rate (f_H), (b) stroke volume (V_S), (c) cardiac output (\dot{Q}), (d) oxygen consumption ($\dot{M}O_2$), and (e) oxygen extraction ($\dot{M}O_2 / \dot{Q}$). Symbols without a letter in common are significantly different ($p < 0.05$) between groups (lowercase) at a particular measurement time point, and between measurements time points within a group (uppercase). Values are means \pm s.e.m. with $n = 6-9$ per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
 Q_{10} values for the effect of chronic (8 vs 1 °C acclimation) and acute cold (8–1 °C over 7h) exposure on cardiorespiratory parameters.

Q_{10}	Chronic	Acute
f_H	2.56	2.62
\dot{Q}	2.73	2.98
$\dot{M}O_2$	2.37	2.86
$\dot{M}O_2 / \dot{Q}$	0.90	0.94

Table 2
 The standard metabolic rate (SMR; prior to drug injections), intrinsic heart rate (f_H), and ‘tone’ on the heart as determined using a series of pharmacological injections. Values without a letter in common are significant at $p < 0.05$; $n = 9$ per group.

	8 °C Acclimated	1 °C Acclimated	8-1 °C
<i>Cardiorespiratory Measurements:</i>			
SMR ($\text{mg O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$)	44.6 \pm 5.5 ^a	18.3 \pm 0.7 ^b	23.0 \pm 1.7 ^b
RMR ($\text{mg O}_2 \text{ kg}^{-1} \text{ hr}^{-1}$)	66.5 \pm 6.2 ^a	21.6 \pm 1.5 ^b	28.5 \pm 3.1 ^b
Intrinsic f_H (beats min^{-1})	50.5 \pm 1.3 ^a	23.6 \pm 0.9 ^b	20.9 \pm 1.1 ^b
<i>Tone (%):</i>			
Cholinergic	12.5 \pm 4.1	15.8 \pm 4.4	17.7 \pm 3.1
Adrenergic	35.7 \pm 3.5 ^a	52.5 \pm 5.5 ^{ab}	69.8 \pm 9.5 ^b

(Fig. S1)]. In fact, this data shows that V_S after propranolol injection was higher in the 1 °C acclimated group as compared to both of the other groups (Fig. S1).

4. Discussion

This study adds greatly to our understanding of how the cardiorespiratory physiology of Atlantic salmon (and likely many other temperate fishes) responds when they are exposed to temperatures approaching 0 °C. This research shows that there are few differences in morphometric and haematological parameters, cardiac function, or the metabolism of resting Atlantic salmon (*Salmo salar*) acclimated to 1 °C as compared to those acutely (over several hours) exposed to this temperature. These data suggest that there is limited thermal compensation when salmon are acclimated to, vs. acutely exposed to, temperatures approaching 0 °C. However, we cannot exclude the possibility that cardiorespiratory parameters ‘reset’ quickly following acute exposure to these temperatures (i.e., these values would be lower if exposure to 1 °C was much quicker; < 1 h). In addition, we report a number of important differences between the groups. First, our data show that, just like exposure to warm temperatures, V_S plays no role in temperature-dependent changes in resting cardiac function. Second, plasma cortisol levels were elevated in both groups at 1 °C as compared to those held at 8 °C, suggesting that salmon at this temperature are under increased stress. Third, although circulating catecholamine levels were not elevated in fish acutely exposed to 1 °C, adrenergic tone appeared to

