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RESEARCH ARTICLE

Upper thermal limits of growth in brook trout and their relationship to stress physiology

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ABSTRACT

Despite the threat of climate change, the physiological mechanisms responsible for reduced performance at high temperatures remain unclear for most species. Elevated but sublethal temperatures may act via endocrine and cellular stress responses to limit performance in important life-history traits such as growth. Here, brook trout (*Salvelinus fontinalis*) subjected to chronically elevated or daily oscillating temperatures were monitored for growth and physiological stress responses. Growth rate decreased at temperatures above 16°C and was negative at 24°C, with an estimated upper limit for positive growth of 23.4°C. Plasma cortisol increased with temperature and was 12- and 18-fold higher at 22 and 24°C, respectively, than at 16°C, whereas plasma glucose was unaffected by temperature. Abundance of heat shock protein 70 (HSP70) in the gill increased with temperature and was 11- and 56-fold higher at 22°C and 24°C, respectively, than at 16°C. There was no relationship between temperature and plasma Cl^-, but there was a 53% and 80% decrease in gill Na^+/K^+-ATPase activity and abundance at 24°C in comparison with 16°C. Daily temperature oscillations of 4°C or 8°C (19–23°C or 17–25°C) were compared with 21°C controls. Growth rate decreased with temperature and was 43% and 35% lower by length and mass, respectively, in the 8°C daily oscillation treatment than in the controls. There was no effect of temperature oscillation on plasma cortisol or glucose levels. In contrast, gill HSP70 abundance increased with increasing daily oscillation and was 40- and 700-fold greater at 4°C and 8°C daily oscillation, respectively, than in the constant temperature controls. In individuals exposed to 17–25°C diel oscillations for 4 days and then allowed to recover at 21°C, gill HSP70 abundance was still elevated after 4 days recovery, but not after 10 days. Our results demonstrate that elevated temperatures induce cellular and endocrine stress responses and provide a possible mechanism by which growth is limited at elevated temperatures. Temperature limitations on growth may play a role in driving brook trout distributions in the wild.

KEY WORDS: Climate change, Temperature tolerance, Osmoregulation, Cortisol, Glucose, Heat shock protein

INTRODUCTION

Climate change is one of the largest ecological challenges that conservationists face today, and will provide many obstacles to those attempting to manage natural resources. In spite of the increasing attention to the ecological impacts of climate change, it is still unclear what physiological mechanisms are important to the response of individual species to climate change (Wikelski and Cooke, 2006; Portner and Farrell, 2008; Portner et al., 2009). Several integrative approaches indicate a decrease in aerobic scope, growth and swimming performance at elevated temperatures that are associated with cellular and physiological stress responses (Portner and Farrell, 2008; Sokolova, 2013). Although somatic growth is a key aspect of population persistence, our understanding of the means by which temperature impacts growth is limited. Sublethal temperatures may act via the stress response to inhibit growth in individuals, thus constraining populations.

The eastern brook trout, *Salvelinus fontinalis* (Mitchill 1814), is an iconic cold-water species of North America and, for many stream systems, the most abundant vertebrate. Brook trout may be particularly sensitive to increased water temperatures in response to climate change, as local populations are spatially constrained to stream networks. Previous brook trout habitat models suggest global warming will lead to a significant loss of habitat throughout the species range, with increasing impacts felt by southern populations (Meisner, 1990; Flebbe et al., 2006). These models currently rely on untested assumptions of the upper temperature limit of persistence and would be improved with information on the upper thermal limits for growth in this species. There are well-defined effects of temperature on growth rates for most freshwater salmonids (Brett, 1979), and a number of studies have found 13–16°C to be optimal for brook trout growth (Baldwin, 1956; McCormick et al., 1972; Hokanson et al., 1973; Dwyer et al., 1983; McMahon et al., 2007), but the upper limits for growth in brook trout have yet to be determined. Recent field studies indicate that brook trout are not present in waters above a 24-day mean maximum temperature of 22°C (Wehrly et al., 2007). Additional work in a lentic system suggests that populations are limited by temperatures above 20°C (Robinson et al., 2010). Laboratory and field-based studies suggest the upper incipient lethal temperature for brook trout is 25.3°C (Fry et al., 1946; Fry, 1951; Wehrly et al., 2007). The disparity between the lethal temperature and the temperature limits seen in nature suggests that sublethal temperature effects play a crucial role in limiting fish populations, perhaps through their impact on food consumption and growth.

The majority of studies designed to investigate the impacts of elevated temperature on physiology and whole-animal performance use acute (hours) or chronic (days or weeks) elevated fixed temperatures. While important, these constant exposure experiments are inconsistent with what most species experience in the wild. Daily variations in temperature are a normal part of life for most habitats and as the climate warms many species will experience daily temperature oscillations that feature stressfully elevated temperatures. Daily temperature oscillations have been shown to affect heat shock proteins (HSPs) and metabolic adjustments in several salmonids (Callaghan et al., 2016). There

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is conflicting evidence on the impact of oscillating temperatures on the growth of fishes (Hokanson et al., 1977; Thomas et al., 1986; Meeuwig et al., 2004; Shrimpton et al., 2007), which may relate to the highest temperature fish experience and the magnitude of thermal variation.

There is increasing interest in utilizing mediators of the stress response, such as increased plasma cortisol, glucose, lactate and HSP expression as indicators of both individual- and population-level responses to environmental stressors, including elevated temperature (Wendelaar Bonga, 1997; Iwama et al., 1999, 2004; Lund et al., 2002; Wikelski and Cooke, 2006; Wendelaar Bonga, 1997). Experimental warming of juvenile Chinook salmon and rainbow trout resulted in elevated circulating cortisol and glucose levels (Meka and McCormick, 2005; Quigley and Hinch, 2006). Similarly, adult sockeye salmon exhibited elevated plasma cortisol and lactate levels in response to increased temperature and exercise (Steinhausen et al., 2008). A variety of studies have demonstrated elevated HSPs in response to elevated temperature in a variety of salmonid species in the laboratory (DuBeau et al., 1998; Smith et al., 1999; Mesa et al., 2002; Lund et al., 2003; Rendell et al., 2006; Stitt et al., 2014) and in the wild (Werner et al., 2005). Recent laboratory and field work from our group indicate that acute temperature thresholds for increased gill HSP70 (20.7°C) and plasma glucose (21.2°C) in brook trout are similar to their proposed thermal ecological limit of 21.0°C (Chadwick et al., 2015). Sublethal temperatures may act via the stress response to inhibit growth in individuals (Iwama et al., 1999), potentially limiting populations (Portner, 2010).

