Do prior diel thermal cycles influence the physiological response of Atlantic salmon (*Salmo salar*) to subsequent heat stress?
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We designed two environmentally relevant thermal cycling regimes using monitoring data from an Atlantic salmon river to determine if exposure to prior diel cycles stimulated protective mechanisms (e.g. heat hardening), and/or resulted in physiological and cellular stress. Wild fish were exposed to three days of diel cycling in the lab and then exposed to an acute thermal challenge near their upper reported critical temperature. We measured routine metabolic rate across the time course as well as indicators of physiological status (e.g. plasma glucose and osmolality) and cellular stress (e.g. heat shock protein 70). We observed that thermal cycling altered physiological and cellular responses, compared to an acute heat shock, but saw no differences between cycling regimes. Unique temperature regime and tissue specific responses were observed in heat shock protein induction, metabolites, haematology and osmotic indicators. Routine metabolic rate was not affected by the thermal cycling and increased according to $Q_{10}$ predictions. While we report unique physiological and cellular responses between all treatment groups, we did not observe a clear indication of a heat hardening response.
INTRODUCTION

The temperature in natural ecosystems is rarely constant; real-world scenarios involve varying magnitudes of diel temperature fluctuation, coupled with sporadic extreme events (Ma et al. 2015). The dynamic nature of these cycles creates variability in temperature parameters such as the mean, rate of change, maximum temperature achieved, magnitude of change and accumulated heat exposure over time (i.e. thermal load). For aquatic ectotherms like fish, whose body temperature is normally within 1 ºC of the surrounding water (Hazel and Prosser 1974), temperature has critical and pervasive effects on biology. Due to logistical difficulties in execution, and prolonged experimental duration, few studies examine the biological effects of environmentally relevant diel thermal cycles. However, those studies that have been conducted in fish show both the metabolic rate (e.g. Lyytikäinen and Jobling 1998; Beauregard et al. 2013, Oligny-Hébert et al. 2015) and physiological responses (e.g. Mesa et al. 2002; Narum et al. 2013; Eldridge et al. 2015) of thermally cycled fish are altered compared to those maintained at a constant temperature. Whether these diel thermal cycles can enhance a fish’s thermal tolerance and provide protection during a subsequent heat event is presently unclear. This idea has parallels to heat hardening, where a short (minutes to hours) exposure to a sublethal temperature increases survivorship following a subsequent more severe heat stress (Loeschcke and Hoffmann 2007) and is reminiscent of ischaemic preconditioning in mammalian (Murry et al. 1986) and fish hearts (Gamperl et al. 2001). If exposure to prior thermal cycling does impact thermal tolerance, then understanding both the nature of the cycle and the induced biological effects is important and may enhance the efficacy of conservation, management, and remediation efforts.
The range of temperatures tolerated by a fish is bounded by an upper and lower critical temperature ($T_{\text{crit}}$) within which lies an optimum temperature range ($T_{\text{opt}}$) where performance is maximal. Metabolic physiology is highly temperature-dependent due to the inextricable link between temperature and the reaction rate of biological processes. Routine metabolic rate (RMR) and maximal metabolic rate (MMR) increase almost exponentially with temperature; however, MMR plateaus and may even decline at high temperatures (Eliason and Farrell 2016). All life processes (e.g. foraging, growth, predator evasion, and reproduction) must occur within an animal’s aerobic scope, which is defined as the difference between MMR and RMR at a given temperature. Aerobic scope is generally considered to be widest at a fishes $T_{\text{opt}}$ and smallest around $T_{\text{crit}}$ (Steinhausen et al. 2008), although this may not always be the case (Clark et al. 2013). A thermal stress response may occur if water temperatures stray outside of a species’ $T_{\text{opt}}$, and particularly if they approach either the upper, or lower $T_{\text{crit}}$ (for review see Currie and Schulte 2014). Such increases in temperature result in increased levels of circulating catecholamines (LeBlanc et al. 2011, 2012; Templeman et al. 2014) that mobilize energy reserves and enhance oxygen delivery. From a cellular perspective, the induction of heat shock proteins (HSPs) are an important adaptive component of the thermal stress response, maintaining the structure and function of cellular proteins that may otherwise be compromised with exposure to high temperatures. Overall, the heat shock response has been primarily studied following exposure to a single heat shock; however, redband trout ($Oncorhynchus mykiss gairdneri$) experiencing a diel thermal cycle show acclimation in expression of $hsp$ mRNA over 6 weeks (Narum et al. 2013). It is unclear how different diel thermal cycles will affect the heat shock response, particularly at the biologically active protein level. This paucity of physiological information on diel temperature cycling over multiple days in any fish species was this impetus
for this study. We used Atlantic salmon (*Salmo salar* Linnaeus), a primarily cold-water adapted
species, as a model to assess indicators of metabolic, physiological, and cellular stress in
response to an experimental design that mimicked the naturally occurring diel temperature cycles
in this species’ river habitat during the summer months.

Freshwater habitats are home to approximately 40% of all identified fish species
(Lundberg et al. 2000) and are particularly vulnerable to climate change (Woodward et al. 2010).
The Miramichi River in New Brunswick, Canada, like many other temperate river systems,
experiences diel cycles in temperature and therefore provides an excellent model to investigate
the biological impacts associated with warming rivers. The Miramichi River is recognized for
having the largest Atlantic salmon run in eastern North America (DFO 2013); but population
numbers are at record lows (ICES 2015) with warming river temperatures in the summer months
thought to be a contributing factor to this overall population decline. The optimum temperature
range for Atlantic salmon is thought to span 6–20 ºC, within which maximal growth occurs at 16–
17 ºC (Jensen et al. 1989). In Atlantic salmon acclimated to 15 ºC, the upper lethal temperature
(survival <10 min) is reported as 32 ºC (Elliot 1991). Data from ongoing conservation efforts
(e.g. Miramichi River Environmental Assessment Committee) for summer 2014 and 2015 shows
the average water temperature in July and August to be 21.0 ± 0.6 ºC, with peak temperatures of
27.9 ± 0.9 ºC. The rate of temperature change is often rapid, warming on average 0.43 ± 0.03
ºC·h⁻¹ (maximum 1.4 ºC·h⁻¹), thus allowing little time for acclimation. Diel thermal cycles are
not always constant, and the mean temperature can increase over several days if overnight
cooling is not sufficient before warming begins again the following day (see Figure 1B).
Exposure to these naturally occurring, repeated, daily fluctuations in temperature may provoke a
heat hardening response, whereby fish are better able to withstand subsequent, possibly more
severe, temperature increases. Alternatively, the accumulated thermal load from these repeated
exposures could cause stress, damage, and depletion of energetic stores, as Atlantic salmon
juveniles are reported to cease feeding above 23 °C (Breau et al. 2011).

Some information on the biological responses of Atlantic salmon exposed to high
temperatures is available. Maximum aerobic metabolic rate in wild 2+ individuals occurs at ~ 24
°C (540 mgO$_2$·kg$^{-1}$·h$^{-1}$), and above this temperature muscle glycogen stores become depleted and
blood and muscle lactate levels increase (Breau et al. 2011). Parr removed from the Miramichi
River at 27 °C had significantly higher whole body HSP70 protein and mRNA levels than fish
catched in the same location at 20 °C indicating that these stress proteins are induced in the wild
(Lund et al. 2002). Thermal tolerance does appear to be plastic in Atlantic salmon, as Elliot
(1991) determined that increased acclimation temperature increased the upper lethal tolerance of
Atlantic salmon. Furthermore, Anttila et al. (2014) showed increases in maximum heart rate and
onset temperature of cardiac arrhythmias in Atlantic salmon reared at warmer (+ 8 °C)
temperatures. Notably, the temperatures chosen in these two studies were held constant and
maintained for weeks to months.