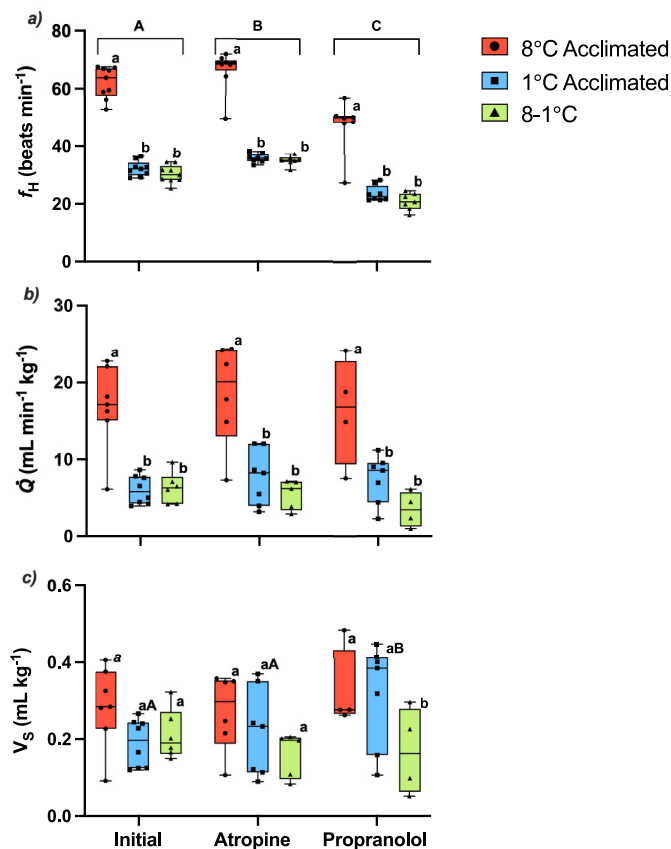


Fig. 5. Changes in cardiac function [(a) heart rate (f_H), (b) cardiac output (\dot{Q}), and (c) stroke volume (V_S)] in Atlantic salmon acclimated to 8 (red, ●) and 1 °C (blue, ■), and acutely cooled from 8 to 1 °C (green, ▲), when injected with the pharmacological antagonists atropine sulfate (to block cholinergic nervous tone) and propranolol hydrochloride (to block β_1 - and β_2 -adrenoreceptors). Symbols without a letter in common are significantly different ($p < 0.05$) between groups (lowercase) and between the pharmacological antagonists within a group (uppercase). Values are means \pm s.e.m. with $n = 4$ –9 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

play a more prominent role in these fish with regards to the control of f_H , and removing this tone on the heart resulted in an increase in V_S in the 1°C-acclimated fish that was not observed in the other two groups. These latter results suggest that important changes in adrenergic control/responsiveness occur in the cardiovascular system of salmon when exposed to cold temperatures (0–1 °C), but that the changes are specific to the duration of exposure.

4.1. Effect of cold temperatures on morphology and blood parameters

4.1.1. Haematocrit, haemoglobin and RVM

An adaptation that helps to decrease blood viscosity (a haematological parameter that increases at low/cold temperatures (Axelsson, 2005; Eginton, 1996)) is the reduction, or in extreme cases the complete absence, of Hct and Hb. These changes are well documented in polar and Antarctic fish species (Farrell and Steffensen, 2005; Holetton, 1970; Ruud, 1954). Further, to counteract the decrease in oxygen carrying capacity due to reduced Hct and [Hb], and to assist in pumping blood at low/cold temperatures, an increase in relative ventricular mass (RVM) is also a common (but not universal) observation (Aho and Vornanen, 2001; Driedzic et al., 1996; Graham and Farrell, 1989; Kent et al., 1988; Klaiman et al., 2011). For example, although rainbow trout (*Oncorhynchus mykiss*) acclimated to 5 °C for 4 weeks had a 50% greater RVM than 15 °C acclimated fish (Graham and Farrell, 1989), Klaiman et al.

(2014) reported no differences in RVM when this species was acclimated to 4, 11 or 17 °C for 8 weeks. It is very unlikely that the acclimation period during our current experiment was not long enough to induce an increase in RVM, and it could be that the extent of the temperature decrease (to 1 °C, a value much lower than the above studies) played a role. For example, a recent study (Gamperl et al., 2020) suggested that the salmonid heart's response to increased temperatures is dependent on the maximum temperature to which it is exposed; i.e., RVM only increases at warm temperatures in salmon that are reared at temperatures very close to their upper thermal limits. Additional research, at a range of temperatures at the lower end of their thermal niche, will be required to understand how the RVM of Atlantic salmon responds to decreasing temperatures.