Based on the studies cited above, we predicted that temperatures above the optimum would result in a relatively steep decline in growth rate, and that oscillating temperatures would result in decreased growth compared with constant temperatures with the same mean temperature. We also predicted that observed decreases in growth due to temperature treatment would be accompanied by increased endocrine (plasma cortisol) and/or cellular (HSP70) stress responses. In order to test these predictions, we exposed brook trout to constant elevated temperatures (target temperatures of 16, 18, 20, 22 or 24°C) or to daily temperature oscillations of 0, 4 or 8°C (constant 21°C, 19–23°C or 17–25°C) for 24 days. In addition to measuring growth, we collected blood and gill tissue to assess biomarkers for stress, including gill HSP70, plasma cortisol and glucose. We also examined gill Na^+/K^+-ATPase (NKA) activity and abundance and plasma Cl^- to explore the relationship between temperature and osmoregulation. A third experiment was conducted to examine acute responses and recovery from daily temperature variations. Brook trout were exposed to the same daily temperature variations as above for 1 day or 4 days, and then sampled 1 h, 2, 4 and 10 days later to determine the recovery period following daily temperature oscillations.

MATERIALS AND METHODS

Fish stock

Juvenile (0+) brook trout were obtained from the Sandwich State Hatchery (Sandwich, MA, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in July 2009 for the chronic elevated temperature study, and in July 2010 and 2011 for the oscillating temperature studies. Fish were housed in 1.7 m diameter tanks (150–200 fish per tank) supplied with 4 l min^{-1} of chilled Connecticut River water (16±2°C) and given supplemental aeration. Fish were fed to satiation (Finfish Starter, Zeigler Bros, Gardners, PA, USA) twice daily with automatic feeders and maintained under natural photoperiod. Rearing and experiments were carried out in accordance with USGS Animal Care Guidelines under IACUC 9070. Fish were used in the same year they were obtained so that 0+ fish of similar size were used in all studies. During all experiments fish were fed a commercial trout pellet (42% protein, 16% fat, Finfish Gold, Ziegler Bros).

Experiment 1: chronic temperature growth study

In August 2009, one week before the start of the experiment, 80 fish [12.4–15.8 cm fork length (FL)] were removed from their rearing tank and lightly anesthetized with buffered and neutralized tricaine methanesulfonate (50 mg MS-222 l^{-1}, pH 7.0). While anesthetized, the fish were measured for length (nearest 0.1 cm) and mass (nearest 0.1 g) and implanted with a passive integrated transponder (PIT) tag inserted into the abdominal cavity via a small (~5 mm) incision in the ventral surface just rostral to the pelvic fins. After recovering from the anesthetic, the fish were divided randomly into five 0.9 m diameter experimental tanks (N=16 per tank). The tanks were supplied with Turners Falls city water at a rate of 0.9 l min^{-1} and the temperature was maintained at 16°C with 800 W titanium bayonet heaters. Each tank was provided with supplemental aeration. Fish were fed to satiation twice daily throughout the experiment. The tanks were isolated from the rest of the laboratory so that the daily feeding was the only disturbance that the fish experienced. At each feeding, fish were carefully observed so that food was offered only until the point where it was no longer being consumed (when one or two pellets would drop to the bottom of the tank). The amount of feed consumed at each feeding was measured to the nearest 0.1 g.

Feed was withheld from the fish for 24 h prior to the start of the experiment and at any other time that length and mass were measured. Seven days after PIT tags were implanted, fish were again measured for length and mass and returned to their appropriate tank. Water temperatures were then elevated at a rate of 2°C h^{-1} until the target temperatures of 16, 18, 20, 22 or 24°C were reached. Each tank was maintained at its specific target temperature for the remainder of the study. The water temperature of each tank was measured and recorded every 15 min using Hobo temperature loggers (Onset Computer Corporation, Bourne, MA, USA). Water flow rate and feeding regime were as described with a 75% water change every 4 days. Dissolved oxygen levels were measured daily and were always above 90% saturation.

The fish were measured for length and mass every 8 days for 24 days. On the eighth day half of the fish (N=8 per tank) were killed using a lethal dose of anesthetic (100 mg MS-222 l^{-1}, pH 7.0) so that blood and tissue samples could be taken. The remainder of the fish (N=8 per tank) were sampled after 24 days. Blood was collected from the caudal vessels using 1 ml ammonium heparanized syringes within 5 min of tank disturbance. The blood was spun at 3200 ***g*** for 5 min at 4°C and the plasma was aliquoted and stored at −80°C. A biopsy of four to six gill filaments was taken from the first arch and immersed in 100 µl of ice-cold SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and stored at −80°C. The liver was removed, weighed (nearest 0.0001 g) and stored at −80°C. After sampling the gills and blood, the remaining carcass was reweighed and then dried to a constant mass (48 h) at 60°C to obtain dry mass for the calculation of conversion efficiency.

Experiment 2: oscillating temperature growth study

In October 2010, 90 fish (12.0–16.9 cm FL) were moved from their rearing tank to one of nine 0.6 m diameter experimental tanks (N=10 per tank) and allowed to acclimate for one week prior to the start of the experiment. The fish were fed to satiation once daily and

the tanks were supplied with 16°C Turners Falls city water at a rate of 0.8 l min^{-1}. The water temperature was increased from 16°C to 21°C over 48 h and then maintained at 21°C for 5 days. Each tank received additional heated (34°C) city water as needed to achieve the desired temperature. The heated water flowed through solenoid valves (Granzow Inc., Charlotte, NC, USA) that were controlled by Omega cn7500 controllers (Omega Engineering Inc., Stamford, CT, USA) with resistance thermometer input installed on each tank. The controllers were optimized to the testing conditions and programmed to pulse the solenoid valves open and shut at varying frequency to either maintain a set point or to achieve a new set point within a predetermined time frame. Each tank was provided with supplemental aeration.

The temperature regimes chosen for this study were based on temperature records from known brook trout streams in western Massachusetts (Chadwick et al., 2015). Feed was withheld from the fish for 24 h prior to the start of the experiment. On the first day of the experiment, all of the fish were lightly anesthetized (50 mg MS-222 l^{-1}, pH 7.0) so that length and mass could be recorded. Additionally, each fish received a unique paint mark on either the anal or caudal fin for individual identification purposes. Fish were allowed to recover before being returned to their experimental tanks. The three temperature treatments (three tanks per treatment) were initiated that evening. Treatment 1 was a control group and was maintained at 21°C throughout the study. Treatment 2 consisted of a daily 4°C oscillation that fluctuated between 19°C and 23°C. Treatment 3 consisted of a daily 8°C oscillation that fluctuated between 17°C and 25°C. Treatments 2 and 3 were designed such that the daily low temperature occurred at 06:00 h and the daily high at 18:00 h so that the daily mean was 21°C. These temperature treatments were repeated daily for 24 days. Water temperatures were measured and recorded every 15 min using Hobo pendent temperature loggers (Onset Computer Corporation). Fish were fed to satiation once daily and the amount of feed offered was measured by weight (nearest 0.1 g). Feeding occurred between 11:00 h and 12:00 h when all treatments were at 21°C. In this and all other experiments dissolved oxygen levels were measured daily and were always above 90% saturation. On day 6, a valve remained open in one of our three control tanks and the subsequent temperature spike killed all of the fish in the tank.