The goal of this study was to assess whether recent exposure to environmentally relevant
diel thermal cycles influences the response to a subsequent acute high temperature event in
Atlantic salmon. Specifically, we were interested in whether or not two distinct, summer water
temperature scenarios involving diel cycling over multiple days stimulated protective
mechanisms (e.g. heat hardening), and/or resulted in physiological and cellular stress. One
thermal profile reflected a greater magnitude of temperature change and higher daily maximum
temperatures (diel cycle A) and one mimicked increasing mean temperatures and a higher
thermal load (diel cycle B). We compared these scenarios to an acute heat shock after a constant
thermal history of 16 °C as well as a 16 °C negative control. While the metabolic rate of Atlantic salmon was not differentially altered by either diel thermal cycle, we report unique physiological and cellular responses between all treatment groups, but did not observe a clear indication of a heat hardening response.

MATERIALS AND METHODS

Animal collection and holding conditions

Wild (1+) Atlantic salmon (Salmo salar) parr were collected using a Smith-Root LR-24 backpack electrofisher, in June, 2013, from the Cains River (N46°25'59.5; W066°01'29.1- N46°26'05.0; W066°01'10.8 ± 15m), a tributary of the Southwest Miramichi River, New Brunswick, Canada. At capture, body masses and fork lengths (FL) were approximately 5 g and 7 cm respectively. Fish were immediately transported to Mount Allison University, Sackville, NB, and held in a 750 L tank of recirculating freshwater maintained at 16 ± 1.0 °C under natural photoperiod conditions. Fish were initially fed a mixture of dehydrated krill and commercial pellet feed (Corey Nutrition Company, Fredericton, NB; 1.0 mm) twice daily until satiation. Subsequently, parr were weaned from krill and fed exclusively pellets to satiation once per day for the remainder of holding. Feeding was ceased 24 h prior to experimentation to limit elevations in metabolic rate due to specific dynamic action. Experiments were conducted between November, 2014 and 2015, prior to which fish had been maintained at 16 ± 1.0 °C for at least 10 months. All experimental fish (n = 28) had undergone smoltification as characterized by
the absence of parr spots and were either 3+ or 4+ animals\textsuperscript{1}. The FL and body mass of experimental fish ranged between 26.5 – 41.5 cm and 165.5 – 802.1 g (these ranges are large due to the extended experimental duration with this experimental design). All experimental protocols were performed in accordance with the Canadian Council on Animal Care guidelines and research was approved by the Mount Allison University Animal Care Committee (Protocol # 14-03).

Swim tunnel respirometry

All temperature manipulations and metabolic rate measures were performed individually within a swim tunnel respirometer. The swim tunnel consisted of a 30 L measurement chamber, within a larger 120 L chamber of aerated water (Loligo Systems, Tjele, Denmark), which was continually passed through a 200 L aquarium bio- and charcoal- filter to prevent accumulation of ammonia and nitrite wastes. Low levels of these waste products were verified periodically using Nutrafin colourimetric kits (Hagen Inc., Canada), and the system was completely drained and refilled between experimental animals. Water velocity, O\textsubscript{2} concentration, and temperature were continuously monitored and controlled by a DAQ-M data acquisition instrument and AutoResp software (Loligo Systems). The water velocity within the measurement chamber was calibrated using a vane wheel probe and handheld flow sensor (Höntzch, Waiblingen, Germany) and a solid blocking correction factor was applied to correct water velocity to body lengths per second (BL·s\textsuperscript{-1}) for each experimental fish. Dissolved O\textsubscript{2} content was measured using a dipping probe mini-sensor (PreSens, Regensburg, Germany) and was calibrated using a 2-point calibration in

\textsuperscript{1} See supplemental table S1
aerated tank water (100%), and a solution of sodium sulphite (0%). Temperature within the measurement chamber was monitored using a platinum resistance temperature probe. During the flush phase a 57 L·min$^{-1}$ submersible aquarium pump, controlled by the DAQ-M, was used to exchange the water inside the measurement chamber with aerated water from the reservoir. This pump was turned off during the closed loop measurement phase. Ambient temperature in the swim tunnel was maintained by continuously circulating water through a chiller (Aqua Logic Inc, San Diego, USA). To manipulate temperature for each experimental condition, a 120 L hot water reservoir was created using a rod immersion heater (Cole-Parmer, Montreal, Canada) on a floating platform. A 10 L·min$^{-1}$ submersible pump also controlled by the DAQ-M pumped hot water into the ambient reservoir of the swim tunnel. By opposing action of the chiller and this hot water pump, the temperature within the swim tunnel could be increased or decreased in a controlled and reproducible manner.

**Experimental procedure**

Fish were removed individually from the holding tank and lightly sedated in buffered anaesthetic solution (125 mg·L$^{-1}$ tricaine methanesulfonate, 250 mg·L$^{-1}$ NaHCO$_3$) to measure body mass, FL, body depth, and width (to the nearest 0.5 cm). An initial blood sample (0.5 mL; $t = 0$ h) was drawn via caudal puncture using a 23 gauge needle and syringe rinsed with heparinised salmonid saline to prevent clotting (in mmol·L$^{-1}$: 124 NaCl, 5.1 KCl, 1.9 MgSO$_4$, 1.5 Na$_2$HPO$_4$, 11.9 NaHCO$_3$, CaCl$_2$, 5.6 glucose, 100 U·mL$^{-1}$ heparin). Salmon were then transferred to the darkened swim tunnel respirometer and allowed to recover overnight on a constant flush cycle at a water speed of 0.5 BL·s$^{-1}$. In a subset of five fish, O$_2$ consumption was monitored
overnight to ensure sufficient recovery time before commencing experiments\(^2\). Following 
recovery, fish were subjected to one of four experimental thermal regimes (see below and Table 
1) and \(O_2\) consumption was measured over 84 h. Salmon were then removed from the swim 
tunnel, sedated, and a second blood sample (~2 mL) taken in the same manner described above. 
White muscle, heart, liver, and gill tissues were sampled following transection of the spinal cord, 
flash frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis.

**Thermal profiles**

Two environmentally relevant thermal profiles were developed to mimic summer water 
temperature scenarios in the Miramichi River based upon monitoring data from the Miramichi 
River Environmental Assessment Committee (Fig. 1). These profiles are referred to as cycle A 
and B throughout the text. Cycle A mimics a river water scenario where the temperatures 
oscillate daily by the same magnitude. In this diel cycle, the change in temperature (\(\Delta T\)) is 8 °C, 
fluctuating between 16 and 24 °C and ending with a temperature ramp to 27 °C over 12 h (\(\Delta T \leq 11\) 
°C), with a cumulative thermal load, or the area under the curve, of 349 °C·h (Table 1). Cycle B 
represents a scenario with a smaller daily magnitude of change (\(\Delta T\) between 3.5 and 6 °C), and a 
larger cumulative thermal load of 415 °C·h (Table 1), mimicking river conditions shown in Fig. 
1B. To compare these thermal profiles with other studies on thermal stress, we conducted an 
acute, single heat shock as a positive control, in which temperature was ramped from 16 to 27 °C 
over a 12 h period. Finally, a negative control experiment was performed in which temperature 
was maintained at 16 °C for 84 h (Table 1).