Blood oxygen carrying capacity (i.e., Hct and Hb) and oxygen delivery to the tissues are generally considered to be large contributors to fish thermal tolerance, particularly at warm temperatures (Anttila et al., 2013; Leeuwis et al., 2021; Muñoz et al., 2018; Pörtner and Knust, 2007; Pörtner, 2010; Wang and Overgaard, 2007). It is quite common for fish to release erythrocytes (red blood cells; RBCs) via splenic contraction within minutes of an acute stress as a secondary stress response (Muñoz et al., 2018; Pearson and Stevens, 1991), and this results in an increase in Hct (i.e., in the number and/or in size of RBCs). However, this did not occur during our experiment. This may be because SMR and RMR at 1 °C were approx. 50% of those at 8 °C (Table 2), and thus, this would have reduced the Atlantic salmon's need for blood oxygen transport.

4.1.2. Plasma cortisol and catecholamine levels

In the present study cortisol, a primary stress hormone released from the interrenal cells in the anterior kidney ('head kidney') due to stimulation of the hypothalamus-pituitary-interrenal (HPI) axis (Faught et al., 2016; Gamperl et al., 1994b), was elevated in fish acclimated to 1 °C and acutely exposed to this temperature. These data suggest that exposure to temperatures approaching 0 °C induces a stress response in Atlantic salmon, independent of the time of exposure. There is a substantial body of literature which suggests that elevated temperatures increase plasma cortisol levels in many fish species (Chadwick and McCormick, 2017; LeBlanc et al., 2011; Pérez-Casanova et al., 2008), including Atlantic salmon (Madaro et al., 2018). However, our study is the first to measure cortisol levels in Atlantic salmon acclimated and acutely exposed to extremely low (cold) temperatures, and it suggests that temperatures approaching this species' lower thermal limit cause sublethal stress. This interpretation is consistent with data on resting Atlantic cod (*Gadus morhua*; Staurnes et al., 1994) where cortisol levels were higher after >1 week of acclimation to 1 °C as compared to ~8 °C acclimated fish, and for common carp (*Cyprinus carpio*) exposed to cold shock (Tanck et al., 2000). Elevated levels of cortisol for prolonged periods of time are known to have detrimental physiological effects on fish via the reallocation of energy substrates, and negative tertiary effects on the immune system, growth and reproduction (Alfonso et al., 2021; Reid et al., 1998; Schreck and Tort, 2016; Wendelaar Bonga, 1997). These may even be passed on to progeny via epigenetic changes (i.e., a quaternary stress response) (Colson et al., 2019; Redfern et al., 2017). Clearly, research that addresses how temperatures near 0 °C affect cortisol synthesis/production, clearance and tissue responsiveness is needed before we can understand the mechanistic basis(es) for the increased plasma [cortisol] levels, and how long-term exposure to such temperatures affects salmon stress physiology, health and welfare.

Catecholamines (CA), predominantly adrenaline (AD) and noradrenaline (NAD), are also released into the circulation from the 'head kidney' of teleost fish as a primary response to stress (Reid et al., 1998; Schreck and Tort, 2016; Wendelaar Bonga, 1997). In this study, CA levels in all the groups and at all time points (mean plasma concentrations less than 2.5 nM) would be considered resting values (Gamperl et al., 1994b). Given the significant increase in circulating cortisol levels in this study at 1 °C, and that plasma CA in rainbow trout can increase by up to ~100-fold during exposure to chronic

physiological and environmental stressors (i.e., from ~ 2 to 5 nM to upwards of 300 nM; Gamperl et al., 1994b; LeBlanc et al., 2011, 2012; Perry et al., 1996), the absence of elevated circulating CA levels in the Atlantic salmon when acutely exposed to cold temperatures was an unexpected result. However, there is little, to no, data to which this study can be compared. For example, although Chen et al. (2002) measured increases in plasma AD and NAD (i.e. by approx. 15 nM) in tilapia (*Oreochromis aureus*) acutely exposed to a temperature drop from 25 to 12 °C (the latter close to this species' lower temperature limit), these fish were netted from their tanks prior to blood sampling, and values of AD and NAD in 'control' fish were reported to be ~25 and 50 nM, respectively (Chen et al., 2002).