Every 8 days the fish were measured for length and mass as described. On day 8, four fish per tank were killed using a lethal dose of anesthetic (100 mg MS-222 l^{-1}, pH 7.0) so that blood, plasma and gill tissues could be sampled. FL (nearest 0.1 cm) and mass (nearest 0.1 g) were recorded for each fish. Blood, plasma, gill tissue and liver were collected as described above. The remaining fish (N=6 per tank) were sampled after 24 days. All sampling occurred between 09:00 h and 11:00 h.

Experiment 3: oscillating temperature acute exposure and recovery

In September 2011, 160 fish (11.4–16.0 cm FL) were moved from their rearing tank to one of 10 0.6 m diameter experimental tanks (N=16 per tank) and allowed to acclimate for 11 days prior to the start of the experiment. The fish were fed to satiation once daily and the tanks were supplied with 18°C Turners Falls city water at a rate of 0.8 l min^{-1}. The water temperature was increased from 18°C to 21°C over 4 days and then maintained at 21°C until the start of the experiment. The water temperature in each tank was regulated as described above. Each tank was provided with supplemental aeration.

Feed was withheld from the fish for 24 h prior to the start of the experiment. There were five temperature treatments (two tanks per treatment) used in this study. Treatment 1 was a control group and was maintained at 21°C throughout the study. Treatment 2 consisted of a daily 4°C oscillation that fluctuated between 19°C and 23°C. Treatment 3 consisted of a daily 8°C oscillation that fluctuated between 17°C and 25°C. Treatments 2 and 3 were repeated daily for 4 days. Treatments 4 and 5 featured the same temperature oscillations as Treatments 2 and 3, but these fish only experienced this fluctuation on 1 day and were otherwise kept at 21°C. On the last day of exposure each treatment reached its peak temperature and was allowed to return to 21°C where they were held for the remainder of the study in order to investigate recovery time from elevated temperatures. Temperature treatments were initiated in the morning, so that peak temperature was reached in the afternoon as would occur in nature.

Four fish per tank (N=8 per treatment) were sampled 1 h after reaching peak temperature on the fourth day of treatment. Fish were killed using a lethal dose of anesthetic (100 mg MS-222 l^{-1}, pH 7.0) so that tissues could be sampled as described. Four fish per tank were sampled 2, 4 and 10 days after initial sampling as described. All fish were fed to satiation on the morning after initial sampling (1 day) and at 3, 5, 7 and 9 days and the amount of feed offered was measured by weight (nearest 0.1 g).

Physiological parameters

Blood for hematocrit measurement was collected in heparanized micro-hematocrit capillary tubes and centrifuged at 13,500 ***g*** for 5 min in a micro-hematocrit centrifuge and read on a micro-capillary reader (Damon/IEC Division, Needham, MA, USA).

Plasma Cl^- was measured by silver titration using a digital chloridometer (Labconco, Kansas City, MO, USA). Plasma glucose was measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase. Plasma cortisol levels were measured by a competitive enzyme immunoassay validated for use in salmonids (Carey and McCormick, 1998). Sensitivity, as defined by the dose–response curve, was 1–400 ng ml^{-1}. The lower detection limit was 0.3 ng ml^{-1}. Using a pooled plasma sample, the mean intra-assay variation was 7.2% (N=6) and the mean inter-assay variation was 11.8% (N=6).

NKA activity in gill homogenates was determined using a temperature-regulated microplate method (McCormick, 1993). Gill biopsies were homogenized in 150 µl of SEID (SEI buffer and 0.1% deoxycholic acid). Ouabain-sensitive ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mmol l^{-1} ouabain. Samples (10 µl) were run in duplicate in 96-well microplates at 25°C and read at a wavelength of 340 nm for 10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA). Protein concentration of the homogenate was determined using a BCA protein assay.

Gill HSP70 and NKA protein abundance were measured as previously described (Pelis and McCormick, 2001; Chadwick et al., 2015). The remaining homogenate from the gill NKA activity assay was diluted with an equal volume of 2× Laemmli buffer, heated for 15 min at 60°C and stored at −80°C. Thawed samples were run on a 7.5% SDS-PAGE gel at 2.5 µg per lane with 5 µg Precision Plus protein standards in a reference lane (Bio-Rad Laboratories, Hercules, CA, USA). For each gel, two additional reference samples were run: one for HSP70 and one for NKA analysis. Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membranes (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mmol l^{-1} Tris and 192 mmol l^{-1} glycine buffer at

Journal of Experimental Biology (2017) 220, 3976-3987 doi:10.1242/jeb.161224

pH 8.3. PVDF membranes were blocked in phosphate-buffered saline with 0.05% Triton X-100 (PBST) and 5% non-fat dried milk for 1 h at room temperature, rinsed in PBST and probed with an HSP70 antibody (AS05061; Agrisera, Vännäs, Sweden) diluted 1:20,000 in PBST and 5% non-fat dried milk for 1 h at room temperature. This antibody is specific to the inducible isoform of salmonid HSP70 and does not recognize the constitutive isoform (Rendell et al., 2006). After rinsing in PBST, blots were exposed to goat anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:10,000 in PBST and 5% non-fat dried milk for 1 h at room temperature, rinsed again in PBST, exposed to chemiluminescent solutions and then exposed to X-ray film (RPI, Mount Prospect, IL, USA). After imaging, blots were rinsed in stripping solution (62.5 mmol l^{-1} Tris, 2% SDS, 100 mmol l^{-1} β-mercaptoethanol, pH 6.7) for 30 min at 50°C to remove antigen. Blots were reblocked and reprobed using an NKA α-subunit antibody (α5; Iowa Hybridoma Bank, Iowa City, IA, USA) diluted 1:10,000 followed by goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:10,000, following the same protocol. This antibody has been widely used in studies on teleost fish and has been previously validated for use in salmonids (McCormick et al., 2009). Digital photographs were taken of individual gels, and band-staining intensity was measured using ImageJ (NIH, Bethesda, MD, USA); protein abundance is expressed as a cumulative 8-bit grayscale value. The HSP70 and NKA reference lanes on each gel were used to correct for inter-blot differences.