\(^2\) See supplemental Figure S1
Oxygen consumption measurements

Routine mass specific metabolic rate ($\dot{M}O_2$) was measured through intermittent closed loop respirometry. Oxygen consumption was calculated from the decline in dissolved oxygen content within the measurement chamber of the swim tunnel over 300 s after a 30 s wait period. Between measurement periods the chamber was flushed with aerated water from the reservoir for 570 s to return oxygen levels to approximately 100% saturation. One full flush/measure cycle therefore took 15 min, allowing 4 $\dot{M}O_2$ readings per hour across the 84 h experimental period. Autoresp software (Loligo Systems) automatically calculated $\dot{M}O_2$ accounting for fish mass, water temperature, salinity, and barometric pressure. To account for background microbial oxygen consumption, oxygen depletion was monitored in an empty chamber, both before and after a fish was placed in the setup. The slope of background oxygen depletion was always negligible and therefore metabolic rates are reported uncorrected. Any $\dot{M}O_2$ values generated from an oxygen depletion slope with an $R^2$ of less than 0.7 were removed in the interest of data integrity. Additionally, any individual $\dot{M}O_2$ values 25% greater than the determined maximum metabolic rate (see below) were considered outliers and were removed ($n = 18$ out of approximately 7300 total measurements).

Maximal metabolic rate

In a separate group of four fish (679.9 ± 52.1 g), maximum and routine metabolic rates were determined at 16°C to derive aerobic scope at this temperature. Fish were individually
placed in the swim tunnel respirometer and allowed to acclimate overnight using the same intermittent-loop respirometry parameters detailed above. Fish were not anesthetized, and no preliminary blood sample was taken from this group of animals. Routine metabolic rate was determined from the average of the final 10 recorded $\dot{M}O_2$ values the following morning. Each fish was then moved to a separate tank and chased to exhaustion (determined as a lack of response to handling) over a period of 5 – 10 min. Each fish was then quickly transferred back to the swim tunnel respirometer and sealed in a closed loop. Respirometry measurements commenced within ~1 min. $O_2$ depletion was continuously recorded over a period of 5 min, and an $\dot{M}O_2$ value was automatically calculated using AutoResp software every 30 s over this measurement period. Maximum metabolic rate was determined by averaging the three highest $\dot{M}O_2$ values immediately following this exhaustive exercise protocol. Aerobic scope was calculated as the difference between MMR and RMR.

Blood and plasma analyses

Within 5 min of blood sampling, haematocrit (Hct, %), and haemoglobin concentration (Hb, g·L$^{-1}$) in whole blood were measured using a SpinCrit Microhaematocrit centrifuge (SpinCrit, Indiana, USA), and HemoCue® Hb 201+ system (Ängelholm, Sweden) according to the manufacturer’s instructions. A correction factor for fish blood was applied to all Hb values as per Clark et al. (2008). Mean corpuscular haemoglobin concentration (MCHC, g·L$^{-1}$) was subsequently calculated from these two parameters. The remaining blood sample was then spun at 14,000 g for 5 min at 4°C, any buffy coat was discarded, and the plasma supernatant and RBC pellet were flash frozen separately in liquid nitrogen and stored at -80 °C. Plasma was
subsequently analysed to determine osmolality (mmol·kg⁻¹) using a Wescor Vapro 5520 Vapour Pressure Osmometer (Logan, Utah, USA), and chloride ion concentration (mmol·L⁻¹) using a Chloride Analyzer 925 (Nelson Jameson Inc., Marshfield, WI, USA). Plasma samples were deproteinized by diluting 1:2 (v:v) with 6% perchloric acid (PCA), and centrifuging at 13,000 rpm for 3 min at 4 °C. The glucose concentration in the supernatant of each sample was determined relative to a standard curve of D-glucose in a combined assay solution with final in well concentrations: 100 mmol·L⁻¹ imidazole, 5 mmol·L⁻¹ MgCl₂, 4 mmol·L⁻¹ ATP, 0.32 mmol·L⁻¹ NADP⁺, 1 U glucose-6-phosphate dehydrogenase, 1 U hexokinase. The reaction was allowed to run to completion at 37°C and the absorbance of generated NADPH measured at 340 nm using a SpectraMax M5 Plate Reader and SoftMax Pro software (Molecular Devices, Sunnyvale, USA).

**Tissue lactate**

Lactate was quantified in plasma, heart, and liver tissues as an indication of anaerobic metabolism. Ground heart, and liver samples were diluted 1:4 (w:v) in 8% PCA containing 1 mmol·L⁻¹ EDTA using a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, Canada). Plasma samples were diluted 1:2 (v:v) in 8% PCA and vortexed to mix. All samples were then centrifuged at 13,000 rpm for 4 min at 4 °C to pellet cell debris and protein. A known volume of supernatant was removed and neutralized using a solution of 2 mmol·L⁻¹ KOH and 0.4 mmol·L⁻¹ imidazole. Samples were centrifuged again at 13,000 rpm at 4 °C for 1 min and the resulting supernatant removed for lactate quantification. Samples were analysed in triplicate relative to a standard curve of L-lactic acid. Final in-well concentrations of all assay components were: 0.16
mol·L⁻¹ glycine, 0.13 mol·L⁻¹ hydrazine, 1.9 mol·L⁻¹ NAD⁺, 10 U lactate dehydrogenase. The reaction was allowed to run to completion at room temperature, after which absorbance of generated NADH was read at 340 nm using a SpectraMax M5 Plate Reader and SoftMax Pro software.

Na⁺/K⁺-ATPase Activity

Gill Na⁺/K⁺-ATPase activity (NKA; µmol·mg total protein⁻¹·h⁻¹) was measured as an indicator of ion regulation. The protocol was as per McCormick (1993) with the exception that gill tissue used for analysis had been frozen and ground over liquid nitrogen (see below).

Protein extraction and quantification

Gill, heart, liver, and white muscle tissues were ground over liquid nitrogen and diluted 10-15x (w:v) in protein extraction buffer (50 mmol·L⁻¹ Tris base, 2% SDS; pH 7.5) with 0.05 µL·mg tissue⁻¹ protease inhibitor cocktail (PIC004.1; Bioshop, Ontario, Canada). Samples were then homogenized using a Fisher Scientific PowerGen 125, and centrifuged at 14,000 g for 10 min at 4 °C, and the supernatant removed and stored at -80°C. Each RBC pellet was diluted 1:1 (v:v) in heparinised salmonid saline with a 1x working concentration of protease inhibitor cocktail (PIC003.1; BioShop) and transferred to a FastPrep MatrixD cell lysing tube (MP Biomedical, California, USA) containing 1.4 mm ceramic beads. Tubes were shaken for 45 s at 4.0 m·s⁻¹ to lyse cells, and then cooled on ice. Tubes were centrifuged at 16,000 g for 3 min at 4 °C to pellet the beads and the soluble protein supernatant was removed and stored at -80 °C.
Soluble protein concentrations in all samples were assayed using a DC Protein Assay kit (Bio-Rad, Mississauga, ON, Canada). Samples and standards (bovine serum albumin; Bio-Rad) were diluted in protein extraction buffer or saline as appropriate and their absorbance read in triplicate at 750 nm with a SpectraMax M5 plate reader and SoftMax Pro software.