4.1.3. Plasma lactate and resting metabolism

Plasma lactate is another haematological parameter that is known to increase in salmonids when exposed to thermal stress, and it is an indicator that anaerobic metabolism is occurring (Clark et al., 2008; Eliason et al., 2013). For example, elevated lactate concentrations have been reported in resting Chinook (*Oncorhynchus tshawytscha*) and Sockeye (*Oncorhynchus nerka*) salmon when exposed to temperatures approaching their upper thermal limits (Clark et al., 2008; Eliason et al., 2013), and this shows that thermal stress can result in a mismatch between oxygen supply and its demand by the tissues (Pörtner and Knust, 2007; Pörtner, 2010). Interestingly, in the current experiment, acute exposure to temperatures approaching the lower limit for Atlantic salmon resulted in a decrease in plasma lactate levels. These data indicate that oxidative phosphorylation is not compromised in Atlantic salmon at temperatures close to 0 °C, and is sufficient to supply the fish's metabolic needs. This finding is consistent with experiments on the effects of acclimation to 0 vs. 10 °C, and an acute drop in temperature from 10 to 1 °C on Atlantic salmon mitochondrial function (Gerber et al. in final prep). The Q_{10} for State 3 respiration (oxidative phosphorylation with pyruvate, malate and succinate as substrates; and with a saturating concentration of ADP) of liver mitochondria for these two groups was approximately 2.7 and 3.0. These values are very comparable to those shown in Table 1 for MO_2 . The mitochondria of many fish species experience dysfunction when exposed to high temperatures due to increased proton leak, a reduced capacity for oxidative phosphorylation, a loss of ADP and substrate affinities, and increased reactive oxygen species (ROS) production (see Ekström et al., 2016b; Iftikar and Hickey, 2013; Penney et al., 2014; Gerber et al., 2020a,b). However, our understanding of how very cold temperatures (i.e., at or close to a fish's lower thermal limit) affect mitochondrial function is extremely limited.

4.2. Cardiorespiratory changes upon exposure to cold temperatures

The physiological response, particularly of cardiorespiratory function, to changes in temperature varies greatly between and within species, and is influenced by the duration of exposure (e.g., acute vs. chronic) (Eliason and Anttila, 2017). Temperature coefficient (Q_{10}) values are used to describe the factorial change in a physiological rate [e.g., heart rate (f_H), cardiac output (\dot{Q}) and oxygen consumption (MO_2)] as temperature is changed by 10 °C, and are typically between 2 and 3. In the present study, the Q_{10} values for f_H , $f_{H_{int}}$, \dot{Q} and MO_2 were extremely similar for fish chronically exposed (acclimated) to 1 °C vs. acclimated to 8 °C and acutely cooled to 1 °C (Table 1). Given the similar Q_{10} values between the two groups and their magnitude (2.4–3.0), and that acclimation normally results in fish chronically exposed to low/cold temperatures having a lower Q_{10} as compared to fish acutely exposed to these temperatures (i.e., cardiorespiratory parameters in cold-acclimated fish would be expected to be higher than in acutely exposed fish; e.g. see Aho and Vornanen, 2001; Graham and Farrell, 1995), one could conclude that the Atlantic salmon has a very limited capacity to acclimate to temperatures near their lower thermal limit. However, Sutcliffe et al. (2020) reported that it takes only 8 h for

winter-acclimated trout to reset their f_H when cooled from 12 to 4 °C, and thus, it is possible that the Atlantic salmon's phenotypic plasticity allows it to rapidly compensate for thermal effects on resting cardiorespiratory function over this temperature range; i.e., there is a rapid 'resetting' of cardiac function. This interpretation would be consistent with Lurman et al. (2012) who showed that intrinsic f_H , and the maximum performance of the *in situ* cod (*Gadus morhua*) heart, were not different between fish acclimated to 10 and 4 °C, and then acutely tested at 4 and 0 °C, respectively, as compared to those acclimated to these latter temperatures. Given that Q_{10} values may not be within the 'typical' range close to an animal's thermal limits, and that cardiorespiratory function has been shown to reset quite quickly in trout following thermal acclimation (Sutcliffe et al., 2020), this question needs to be examined further. For example, by very quickly (i.e., in <1 h) exposing fish to the 8-1 °C decrease and comparing values with 1 °C acclimated fish shortly after the temperature change. If the Atlantic salmon can compensate/acclimate, or partially acclimate, to temperatures of 0–1 °C, one would expect cardiorespiratory parameters after acute transfer to 0–1 °C to be significantly lower than in acclimated fish.