Statistics

Daily growth rate in mass was calculated as 100{[(natural log of end mass)–(natural log of start mass)]/number of days}. Daily growth rate in length was calculated as [(end fork length)–(start fork length)]/number of days. Hepatosomatic index was calculated as 100(liver mass/body mass). All data are presented as means±s.e.m. Individually marked fish were used in the study and individuals were treated as an independent replicate in all ANOVA analyses. Growth rate as a function of experimental treatment was similar in the first 8-day and last 16-day intervals, so only growth for the entire 24 day experiment is presented. For this reason, one-way ANOVA was used to examine the impact of temperature treatment on growth at 24 days for Experiments 1 and 2. Two-way ANOVA was used to examine the impact of temperature treatment and duration on physiological parameters for all three experiments. Several of the physiological parameters (plasma cortisol, gill HSP70 and NKA abundance) did not meet the assumption of homogeneity of variance, so the data for these parameters were ranked prior to ANOVA. In Experiments 2 and 3, we tested for tank effects using a nested ANOVA and found no significant tank effects (P>0.3), with the exception of plasma Cl^- in Experiment 2 (tank effect, P=0.006). As there was minor variation in this parameter (range 128–141 mmol l^{-1}) and no significant treatment effect, we judged this tank effect to be biologically unimportant. For all analyses, the probability of establishing statistical significance was $P \leq 0.05$. Prior research has established non-linear responses to temperature for some of the physiological parameters in this study (Chadwick et al., 2015). Therefore, when significant effects were detected by ANOVA, we subsequently conducted both linear and non-linear (first- and second-order) regression analyses using individual data (not means) and presented the result with the highest R^2 value. Where appropriate, Tukey's HSD *post hoc* test was used to determine differences among individual groups. All statistical analyses were performed using Statistica 6.0 (Statsoft, Inc., Tulsa, OK, USA).

RESULTS

Experiment 1: chronic temperature growth study

Mean water temperatures varied slightly from the target temperatures and were 15.5, 17.7, 20.0, 22.4 and 24.4°C during the 24 days (Fig. 1A). There was one mortality in the 20°C treatment on day 16 and one in the 24°C treatment observed on day 23. Throughout 24 days, daily growth rate in length was highest at 16°C (0.104 mm day^{-1}) and decreased significantly with temperature to a low at 24°C (−0.017 mm day^{-1}) (Fig. 1B). Similarly, daily growth rate in mass was highest at 16°C (3.2 % mass day^{-1}) and decreased significantly with temperature to a low at 24°C (−0.9 % mass day^{-1}) (Fig. 1C). Our regression model indicates that the

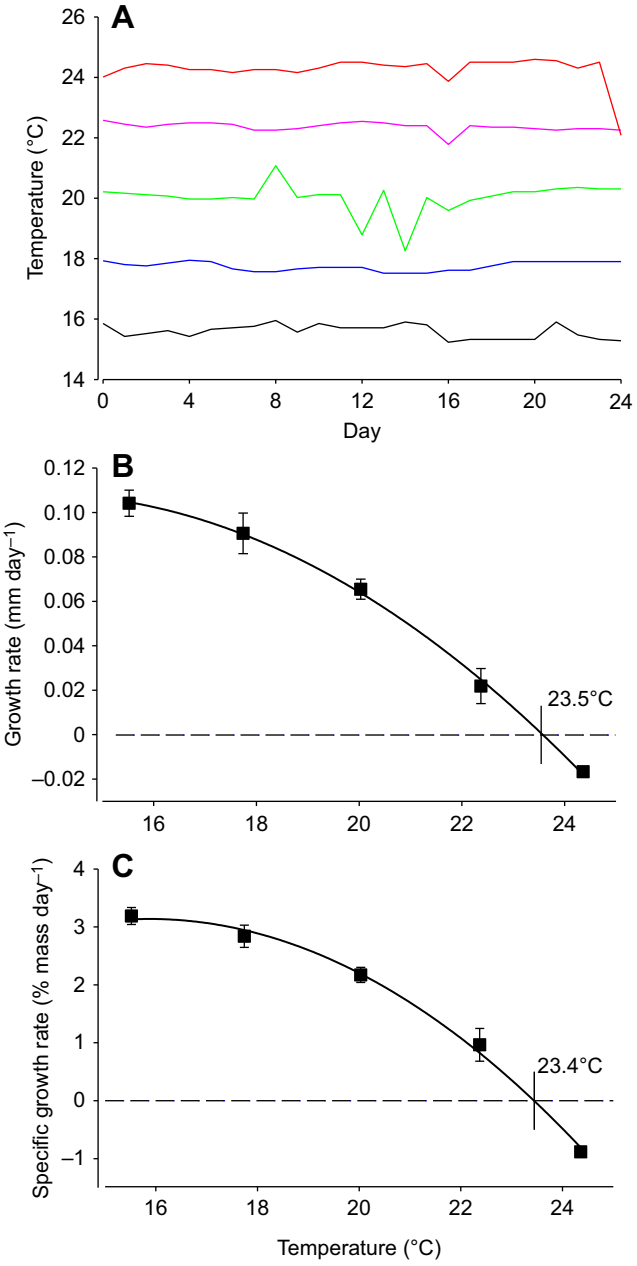

Fig. 1. Influence of temperature on growth rate of brook trout. Water temperature recorded every 20 min in each of the five temperature treatments (A) and their impact on linear (B) and specific growth rate in mass (C) in brook trout. Values are means±s.e.m. of 7–8 fish per treatment. Regression lines for B and C were R^2=0.82 and R^2=0.81, respectively (P<0.00001).

Journal of Experimental Biology (2017) 220, 3976-3987 doi:10.1242/jeb.161224

upper limit for positive growth by length (0.0 mm day^{-1}) and mass (0.0 % mass day^{-1}) for juvenile brook trout is 23.5°C and 23.4°C, respectively (Fig. 1B,C). As temperature increased, the amount of feed consumed decreased (86.1, 77.4, 57.0, 45.8 and 25.0 g dry feed tank^{-1}) as did conversion efficiency (0.432, 0.401, 0.347, 0.347, 0.213 and –0.931 g dry fish g dry feed^{-1}). Hepatosomatic index decreased with temperature and was 55% lower at 24°C than at 16°C (Table 1). There was no effect of treatment length on hepatosomatic index (R^2=0.50, temperature, P<0.01; treatment length, P=0.21).

Plasma cortisol levels were lowest at 16°C (1.3 ng ml^{-1}) and increased with temperature to a peak of 23.4 ng ml^{-1} at 24°C (Fig. 2A). There was a significant effect of temperature (P<0.0001), no effect of treatment duration (P=0.54) but a significant interaction on plasma cortisol (P<0.001). Although there was a significant effect of temperature on plasma glucose (P=0.001), there was no distinct pattern and no effect of treatment duration (P=0.60; interaction, P=0.011; Fig. 2B). Abundance of the inducible isoform of HSP70 in gill tissue increased with temperature and was 10.7- and 56.0-fold higher after 24 days at 22°C and 24°C, respectively, than at 16°C (Fig. 2C). There was no effect of treatment length on gill HSP70 abundance (temperature, P<0.0001; treatment duration, P=0.87; interaction, P=0.35).