Heat shock protein immunoblotting

As an indicator of cellular stress, we measured HSP70 in salmon RBCs, and tissues. 4 µg of soluble protein sample was loaded per well, and for quantification purposes a four-point standard curve of ‘supersample’ containing a mixture of all tissues from a representative fish was run on each gel. Electrophoresis was performed using Bolt™ 4-12% Bis-Tris mini gels (ThermoFisher Scientific, Ontario, Canada) and MOPS SDS running buffer (ThermoFisher Scientific). Gels were run at 200V and separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 20V using the Bolt™ Mini Blot module and transfer buffer (25 mmol·L⁻¹ bicine, 25 mmol·L⁻¹ bis-tris, 1 mmol·L⁻¹ EDTA; pH 7.2). Membranes were then blocked for 1 h at room temperature, or overnight at 4 °C, in 5% milk powder dissolved in Tris-buffered saline with Tween 20 (TBS-T; 25 mmol·L⁻¹ Tris, 138 mmol·L⁻¹ NaCl, 0.1% Tween 20, pH 7.6). Membranes were incubated in polyclonal rabbit affinity purified HSP70 primary antibody (AS05 061A, Agrisera, Vännäs, Sweden) at a concentration of 1:10,000 in 1% milk powder TBS-T solution for 1 h at room temperature. This antibody is specific to the inducible isoform of salmonid HSP70, and does not detect the constitutive protein³ and therefore provides a powerful tool to assess induction of HSP70 with thermal stress. Secondary antibody (goat anti-

³ See supplemental figure S2 for representative western blot images
rabbit IgG-HRP; SAB 300, Enzo Life Sciences, New York, USA) was also used at 1:10,000 in 1% milk powder TBS-T solution for 1 h at room temperature. Chemiluminescent detection of protein bands was performed using ECL Select (GE Healthcare, Buckinghamshire, UK). Blots were imaged using a VersaDoc™ MP 4000 Molecular Imager (Bio-Rad) and analysed using Image Lab® software. HSP70 in each sample was interpreted relative to a fit of the ‘supersample’ curve. Equal protein loading was visually assessed by Coomassie staining PVDF membranes following immunodetection as per Welinder and Ekblad (2011).

Protein turnover / damage

Tissue and RBC ubiquitin was measured as an indirect indicator of protein turnover and/or damage, using dot blots. Sample protein (0.2 µg·µL⁻¹) and a standard curve of ‘supersample’ (4x – 0.25x) was loaded onto a nitrocellulose membrane (Bio-Rad) and blocked in 5% BSA/TBS-T. Membranes were probed for ubiquitin with a mouse primary antibody (BML-PW8805-0500, Enzo Life Sciences; 1:2,500 in 5% BSA/TBS-T), which detects polyubiquitinylated proteins (i.e. those targeted to the proteosome for degradation) but not mono or free ubiquitin. The secondary antibody was a goat anti-mouse IgM (ab97230, AbCam Inc, California, USA; 1: 20,000 in 0.1% BSA/TBS-T). Levels of ubiquitin in each sample were visualized and quantified as detailed for immunoblotting above. Equal protein loading was visually assessed via staining with Ponceau S following immunodetection.

Statistical analyses
All statistical analyses were performed using R-studio (version 3.2.1). All experimental data were assessed, and appropriately transformed when required, to meet the parametric assumptions of homogeneity of variance (Levene’s test), and normality of residuals (Shapiro-Wilk test). Tissue HSP70 and ubiquitin data were analysed separately using two-way ANOVAs (tissue - 5 levels; treatment – 4 levels). However, due to a statistically significant tissue x treatment group interaction, the data for both parameters were split by tissue to analyse the response between treatments in each tissue separately using a one-way ANOVA (α-critical = 0.05). Fish masses, gill NKA, $\dot{M}O_2$ at 27 ºC, and tissue lactate concentrations were also analysed using one-way ANOVAs to compare between treatment groups (α-critical = 0.05). When a significant effect was detected, Tukey post-hoc tests were performed to assess where treatment groups differed from one another.

All variables where repeated samples were taken over time (plasma osmolality, chloride, glucose, lactate, $\dot{M}O_2$ at 21 ºC, Hb, Hct, and MCHC) were analysed using a split-plot ANOVA to compare the effects of fish nested within treatment group (between-subjects factor), and sampling time point (within-subjects factor). When an overall significant effect of time was detected, a Tukey’s post-hoc test was used (α-critical = 0.05). When a significant interaction was detected, data were split to compare both factors across treatments (4 levels, randomized block ANOVA with Tukey post-hoc tests), and over time (2 levels, two-tailed paired t-test); the α-critical level for both analyses was therefore adjusted to 0.025.

RESULTS
The rate of O$_2$ consumption was measured using intermittent-loop respirometry to characterize the effects of four different thermal profiles on aerobic metabolic rate (Fig. 2). Metabolic rate data were not scaled to the mean mass of all fish used in the study as we found no relationship between ln fish mass and ln $\dot{M}O_2$ ($R^2 = 0.003$) at a given temperature. Similarly, we found no correlation between fish mass and either: osmolality, chloride, glucose, lactate, or HSP70 in any tissue. Predictably, $\dot{M}O_2$ increased and decreased with increases and decreases in water temperature, respectively. In the negative control group, $\dot{M}O_2$ remained constant across the 84 h experimental period at $97.4 \pm 0.6$ mgO$_2$·kg$^{-1}$·h$^{-1}$, a value consistent with those obtained in other studies on salmon (Deitch et al. 2006; Eliason and Farrell 2016). Baseline $\dot{M}O_2$ was similar in the positive control group until 72 h ($107.5 \pm 0.8$ mgO$_2$·kg$^{-1}$·h$^{-1}$), and then increased with temperature, peaking at 27 °C ($268.3 \pm 21.5$ mgO$_2$·kg$^{-1}$·h$^{-1}$). In diel thermal cycle A, the highest $\dot{M}O_2$ readings coincided with the daily temperature peak. In this group, variability between individuals was low, and $\dot{M}O_2$ peaked at $264.5 \pm 16.1$ mgO$_2$·kg$^{-1}$·h$^{-1}$ when the temperature was 27 °C. In diel thermal cycle B, variability between individuals was higher, making the overall $\dot{M}O_2$ response harder to interpret; however, it appears the daily peak in $\dot{M}O_2$ occurs before the temperature peak. At 84 h and 27 °C, $\dot{M}O_2$ was $281.5 \pm 23.3$ mgO$_2$·kg$^{-1}$·h$^{-1}$. The $\dot{M}O_2$ values at the end of the experiment (at 27 °C) in each treatment group were not significantly different from one another ($p = 0.83$; Fig. 3A). When $\dot{M}O_2$ at a common temperature of 21 °C was examined within the two diel cycling groups over days 1 – 4, no differences were observed ($p = 0.73$). However, overall, fish in cycle B had significantly higher $\dot{M}O_2$ at 21 °C than those in cycle A ($p = 0.02$; Fig 3B). This 21 °C temperature was chosen as it was the highest temperature common to
all treatment groups across all four experimental days.

We also determined aerobic scope in a subset of Atlantic salmon at 16 °C. An aerobic scope of 278.4 ± 3.9 mgO₂·kg⁻¹·h⁻¹ was determined from a resting $\dot{M}O_2$ of 81.7 ± 3.5 mgO₂·kg⁻¹·h⁻¹ and a maximal value following exhaustive exercise of 360.3 ± 7.3 mgO₂·kg⁻¹·h⁻¹.