In this study, V_S did not change with acute or chronic exposure to 1 vs. 8 °C, but fish acclimated to 1 °C increased V_S considerably when f_H was lowered by adrenergic blockade (Fig. 5). The former result is in contrast to recent data collected on myocardial strips (Gamperl et al., 2022) which suggests that the effects of cool temperatures on myocardial contraction would greatly constrain f_H , and that this could make increasing V_S much more favourable than increasing f_H to elevate \dot{Q} at low temperatures. Given that we only assessed resting cardiorespiratory function in this study, we are planning swimming (i.e., critical swimming speed; U_{crit}) experiments designed to examine how acute and chronic exposure to 1 °C affect maximum values for f_H , \dot{Q} , V_S and MO_2 . We are particularly interested in what the effects of exposure to this cold temperature have on the capacity of this species to increase f_H versus V_S .

It is difficult to reconcile why V_S increased in salmon acclimated (chronically exposed) to 1 °C following β -adrenergic blockade, but not in fish that were acutely exposed to 1 °C. However, the most likely explanation is an increase in filling pressure [central venous pressure (CVP), 'preload']. This is based on: the observation that the decrease in f_H following the addition of the propranolol was not vastly different between the two cold-exposed groups; and that although Sandblom and Axelsson (2007) did not find any differences in CVP in trout acclimated to 10 and 16 °C (a relatively narrow temperature range, and close to the optimal temperature for this species), Sandblom and Axelsson (2005) showed that this mechanism was responsible for increasing V_S in rainbow trout during hypoxia and hypothesized that increased venous tone allows more blood to enter the central venous compartment, and therefore, increases venous pressure (Sandblom and Axelsson, 2005). Venous capacitance in fish is largely controlled by α -adrenergic mediated mechanisms (Sandblom and Axelsson, 2007). It is possible/probable that bretylium tosylate (after 45 min) did not completely block nervous adrenergic control of the venous vasculature, and that enhanced α -adrenergic nervous control of venous capacitance in fish acclimated to 1 °C contributed to the higher V_S following propranolol injection. Alternatively, it is possible that this α -adrenergic tone (which would increase tension in these vessels and decrease capacitance) was opposed by a β -adrenergic tone in 1°C-acclimated Atlantic salmon. Finally, propranolol has a low affinity for β_3 -adrenoreceptors (Schna and Caplan, 2019), and the blocking of β_1 - and β_2 -adrenoreceptors with this pharmacological agent might have enhanced β_3 -adrenoreceptor-mediated lusitropic effects on the heart (i.e., improved myocardial relaxation), and thus cardiac filling. Clearly, determining which of these hypotheses about the mechanism(s) responsible for the considerable difference in V_S in fish tested at 1 °C will require further research.

4.3. Important changes to cardiac control

4.3.1. Cholinergic vs adrenergic tone

The contribution of cholinergic and adrenergic tone to cardiac function varies widely between species and with temperature (Altimiras et al., 1997; Axelsson, 1988; Axelsson et al., 1987; Mendonça and Gamperl, 2009). For example, the cholinergic and adrenergic tones on the Atlantic cod heart at 10–12 °C were 37.7% and 21.1%, respectively (Axelsson, 1988), as compared to 11.1% and 25.3% for the short-spined sculpin (*Myoxocephalus scorpius*) at 11–12 °C (Axelsson et al., 1987). In the present study, cholinergic tone (~12–17%) was quite low in all groups. However, adrenergic tone ranged from approximately 35–70% between the groups, and was significantly higher in fish acutely exposed to 1 °C as compared to 8 °C acclimated fish (Table 2). Interestingly, the contributions of these modulators of f_H are much different than seen in Antarctic nototheniids that spend their whole life at these temperatures. The cholinergic tonus on the heart is very high under “resting” conditions (up to 80%) in this taxon, and changes in cardiac performance in this group appear to depend chiefly on modulation of this tonus (Davison et al., 1997; Egginton et al., 2006; Campbell et al., 2009). However, this may not be unexpected as the heart of these species apparently lacks adrenergic innervation, and they have low-post-stress catecholamine levels under all but the most severe stressors (Egginton et al., 2006) (i.e., the capacity to influence cardiac function via the release of catecholamines from the chromaffin cells might be limited). That the salmon acutely exposed to 1 °C had an enhanced adrenergic tone was somewhat surprising as circulating catecholamine levels (i.e., of both AD and NAD) were very low (~0.5–6 nM), and similar, in both groups exposed to 1 °C. However, the fish heart is also under neural adrenergic (sympathetic) tone, and this may have been different in fish acclimated vs. acutely exposed to 1 °C. This would need to be investigated by injecting fish with bretylium at least 24 h prior to f_H measurements (see Methods section). Alternatively, exposure to 1 °C for this period could have resulted in the pacemaker cells being populated by a larger number of cardiac β_2 -adrenoreceptors, and/or by β_2 -adrenoreceptors with lower binding affinity (i.e., an increased sensitivity to AD and NAD). Changes in both cell surface β_2 -adrenoceptor density and affinity have been described with temperature acclimation in fishes. For example, Keen et al. (1993) found that acclimation of rainbow trout to 8 vs 18 °C resulted in an increase in myocardial sensitivity to adrenaline by 10-fold, and Shiels et al. (2003) reported that acute exposure of rainbow trout to 7 °C increased the heart’s adrenergic sensitivity and determined that this was a critical maintenance mechanism in fish cardiomyocytes (Shiels et al., 2003).