Gill NKA activity decreased with temperature and after 24 days was 53% lower at 24°C than at 16°C (Fig. 3A). Gill NKA activity

Table 1. Hematocrit, plasma [Cl$^-$] and hepatosomatic index (HSI) in brook trout after 8 and 24 days of temperature treatment

Temperature (°C)	Hematocrit (%)		Plasma [Cl$^-$] (mmol l^{-1})		HSI (%)
	Day 8	Day 24	Day 8	Day 24	day 24
15.5	32±0.8	32±0.8	124±1.7	130±0.8	2.7±0.11
17.7	34±0.9	34±1.1	126±1.9	128±0.8	2.5±0.08
20.0	35±0.8	31±0.7	129±2.1	131±1.6	2.9±0.13
22.4	32±1.1	30±0.7	126±2.6	131±1.7	1.8*±0.21
24.4	34±0.9	18*±1.2	124±2.5	126±3.2	1.2*±0.08

Temperature is the mean water temperature over the 24 days (Experiment 1). Values are means±s.e.m. of 7–8 fish per treatment. The asterisks indicate a significant difference from the 15.5°C control group.

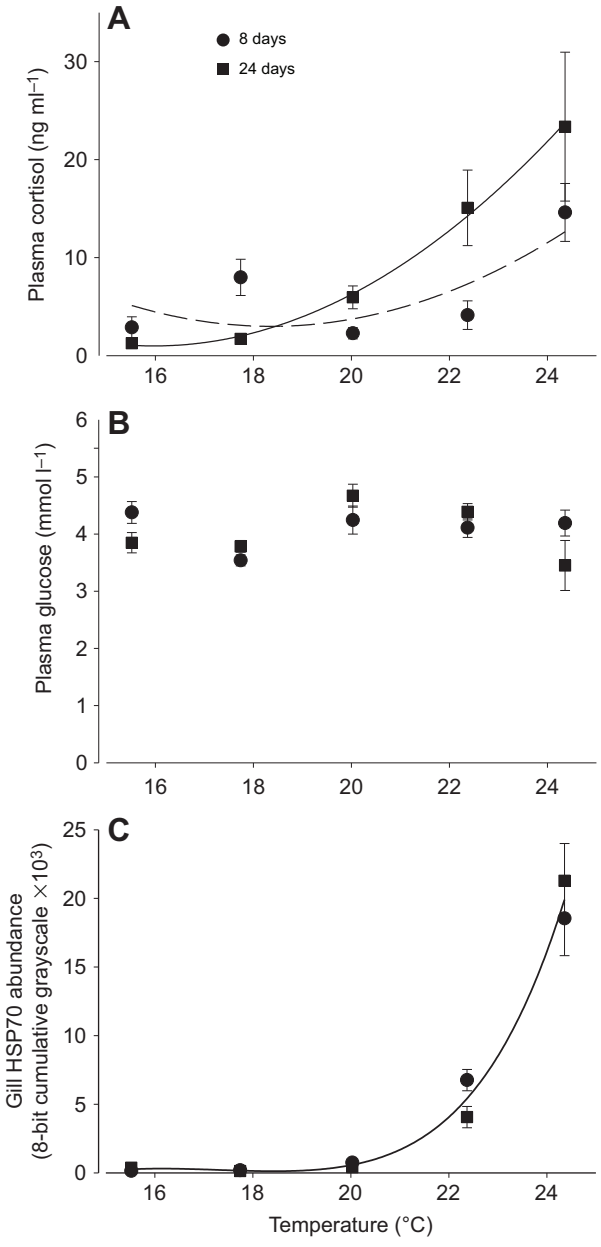

Fig. 2. Influence of temperature on physiological stress responses in brook trout. Plasma cortisol (A), glucose (B) and gill HSP70 (C) levels in brook trout subjected to constant temperature treatments for 8 and 24 days. Values are means±s.e.m. of 7–8 fish per treatment. Only significant regression lines are shown, and had R^2=0.18, P=0.0075 (plasma cortisol day 8), R^2=0.50, P<0.00001 (plasma cortisol day 8) and R^2=0.57, P<0.00001 (gill HSP70).

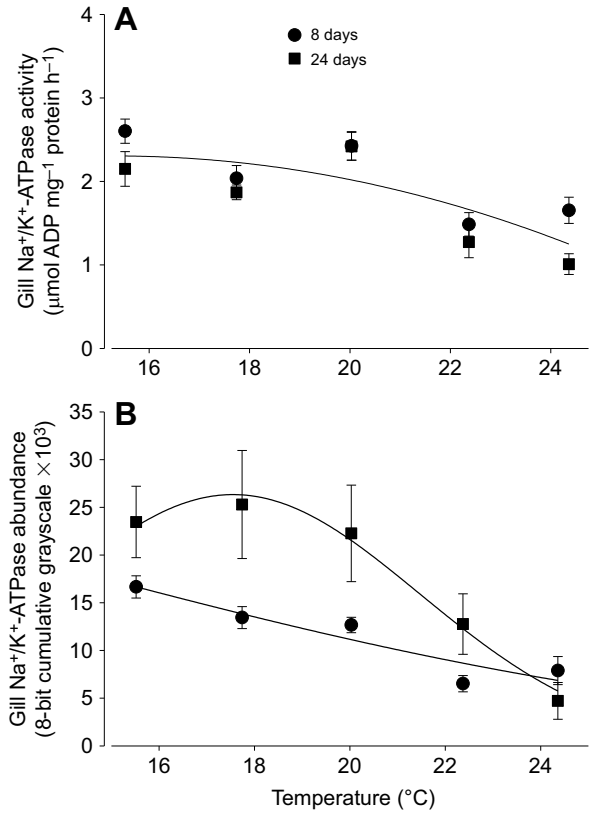

Fig. 3. Influence of temperature on gill Na$^+$/K$^+$-ATPase (NKA) in brook trout. Gill Na$^+$/K$^+$-ATPase (NKA) activity (A) and abundance (B) in brook trout subjected to constant temperature treatments for 8 and 24 days. Values are means±s.e.m. of 7–8 fish per treatment. Only significant regression lines are shown, and had R^2=0.37, P<0.00001 (gill NKA activity), R^2=0.21, P=0.0030 (gill NKA abundance day 8) and R^2=0.58, P<0.00001 (gill HSP70).

Journal of Experimental Biology (2017) 220, 3976-3987 doi:10.1242/jeb.161224

also decreased with treatment length (temperature, $P<0.001$; treatment duration, $P<0.001$; interaction, $P=0.52$). Similarly, gill NKA abundance decreased with temperature and was 80% lower at 24°C than at 16°C after 24 days (Fig. 3B). There was no relationship between treatment length and gill NKA abundance (temperature, $P<0.001$; treatment duration, $P=0.16$; interaction, $P=0.034$). Hematocrit levels were between 30% and 35% in all treatments except after 24 days at 24°C where they decreased to a low of 18% (Table 1; temperature, $P<0.001$; treatment duration, $P<0.001$; interaction, $P<0.001$). There was no relationship between temperature and plasma Cl^- levels, which were similar among all treatments (Table 1; temperature, $P=0.26$; treatment duration, $P=0.02$; interaction, $P=0.73$).