**Plasma and tissue metabolites**

We found a highly significant treatment group x time interaction (p < 0.001) in plasma glucose suggesting that responses were distinct among temperature treatments (Table 2). Plasma glucose significantly decreased over time (from t = 0 to t = 84 h) in the negative control group (p = 0.014), and significantly increased in cycle A (p = 0.005). At t = 0 h plasma glucose was the same across all treatment groups (p = 0.30), but at t = 84 h it was significantly elevated in the positive control (p < 0.001), cycle A (p < 0.001), and cycle B (p = 0.006) compared to the negative control.

Neither heart (p = 0.36), nor white muscle (p = 0.26) lactate concentration significantly changed across treatments (Fig. 4). However, as with plasma glucose, there was a significant treatment x time interaction (p < 0.001) in plasma lactate (Table 2). The concentration of lactate in plasma at t = 84 h was significantly elevated above the negative control in both positive control (p < 0.001) and cycle B (p = 0.020) treatments. In fish subjected to thermal cycle B, plasma lactate increased from t = 0 h to t = 84 h (p = 0.005), while approaching significance (p = 0.029, α-critical = 0.025) in fish subjected to thermal cycle A.

**Ionoregulation**
To assess whether exposure to elevated temperatures led to osmotic stress, we measured plasma osmolality and plasma chloride concentrations (Table 2). Plasma osmolality changes were dependent on thermal group, as indicated by a significant treatment x time interaction ($p = 0.003$). Plasma osmolality remained unchanged over time (from $t = 0$ h to $t = 84$) in the negative control ($p = 0.41$), but increased significantly in the positive control ($p = 0.007$), thermal cycle A ($p = 0.007$), and thermal cycle B ($p = 0.005$) treatments. Initially, at $t = 0$ h, plasma osmolality was the same in all fish across thermal groups ($p = 0.062$). However, after exposure to $27 \, ^\circ C$ at $t = 84$ h, plasma osmolality was significantly elevated above negative control levels in both the positive control and cycle A ($p < 0.001$) fish. The plasma osmolality of fish in cycle B was not significantly different from any other treatment group ($p > 0.05$). Plasma chloride concentration was the same in all fish, across all treatment groups at both $t = 0$ h ($p = 0.74$) and at $t = 84$ h ($p = 0.038$; $\alpha$-critical = 0.025). In thermal cycle B, plasma chloride concentration was significantly higher than $t = 0$ h levels at the end of the experiment ($t = 84$ h; $p = 0.004$).

Haematology

Neither Hb or Hct was significantly different among treatment groups, but both did decrease significantly over time from $t = 0$ h to $t = 84$ h in the negative control ($Hb - p < 0.001$; $Hct - p = 0.01$), and thermal cycle A ($Hb - p = 0.023$; $Hct - p = 0.028$) groups. Changes in both Hb and Hct approached significance in the positive control group ($p = 0.066$ and $p = 0.055$ respectively). We calculated MCHC from Hb and Hct (Table 2) and did not observe significant differences among any of the temperature groups ($p = 0.92$), nor did MCHC change within any
treatment group from $t = 0$ h to $t = 84$ h ($p = 0.95$).

**Cellular Stress and Damage**

The expression of indicators of cellular stress (HSP70) and damage (ubiquitin) were highly tissue-specific as shown by a significant treatment group x tissue type interaction ($p < 0.001$) for both variables. Consequently, the data were split to analyse the stress response in each tissue separately (Fig. 5). HSP70 was undetectable in the negative control group (16 ºC) and for all tissues at $t = 0$ (16 ºC) regardless of treatment group (data not shown). In the positive control, HSP70 was induced in white muscle, heart, and liver (Fig. 5 E, G, I) but not in RBCs or gill (Fig 5 A, C). HSP70 was induced in all tissues in both diel thermal cycling groups with the same magnitude of induction ($p > 0.05$). The induction of HSP70 in gill, white muscle, and RBC was greatest in diel cycles A and B (Fig 5A, E, G). In comparison, the greatest HSP70 increase in heart and liver occurred in the positive control group (Fig. 5G, I).

We measured ubiquitin for a gross indication of whether the HSP70 induction correlated with changes in protein damage/turnover. We observed increases in ubiquitin in at least one of the treatment groups in all tissues except liver ($p = 0.35$, Fig. 5J). In gill, only thermal cycle A resulted in a significant increase in ubiquitin compared to the negative control ($p = 0.006$, Fig. 5B). In the positive control group, the acute heat shock resulted in a significant increase in muscle, RBC, and heart ubiquitin ($p < 0.05$, Fig. 5D, F, H). As was observed with HSP70, the magnitude of ubiquitin expression in both diel thermal cycling groups was statistically similar ($p > 0.05$) in all tissues, with the exception of white muscle where cycle B was significantly higher ($p = 0.031$) than cycle A.
DISCUSSION

Although the response of fishes to increases in temperature has been extensively studied in a variety of species, little work has focused on environmentally relevant diel thermal cycles, particularly over multiple days. The goal of this study was to use Atlantic salmon (*Salmo salar*) as a model to investigate ecologically relevant thermal profiles and assess their effects on indicators of metabolic, physiological, and cellular stress. We designed our thermal profiles using data from the Miramichi River and used wild fish caught from the same location to ecologically ground our laboratory experiments. Two likely outcomes from exposure to repeated fluctuations in temperature are possible and not necessarily mutually exclusive: 1) a protective heat hardening response that increases thermal tolerance, or 2) a detrimental accumulation of heat exposure-related stress, cellular damage, and depletion of energetic stores. Salmon in the Miramichi River survive at mean summer water temperatures (21 °C) which exceed the reported upper optimal temperature for salmon (20 °C; Jensen et al. 1989), thus enhanced thermal tolerance due to chronic exposure to such sub-optimal thermal conditions seems plausible. Conversely, Atlantic salmon population numbers are currently well below historical levels, suggesting a potential detrimental impact of high temperature exposures. We did not find clear evidence of a heat hardening response, nor did exposure to any thermal treatment group appear detrimental or induce a severe stress response, at least over the time course tested. It is possible, although currently unexamined in the literature, that a longer period of thermal cycling may induce a heat hardening response in this species. As predicted, we did observe clear differences
in the physiological and cellular responses of fish exposed to diel thermal cycling in comparison
to fish kept at a stable temperature. Given that these two different diel thermal cycles had similar
overall mean temperatures (Table 1), we are confident that our findings are a result of
temperature cycling and not differences in mean temperature. We observed unique responses in
some physiological parameters with thermal cycling, although the changes noted were subtle.
This suggests that thermal cycling or temperature variance is more important than the nature of
the diel cycling (e.g. accumulated thermal exposure, magnitude, rate of temperature change, or
mean), at least with regard to the two different cycling profiles tested here.

In general a 10 °C increase in temperature doubles or triples RMR in fish (i.e. a Q_{10} of 2-3; Reid et al. 1997). Increasing aerobic scope either through decreased RMR and/or increased
MMR increases the amount of available energy and thus provides an overall benefit to the fish.
We saw no evidence that a prior exposure to environmentally relevant thermal cycles could
lower RMR, and thus increase aerobic scope, during a subsequent acute thermal shock. At 27 °C
(84 h), RMR was statistically similar in all groups (Fig. 4A), and was also the same on days 1 - 4
at a common temperature of 21 °C in diel cycles A and B (Fig. 4B). The Q_{10} of \dot{MO}_2 for the 12 h
ramp to 27 °C on day 4 did not vary either, fluctuating only from 2.1 to 2.5 between experimental
groups. Therefore, it appears that RMR is not modulated by diel thermal cycling, and that Q_{10}
effects are the primary determinant of RMR under the conditions tested. We did notice that the
variability in \dot{MO}_2 between individuals was higher in diel cycle B; seasonal effects offer a
potential explanation. Evans (1984) determined RMR to be maximal in the spring and to shift
independently from changes in environmental temperature. Notably, four of six individuals in
diel thermal cycle B were tested in the spring.