4.4. Conclusions and perspectives

Overall, our results show that there are few differences in the Atlantic salmon’s cardiorespiratory response when these fish are acutely exposed vs. acclimated to 1 °C; a temperature they are exposed to in aquaculture cages/coastal waters in Atlantic Canada and Iceland. These data strongly suggest that these fish are able to appropriately regulate cardiac function and blood oxygen delivery when acutely exposed (over several hours) to cold temperatures, and that a loss of cardiac function/regulatory capacity is not likely related to (be the cause of) recent episodes of ‘winter kill’ at marine cage-sites in these areas. However, we did find that plasma cortisol levels were elevated in fish exposed to 1 °C, and this suggests that fish welfare (i.e., growth, disease susceptibility etc.) could be impacted at these temperatures. Further, we did have a number of very interesting findings that should be further investigated. These include that: (1) Resting V_S did not change when fish were acclimated or acutely exposed to 1 °C, as compared to their 8 °C conspecifics, and this suggests that temperature-dependent changes in \dot{Q} are solely mediated by f_H across the Atlantic salmon’s entire temperature range. (2) While acute exposure to 1 °C results in a change in adrenergic tone, it is unclear

whether this is due to enhanced neural sympathetic tone or pacemaker β -adrenoreceptor sensitivity. (3) It appears that acclimation to this temperature alters the balance between α - and β -adrenergic control of CVP/venous capacitance, and thus, V_S . Understanding the mechanistic basis of the latter finding will require direct measurements of these parameters in fish exposed to these temperatures, as will determining whether increases in f_H and/or V_S are most important for increasing \dot{Q} in fishes at these temperatures. The recent data of Gamperl et al. (2022), based on mechanistic studies of heart function, would suggest that it is the latter. However, this hypothesis also needs experimental verification. Finally, this study was performed on male fish from a commercial farm that has held salmon for 25 years (approx. 8 generations) in land-based systems where the lowest temperature they experience is 10 °C (the population of salmon available to us at the time). Long-term rearing conditions can have a significant effect a fish’s response to environmental conditions (e.g., Faust et al., 2004; Adams et al., 2022) and several studies have reported that sex has a significant effect on temperature- and oxygen-dependent cardiac function and control in fishes (Clark et al., 2009; Rodnick et al., 2007; Sandblom et al., 2009). Thus, future research should include studies that include both male and female fish.

In conclusion, this research significantly improves our understanding of how salmon (fish) physiology (especially their stress and cardiorespiratory physiology) is impacted by exposure to very cold temperatures and provides important information with regards to the temperature-dependent biology of this taxon, and where research and management efforts should be focused (or not focused) with regards to improving their survival and welfare during the winter months.

CRedit authorship contribution statement

E.S. Porter: Formal analysis, Writing – original draft, Writing – review & editing. **K.A. Clow:** Formal analysis. **R.M. Sandrelli:** Writing – review & editing. **A.K. Gamperl:** Funding acquisition, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crphys.2022.03.002>.

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