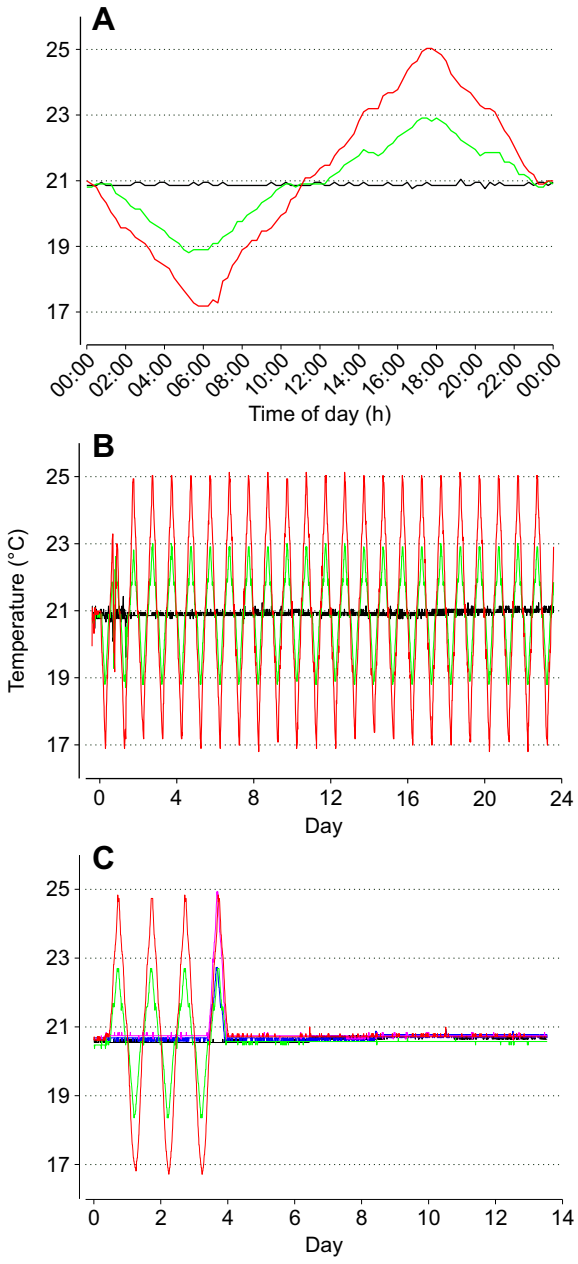

Fig. 4. Oscillating temperature treatments used in experiments 2 and 3. Water temperature recorded every 10 min in each representative tank of each of the three temperature treatments. (A,B) Experiment 2; (C) Experiment 3.

Experiment 2: oscillating temperature growth study

On the first day of the growth study our heating system malfunctioned and peak temperatures were not achieved; however, this was the only time when there was a significant deviation from our target temperatures (Fig. 4A,B). There were two mortalities during the experiment: one occurred on day two in one of our control tanks, and the other on day eight in one of the 8°C oscillation tanks.

In individuals sampled at 24 days, growth rate (mm day^{-1}) declined with increased temperature oscillation (Fig. 5A; $P=0.03$) and was 23% and 43% lower at 4°C and 8°C oscillation, respectively, than in the 21°C control. Specific growth rate was 10% and 35% lower in the 4°C (1.76 % mass day^{-1}) and 8°C (1.28 % mass day^{-1}) oscillations than in the 21°C (1.95 % mass day^{-1}) control; statistical significance was not detected by ANOVA ($P=0.07$) but was detected by regression analysis (Fig. 5B; $P=0.026$). Feed consumed decreased with increasing temperature oscillation (104.3±10.5, 99.4±3.5 and 81.4 ±1.1 g dry feed $tank^{-1}$; $P=0.041$). Conversion efficiency was similar at 0°C and 4°C (0.259±0.001 and 0.278±0.009 g dry fish g^{-1} dry feed, respectively) but was lowest at 8°C oscillation (0.218± 0.033 g dry fish g^{-1} dry feed), 16% lower than in the 21°C control ($P=0.22$). Hepatosomatic index increased over the duration of the study, but there were no differences between any of the treatment groups (Table 1; temperature, $P=0.34$; treatment duration, $P<0.01$).

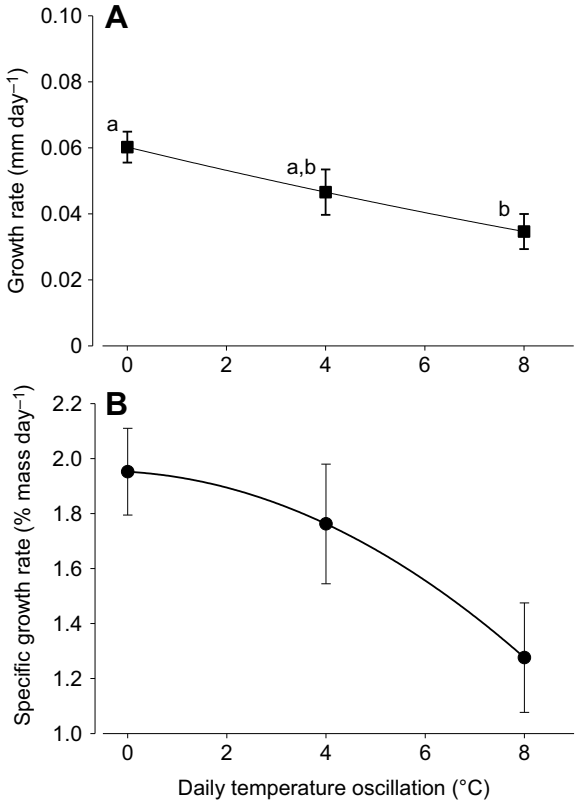

Fig. 5. Influence of oscillating temperatures on growth rate of brook trout. Effect of oscillating temperature treatments for 24 days on linear (A) and specific growth rate in mass (B) in brook trout. Values are means± s.e.m. of 14–15 fish per treatment (4–5 fish per tank). Means with the same letters were not significantly different from one another ($P>0.05$, Tukey's HSD *post hoc* test). Regression lines for A and B were $R^2=0.16$ ($P=0.0066$) and $R^2=0.11$ ($P=0.026$), respectively.