Aerobic scope can be expressed as an absolute valu e, or as a ratio termed factorial
aerobic scope (FAS = MMR/RMR). A FAS value ≤ 1 indicates MMR has been reached or exceeded, and the fish is relying on anaerobic processes to fuel function; the duration of survival under such conditions is limited (Portner and Knust 2007). Limitations in fish availability only allowed routine and maximum metabolic rate to be determined in the same individual (n = 4) at one temperature (16 ºC), and FAS in this group was 4.4 ± 0.1. If we use the MMR at 16 ºC to calculate FAS at 27 ºC in the positive control and both diel thermal cycling groups, FAS drops to ~1.4. This is almost certainly an underestimate of actual FAS since MMR (the numerator) also increases with temperature, although to an unknown extent before plateauing. Regardless, it appears that even in this worst-case-scenario at 27 ºC, Atlantic salmon are still not fully reliant upon anaerobic metabolism to fuel function. This finding is supported by the overall lack of lactate accumulation in any tissue, although without data on lactate flux (i.e. production and utilization rates) we cannot conclusively rule out the activation of anaerobic metabolism. The literature value for the upper incipient lethal temperature (at which fish can survive for at least 7 days) in young (0 - 1+) Atlantic salmon acclimated to 15 ºC is 27.6 ± 0.27 ºC (Elliot 1991). Collectively, our data suggest that at 27 ºC, fish do retain aerobic metabolic capacity and no mortality was observed under the conditions tested. That said, a preliminary experiment holding fish overnight at 27 ºC resulted in mortality after only ~5 h. Juvenile fish are generally considered to have higher thermal tolerance than adults (Fowler et al. 2009; Breau et al. 2007). It is possible that the older and larger fish used here compared to Elliot (1991; 0 – 1+) may account for differences in upper incipient lethal temperatures.

Enhanced oxygen demand due to exercise or elevated water temperatures necessitates increased oxygen uptake at the gill. The mechanisms for increasing oxygen uptake (e.g. increased ventilation, gill surface area, blood perfusion, etc.) are at odds with those required to
minimize ion loss. This conflict has been termed the osmo-respiratory compromise (see Sardella and Brauner 2007 for review). Thus, a teleost fish in freshwater exposed to high temperature would be predicted to gain water and lose ions to their hypotonic surroundings. Anticipated physiological ramifications may include decreased plasma osmolality and ions in addition to lowered Hb and Hct due to haemodilution. Contrary to these predictions, we observed a significant increase in plasma osmolality over time in all experimental treatment groups. This increase is largely accounted for by increases in plasma glucose, lactate, and ions (chloride measured here, and presumably its primary counter ion sodium). NKA activity was the same across all experimental groups (p = 0.122; data not shown). Collectively, these data suggest that an osmo-respiratory compromise is not occurring following exposure to 27 ºC in Atlantic salmon.

Haematocrit and Hb decreased over time in all experimental groups, although only significantly in the negative control and diel cycle A. We propose that these haematological effects are due to an effect of fasting (Gillis and Ballantyne 1996; Djordjevic et al. 2012) rather than a result of haemodilution; but they may reduce oxygen carrying capacity. Any fasting responses here are an unavoidable by-product of fish not being fed during experimentation to avoid post prandial increases in $\dot{M}O_2$.

Heat shock proteins have been implicated in the acquisition and decay of thermotolerance in fish since the *in vitro* work of Mosser and Bols (1988) in rainbow trout fibroblasts. The induction of HSPs is a plastic response, and one that acclimation temperature can modulate. For example: increasing the acclimation temperature of the eurythermal goby (*Gillichthys mirabilis*) by 20 degrees (from 10 to 30 ºC) increased the induction temperature for both HSP70 and HSP90 significantly (Dietz 1994; see also Oda et al. 1991; Dietz and Somero 1992; Nakano
We show that both the HSP70 and ubiquitin responses in Atlantic salmon are tissue specific and dependent on the thermal profile experienced by the fish. The undetectable levels of HSP70 in any tissue at 16 °C reinforce that this was an appropriate holding temperature for Atlantic salmon and that confinement in the swim tunnel was not stressful. Not surprisingly, in all tissues studied we saw marked differences in HSP expression between the two diel thermal cycling groups and the positive control group, where the temperature was maintained at a constant 16 °C. However, in no tissue was there a significant difference in HSP70 between diel thermal cycles A and B. Perhaps the accumulated thermal loads experienced by fish in these two groups (349 and 415 °C·h$^{-1}$ respectively) were not sufficiently different to differentially influence the HSP70 response.

In addition to different thermal loads, each experimental treatment is distinct in the rate and magnitude of temperature change, and the presence or absence of diel cycling, all of which have the potential to influence the cellular stress response. In RBC, gill, and white muscle, increases in HSP70 appeared to mirror increases in thermal load. HSP70 induction was highest in the diel thermal cycling groups (349 - 415 °C·h) in these tissues. This pattern of HSP70 induction was not consistent in the heart and liver, where HSP70 was ~2.5 times higher in the positive control than in either thermal cycling group. Of the tissues examined here, heart and liver are arguably the most likely to experience oxygen limitation due to the single series circulatory anatomy of fish (Satchell 1991). If the unique HSP70 response in heart and liver is due to their susceptibility and/or decreased tolerance to hypoxia, then the greater rate of temperature change in the positive control compared to the thermal cycling groups, coupled with the lack of prior diel cycling, may explain this HSP70 induction pattern.
Dot blotting was successfully used by Hofmann and Somero (1995) to correlate ubiquitin conjugated proteins with HSP70 levels in the intertidal mussel (*Mytilus trossulus*) and similarly by MacLellan et al. (2015) in the gill of an elasmobranch fish (*Squalus acanthias*). However, increases in protein degradation can also result from overall increases in temperature, particularly when coupled with starvation (Houlihan 1991; McCarthy and Houlihan 1997). In general, the level of ubiquitination corresponded to the amount of HSP70 in a given tissue (e.g. ubiquitin levels were lowest in RBCs and gill as were HSP70 levels). One notable exception occurred in white muscle, where ubiquitin levels were high while HSP70 was relatively low. We speculate that the high ubiquitin levels in this tissue were due to increases in protein turnover. White muscle constitutes ~70% of salmonid body mass (Eddy and Handy 2012) and thus offers the largest, most accessible source of protein for degradation to fuel energy metabolism.