Journal of Experimental Biology (2017) 220, 3976-3987 doi:10.1242/jeb.161224

There was no effect of temperature treatment or duration on plasma cortisol levels (Fig. 6A; temperature, P=0.85; duration, P=0.087; interaction, P=0.55). There was no effect of treatment temperature or duration on plasma glucose (Fig. 6B; temperature, P=0.36; treatment duration, P=0.06; interaction, P=0.27). Gill HSP70 increased with magnitude of temperature oscillation (Fig. 6C; temperature, P<0.001; treatment duration, P=0.005; interaction, P=0.072) and was 40- and 700-fold greater at 4°C and 8°C oscillation, respectively, than in the 21°C control. There was no significant effect of oscillating temperature treatment on gill NKA activity, plasma chloride or hematocrit (P>0.05; data not shown).

Experiment 3: oscillating temperature acute exposure and recovery

We were close to achieving our target temperatures and there was minimal deviation from the planned daily temperature fluctuations (Fig. 4C). There was no effect of temperature treatment on plasma cortisol (Fig. 7A; temperature, P=0.25; treatment duration, P=0.09) or plasma glucose (Fig. 7B; temperature, P=0.25). Plasma glucose levels did increase over the course of the study (Fig. 7B; treatment duration, P<0.01), but this occurred in all temperature treatments. There was a significant effect of temperature treatment and duration on gill HSP70 abundance (Fig. 7C; temperature, P<0.001; treatment duration, P<0.001; interaction, P<0.001). At 1 h, gill HSP70

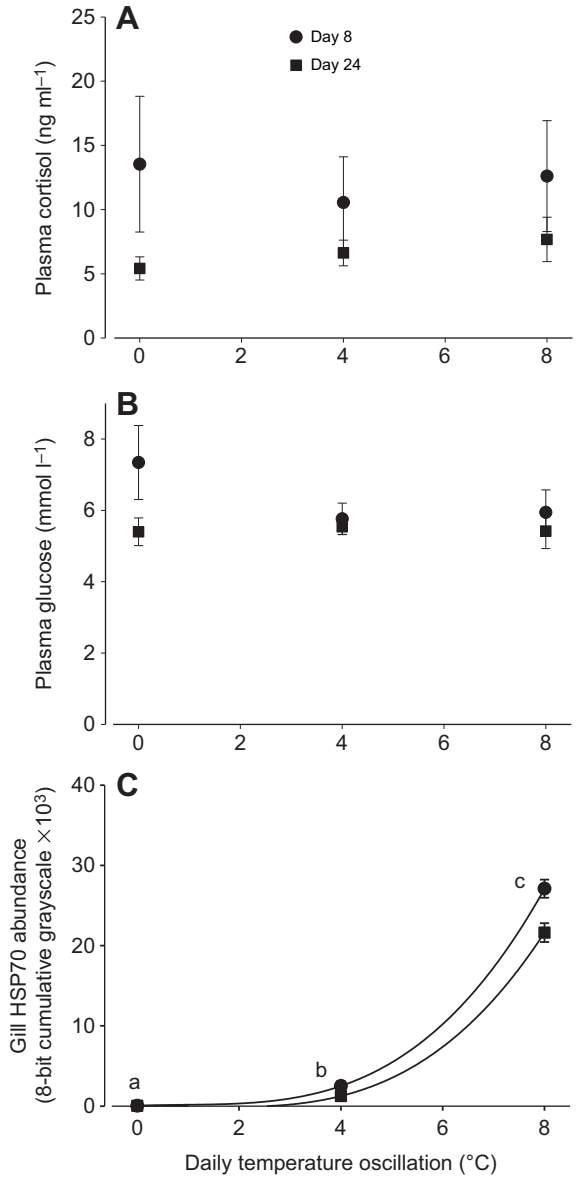

Fig. 6. Influence of oscillating temperatures on physiological stress responses of brook trout. Plasma cortisol (A), glucose (B) and gill HSP70 (C) levels in brook trout subjected to oscillating temperature treatments for 8 and 24 days. Values are means±s.e.m. of 7–8 fish per treatment. Means with the same letters were not significantly different from one another (P>0.05, Tukey's HSD *post hoc* test). Regression lines for HSP70 at day 8 and 24 were R^2=0.80 (P<0.0001) and R^2=0.77 (P<0.0001), respectively.

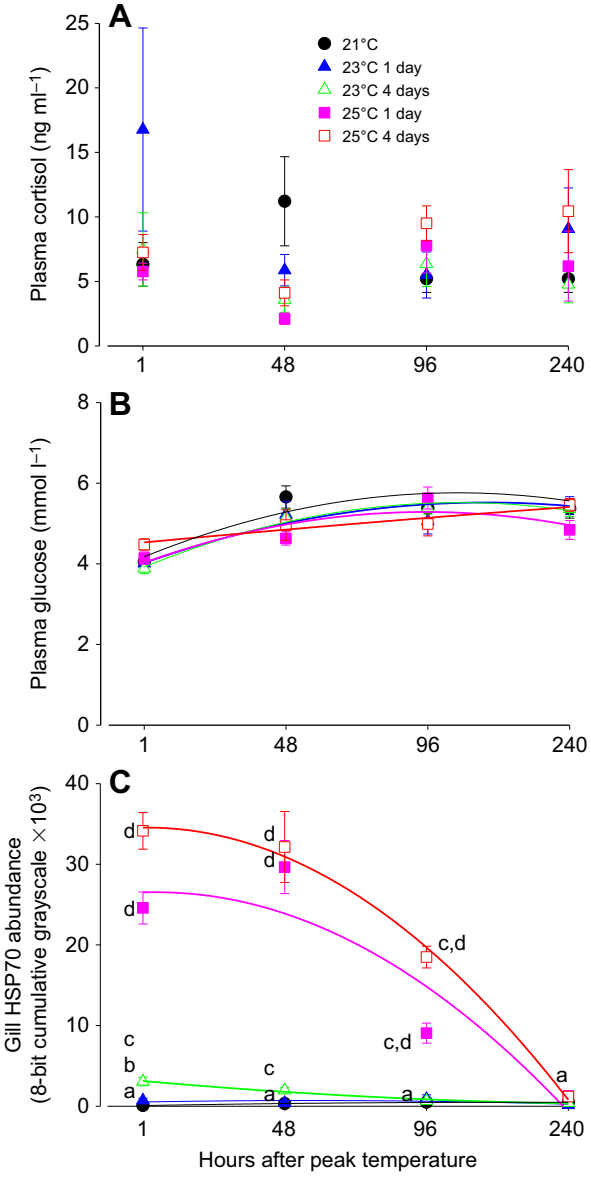

Fig. 7. Recovery of physiological stress responses from oscillating temperatures. Plasma cortisol (A), glucose (B) and gill HSP70 (C) levels in brook trout subjected to oscillating temperature treatments 1 and 4 days and then sampled 1, 48, 96 and 240 h after treatment. Values are means±s.e.m. of 7–8 fish per treatment. Means with the same letters were not significantly different from one another (P>0.05, Tukey's HSD *post hoc* test). All drawn regression lines were statistically significant (glucose P<0.0001; HSP70 P<0.018).