Although this study was designed around the Miramichi River system, temperature fluctuations are a broadly occurring natural phenomenon in the aquatic environment, and thus conclusions drawn here may also be extended to other ecosystems and species. We made several predictions at the outset of this study: 1) that fish exposed to diel thermal cycling would respond differently to a 27 °C exposure than fish maintained at 16 °C, 2) that the two different diel cycles tested would result in unique biological effects, and 3) that diel cycling would result in either beneficial heat hardening, and/or detrimental cellular stress and damage. We did indeed observe differential responses between thermal cycled and non-cycled fish, as well as subtle differences between the two different thermal cycling treatments. We did not see clear evidence of a heat hardening response, nor did exposure to 27 °C appear to induce a severe physiological stress response under the conditions tested. Resting metabolic rate adhered to predicted increases based on $Q_{10}$ effects, indicating that salmon have little capacity to regulate RMR in response to
fluctuations in temperature; metabolic shifts at the cellular and signalling levels remain possible but were not examined here. Collectively, our data suggest that three days of environmentally relevant diel temperature fluctuation does not substantially alter the response of Atlantic salmon to temperatures approaching their $T_{\text{crit}}$. It is possible that diel thermal cycling impacts cardiorespiratory function in Atlantic salmon and improves function in a way not assessed in the current study. Atlantic salmon do exhibit cardiorespiratory plasticity which can contribute to increased thermal tolerance and survival at elevated temperatures (Anttila et al. 2014).

A direct application of this study is refinement of the regulatory policies in Atlantic salmon river systems. At present the Miramichi River is closed to angling activities when the minimum water temperature for two consecutive days is $\geq$20 °C (DFO 2012). Neither diel thermal cycling group tested here meets these closure criteria, yet fish exhibit evidence of cellular, haematological, and osmotic changes that would presumably also occur in the wild. Bringing together our reported physiological changes with the knowledge that post release mortality following angling in warm water (20 °C) is high in Atlantic salmon (~40%; Wilkie et al., 1996), suggests that re-evaluation of river closure criteria is warranted. Furthermore, the differences shown here between constant and cycling thermal histories suggest that conclusions drawn from acute laboratory studies conducted at stable acclimation temperatures may not be reflective of responses in the wild. Moving forward, researchers should make every effort to incorporate ecological relevance into their experimental designs.

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REFERENCES


Table 1: Detailed time and temperature parameters for each experimental treatment group

<table>
<thead>
<tr>
<th>Thermal Profile</th>
<th>Time (h)</th>
<th>Range (°C)</th>
<th>Δ °C</th>
<th>Ramp Rate (°C·h⁻¹)</th>
<th>Overall Mean (°C)</th>
<th>Thermal Load (°C·h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NEGATIVE CONTROL</strong></td>
<td>0 - 84</td>
<td>16</td>
<td>0</td>
<td>--</td>
<td></td>
<td>15.7</td>
</tr>
<tr>
<td><strong>POSITIVE CONTROL</strong></td>
<td>0 – 72</td>
<td>16</td>
<td>0</td>
<td>--</td>
<td></td>
<td>16.7</td>
</tr>
<tr>
<td><strong>DIEL CYCLE A</strong></td>
<td>0 – 11</td>
<td>16 - 24</td>
<td>8</td>
<td>+ 0.73</td>
<td></td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>12 – 23</td>
<td>24 - 16</td>
<td>8</td>
<td>- 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 – 35</td>
<td>16 - 24</td>
<td>8</td>
<td>+ 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>36 – 47</td>
<td>24 - 16</td>
<td>8</td>
<td>- 0.73</td>
<td>20.2</td>
<td>349</td>
</tr>
<tr>
<td></td>
<td>48 – 59</td>
<td>16 - 24</td>
<td>8</td>
<td>+ 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60 – 71</td>
<td>24 - 16</td>
<td>8</td>
<td>- 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 - 84</td>
<td>16 – 27</td>
<td>11</td>
<td>+ 0.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DIEL CYCLE B</strong></td>
<td>0 - 10</td>
<td>16 - 21</td>
<td>5</td>
<td>+ 0.50</td>
<td></td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>13.5 – 20.5</td>
<td>21 – 17.5</td>
<td>3.5</td>
<td>- 0.50</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>24 - 34</td>
<td>17.5 – 22.5</td>
<td>5</td>
<td>+ 0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.5 – 44.5</td>
<td>22.5 - 19</td>
<td>3.5</td>
<td>- 0.50</td>
<td>21.0</td>
<td>415</td>
</tr>
<tr>
<td></td>
<td>48 – 58</td>
<td>19 - 24</td>
<td>5</td>
<td>+ 0.50</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>61.5 – 68.5</td>
<td>24 - 21</td>
<td>4</td>
<td>- 0.57</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>72 - 84</td>
<td>21 - 27</td>
<td>6</td>
<td>+ 0.50</td>
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</tr>
</tbody>
</table>

* Thermal load is calculated as the cumulative area under the curve of each treatment’s thermal profile where the temperature is above 16°C.
Table 2: Plasma (osmolality, chloride, glucose, and lactate) and RBC (Hb, Hct, and MCHC) parameters in Atlantic salmon among four experimental temperature groups at two sampling time points (t = 0 h and t = 84 h), and the calculated difference (∆) between sampling times.

<table>
<thead>
<tr>
<th></th>
<th>TIME</th>
<th>NEGATIVE CONTROL</th>
<th>POSITIVE CONTROL</th>
<th>DIEL CYCLE A</th>
<th>DIEL CYCLE B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLASMA</strong></td>
<td></td>
<td></td>
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<tr>
<td>Osmolality (mmol·kg⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0 h</td>
<td>298.5 ± 5.2</td>
<td>302.5 ± 2.7</td>
<td>302.6 ± 1.2</td>
<td>291.3 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>t = 84 h</td>
<td>295.1 ± 3.0 a</td>
<td>320.7 ± 3.4 b*</td>
<td>318.3 ± 2.1 b*</td>
<td>308.8 ± 4.8 ab*</td>
<td></td>
</tr>
<tr>
<td>∆</td>
<td>-3.4 ± 3.4</td>
<td>+18.2 ± 3.7</td>
<td>+15.6 ± 1.8</td>
<td>+17.5 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>Chloride (mmol·L⁻¹)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>t = 0 h</td>
<td>133.0 ± 1.6</td>
<td>136.3 ± 2.5</td>
<td>135.3 ± 3.1</td>
<td>135.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>t = 84 h</td>
<td>138.3 ± 2.0</td>
<td>140.0 ± 2.0</td>
<td>143.3 ± 2.9</td>
<td>149.0 ± 3.0 *</td>
<td></td>
</tr>
<tr>
<td>∆</td>
<td>+5.3 ± 2.7</td>
<td>+3.8 ± 3.7</td>
<td>+8.2 ± 5.7</td>
<td>+13.5 ± 2.6</td>
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<tr>
<td>Glucose (mmol·L⁻¹)</td>
<td></td>
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<tr>
<td>t = 0 h</td>
<td>5.3 ± 0.5</td>
<td>4.8 ± 0.1</td>
<td>4.8 ± 0.2</td>
<td>5.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>t = 84 h</td>
<td>4.0 ± 0.1 a*</td>
<td>5.6 ± 0.5 b</td>
<td>5.6 ± 0.3 b*</td>
<td>5.2 ± 0.2 b</td>
<td></td>
</tr>
<tr>
<td>∆</td>
<td>-1.4 ± 0.4</td>
<td>+0.9 ± 0.5</td>
<td>+0.8 ± 0.2</td>
<td>-0.06 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Lactate (mmol·L⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>t = 0 h</td>
<td>5.8 ± 0.5</td>
<td>5.1 ± 0.4</td>
<td>3.8 ± 0.7</td>
<td>4.7 ± 0.2</td>
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<tr>
<td>t = 84 h</td>
<td>3.1 ± 0.6 a</td>
<td>9.6 ± 1.8 b</td>
<td>6.4 ± 0.8 a</td>
<td>7.0 ± 0.7 b*</td>
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</tr>
<tr>
<td>∆</td>
<td>-2.8 ± 0.7</td>
<td>+4.5 ± 1.0</td>
<td>+2.6 ± 0.9</td>
<td>+2.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td><strong>RBC</strong></td>
<td></td>
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<tr>
<td>Haemoglobin (g·L⁻¹)</td>
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<td></td>
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<tr>
<td>t = 0 h</td>
<td>86.6 ± 4.7</td>
<td>94.1 ± 2.9</td>
<td>99.1 ± 2.6</td>
<td>87.6 ± 9.2</td>
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<tr>
<td>t = 84 h</td>
<td>62.6 ± 7.3 *</td>
<td>83.8 ± 2.1</td>
<td>79.3 ± 4.8 *</td>
<td>75.0 ± 5.7</td>
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</tr>
<tr>
<td>∆</td>
<td>-24.0 ± 5.3</td>
<td>-10.3 ± 4.4</td>
<td>-19.8 ± 6.1</td>
<td>-12.7 ± 12.0</td>
<td></td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 0 h</td>
<td>44.8 ± 3.2</td>
<td>45.8 ± 1.5</td>
<td>49.4 ± 2.0</td>
<td>43.0 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>t = 84 h</td>
<td>31.3 ± 3.7 *</td>
<td>40.7 ± 1.8</td>
<td>39.7 ± 2.6 *</td>
<td>38.9 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>∆</td>
<td>-13.5 ± 3.5</td>
<td>-5.1 ± 2.1</td>
<td>-9.7 ± 3.2</td>
<td>-4.1 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>MCHC (g·L⁻¹)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>t = 0 h</td>
<td>197.1 ± 13.3</td>
<td>205.8 ± 4.9</td>
<td>201.9 ± 7.1</td>
<td>201.5 ± 8.7</td>
<td></td>
</tr>
<tr>
<td>t = 84 h</td>
<td>202.8 ± 15.7</td>
<td>207.0 ± 4.7</td>
<td>201.1 ± 7.1</td>
<td>197.2 ± 11.8</td>
<td></td>
</tr>
<tr>
<td>∆</td>
<td>+5.7 ± 7.6</td>
<td>+1.1 ± 3.8</td>
<td>-0.8 ± 10.2</td>
<td>-4.2 ± 10.4</td>
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Notes:

All data presented as mean ± S.E.M. n = 6 in each treatment group. Except t = 0 h lactate (n = 5). Different letters show significant difference between treatment groups at that sampling time. Asterisk (*) indicates significant difference between t = 0 h and t = 84 h within treatment group.
For all plasma parameters $\alpha$-critical $= 0.025$. For all RBC parameters $\alpha$-critical $= 0.05$.

FIGURE LEGENDS

**Figure 1:** Hourly river water temperatures in the summer of 2015, from Doaktown monitoring station on the Miramichi River, NB. Data generously provided by the Miramichi River Environmental Assessment Committee. Panel A is representative of a consistent daily oscillation in temperature (~6.5 °C). Panel B is representative of a natural increase in temperature over several days creating a ramp.

**Figure 2:** Atlantic salmon mean $\dot{MO}_2$ (mgO$_2$·kg$^{-1}$·h$^{-1}$ ± SEM) over time (black) in response to temperature fluctuation (grey) in four treatment groups: negative control (A), positive control (B), diel cycle A (C), and diel cycle B(D). n = 6 fish within each treatment group.

**Figure 3:** $\dot{MO}_2$ at 27 ± 0.5 °C in positive control and diel cycles A and B at the end of the experimental time course (A) and at 21 ± 0.5 °C on days 1 – 4 in both diel cycling groups (B). A. n = 6 in each treatment group. No significant difference between treatment groups (p = 0.83; 1W ANOVA). B. For each time within each treatment group n = 6, with the exception of diel cycle B on day 4 where n = 5. Asterisk indicates that treatment groups are significantly different from one another (p = 0.019; split-plot ANOVA).

**Figure 4:** Heart (A), and white muscle (B) lactate concentrations (mmol • L$^{-1}$; mean ± S.E.M) in Atlantic salmon. n = 6 in each treatment group. A. Heart lactate concentrations do not differ significantly between treatment groups (p = 0.26; 1W ANOVA), B. White muscle lactate concentrations do not differ significantly between treatment groups (p = 0.36; 1W ANOVA).
Figure 5: HSP70 and ubiquitin in gill (A, B), white muscle (C, D), RBCs (E, F), heart (G, H), and liver (I, J) in Atlantic salmon (Salmo salar) exposed to four temperature treatments: negative control, positive control, and diel cycles A and B. All graphs show relative band/dot density (mean ± SEM). n = 6 fish within each treatment group for each tissue type. RBC data shown in panels E + F were sampled at 84 h prior to terminal sampling. Due to a significant treatment x tissue interaction (p < 0.001; 2W ANOVA) data were split to analyse change between treatment groups in each tissue separately using 1W ANOVAs. Different letters indicate statistically significant (p < 0.05) differences between treatment groups. A. Significant treatment group effect (p = 0.023). B. Significant treatment group effect (p = 0.001). C. Significant treatment group effect (p < 0.001). D. Significant treatment group effect (p < 0.001). E. Significant treatment group effect (p < 0.001). F. Significant treatment group effect (p = 0.003). G. Significant treatment group effect (p < 0.001). H. Significant treatment group effect (p = 0.003). I. Significant treatment group effect (p < 0.001). J. No significance treatment group effect (p < 0.35).
Figure 2

190x121mm (300 x 300 DPI)
Figure 3

164x231mm (300 x 300 DPI)
Figure 4

151x254mm (300 x 300 DPI)
Figure 5

158x268mm (300 x 300 DPI)
Figure S1: Mean $\text{MO}_2$ (mgO$_2$ • kg$^{-1}$ • h$^{-1}$ ± SEM) during overnight acclimation to swim tunnel and recovery from initial blood sample in five Atlantic salmon. One fish from the negative control and two diel cycling groups, and two fish from the positive control. For comparison, the shaded region shows the overall mean (solid line) plus/minus 1 std. deviation (dashed lines) of the negative control group across the 84 h experimental time course.
**Supplemental Table 1:** Sample size (n), experiment start date, length (cm), body mass (g), percent decrease in body mass at experiment cessation (%), and treatment group (mean ± S.E.M) length, mass, and percent mass change in all Atlantic salmon used in this study.

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- No available data indicated by (-)
- Different lowercase letters indicate statistically significant (α-critical = 0.05) differences in fish masses between treatment groups.
Figure S2: Representative western blot images for the HSP70 salmonid specific antibody (polyclonal rabbit affinity purified; AS05 061A, Agrisera) **A.** Lanes 1 – 4: supersample standard curve (2x – 0.25x), lane 5: negative control gill, lane 6: positive control gill, lane 7: diel cycle A gill, lane 8: diel cycle B gill, lane 9: negative control heart, lane 10: positive control heart, lane 11: diel cycle A heart, lane 12: diel cycle B heart. **B.** Lanes 1 – 4: supersample standard curve (2x – 0.25x), lane 5: negative control liver, lane 6: positive control liver, lane 7: diel cycle A liver, lane 8: diel cycle B liver, lane 9: negative control muscle, lane 10: positive control muscle, lane 11: diel cycle A muscle, lane 12: diel cycle B muscle.