Journal of Experimental Biology (2017) 220, 3976-3987 doi:10.1242/jeb.161224

abundance corresponded with magnitude and duration of daily temperature oscillation (control <4°C oscillation 1 day <4°C oscillation 4 days <8°C oscillation 1 day <8°C oscillation 4 days). After fish were returned to control conditions, gill HSP70 abundance decreased over time to those of the control treated individuals. At 4 days, HSP70 abundance in individuals exposed to 4°C oscillation had recovered to control levels, whereas those exposed to 8°C oscillation remained elevated. At 10 days, there were no significant differences among the treatment groups. There was no effect of oscillating temperature on hematocrit (temperature, P=0.16; data not shown).

DISCUSSION

Our results indicate a decline in growth rate as temperature increases above 16°C in brook trout, and that the upper limit for their positive growth is 23.5°C. These results are in line with an extensive literature suggesting optimal growth of brook trout between 13°C and 16°C (Baldwin, 1956; Hokanson et al., 1973; Dwyer et al., 1983). None of these studies incorporated enough treatments above the optimal temperature to adequately describe brook trout growth at elevated temperatures. Nor did they test temperatures high enough to determine the upper limit for growth in brook trout. Wehrly et al. (2007) reported that brook trout are not found in waters above a 24 day mean maximum temperature of 22°C despite the fact that the lethal temperature in this species is 25.3°C (Fry et al., 1946; Fry, 1951; Wehrly et al., 2007). The fact that the ecological limit is more closely associated with temperature limitations on growth than it is with the lethal temperature suggests that temperature limitations on growth may play a key role in determining brook trout distributions.

In the current study, individually marked fish and near continuous monitoring of temperature were used to provide a clear indication of the relationship between temperature and growth. It should be noted that 'tank effects' (uncontrolled variables that affect an entire tank) can have impacts on growth rate in long-term experiments (Speare et al., 1995). Because even small differences in temperature accumulated over several weeks can impact growth, we chose to use a broad range of temperatures and a regression approach rather than replicate a small number of temperatures. This allowed us to more accurately determine the shape of the growth curve and relate it to physiological parameters. Using the same tank system as in Experiment 1, we found no evidence of tank effects in Experiment 2 at either constant or fluctuating temperatures. The accuracy of this approach is further supported by the strong correspondence between the specific growth rate of 1.95% day^{-1} found for nominal temperature 21°C in Experiment 2 and 1.75% day^{-1} that was predicted for this temperature by the growth curve from Experiment 1 (Fig. 2C).

It should also be noted that behavior of fish within tanks may have a role to play in growth and physiological responses. Individual hierarchies can develop within tanks (Johnsson et al., 1996), and temperature itself may affect these hierarchies. This may explain the greater variation in growth at intermediate temperatures used in the present study (22°C; Fig. 1C) than at the optimal (16°C) or highest temperatures (24°C). However, we found no fin damage in fish at higher temperatures that would indicate a high level of aggression was occurring under these conditions.

In spite of a well-developed literature in fish demonstrating decreased growth at elevated temperatures, the mechanism for temperature control of growth remains unclear. Induction of the endocrine stress response is likely to be involved in limiting growth at high temperature. In the current study, we observed an increase in plasma cortisol levels as temperature increased above 16°C, with highest plasma cortisol levels at 22°C and 24°C. This effect of temperature on plasma cortisol was greater at 24 days than it was at 8 days. Increased cortisol in response to elevated temperatures (Mesa et al., 2002; Quigley and Hinch, 2006), elevated temperature and angling (Meka and McCormick, 2005), elevated temperature and salinity (Gonçalves et al., 2006), and elevated temperature and exercise (Steinhausen et al., 2008) has been reported in a number of salmonids, and together with our findings implicate temperature as an endocrine stressor. In the current study, plasma cortisol levels were elevated at temperatures where growth was decreased. Cortisol is an important regulator of metabolism and elevated levels may impact growth through several pathways. Elevated plasma cortisol is known to increase metabolism as it heightens gluconeogenesis in the liver and raises rates of catabolic pathways such as glycolysis and proteolysis (Vanderboon et al., 1991; Mommsen et al., 1999). This increased metabolic rate is important during times of stress as it provides the necessary energy needed by vital organs to maintain homeostasis, but it also diverts resources away from anabolic pathways necessary for growth. In the laboratory, cortisol administration has been shown to increase aerobic and anaerobic metabolism in cutthroat trout and rainbow trout (Morgan and Iwama, 1996; De Boeck et al., 2001). More recently, cortisol injection resulted in decreased growth in wild largemouth bass and increased standard metabolic rate in laboratory-reared largemouth bass (O'Connor et al., 2011). Furthermore, exposure to a daily stressor resulted in increased metabolic rate and decreased aerobic scope in green sturgeon (Lankford et al., 2005).

In the current study, we observed decreased feeding at temperatures that were high enough to induce a cortisol response. This finding is in agreement with a growing literature that demonstrates a negative relationship between cortisol and appetite and feeding rates in fish. In rainbow trout and channel catfish exogenous cortisol administration resulted in decreased feeding and growth rates (Gregory and Wood, 1999; Peterson and Small, 2005; Madison et al., 2015). We have found that 30 day exogenous cortisol treatment of 8 μg g^{-1} resulted in a 75% reduction in growth rate of juvenile brook trout (L. Vargas-Chacoff and S.D.M., unpublished results). The estimated plasma cortisol at the midpoint of this treatment was 26 ng ml^{-1}, similar to the levels seen with the highest temperature treatment in the present study (22 ng ml^{-1}), indicating that the observed levels of plasma cortisol in the current study have the potential to affect growth in brook trout. Chronic stress and dietary cortisol reduced feed intake and conversion efficiency in sea bass (Leal et al., 2011). Atlantic salmon smolts and rainbow trout exhibited suppressed feeding following an acute confinement stressor (Pankhurst et al., 2008a,b). The exact mechanism for the suppression of feeding by cortisol is still under investigation, and while there is evidence that cortisol reduces plasma ghrelin levels (Pankhurst et al., 2008a,b), it is likely that other neuroendocrine factors controlling appetite are also affected. In addition to the direct effects of cortisol, it is possible that stimulation of corticotropin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH) in response to thermal stress may also affect appetite and growth in fish (Bernier and Peter, 2001).

Cortisol, elevated in response to high temperature, may also influence growth through the growth hormone/insulin-like growth factor I (GH/IGF-I) axis. Exogenous cortisol has been shown to reduce IGF-I mRNA and plasma levels in tilapia (Kajimura et al., 2003). In channel catfish, cortisol administration resulted in reduced growth and plasma IGF-I and increased plasma GH (Peterson and Small, 2005). In contrast, Madison et al. (2015) found that cortisol

treatment reduced growth in rainbow trout while decreasing plasma GH and with no change in plasma IGF-I. These findings add to an extensive literature on the effects of starvation in salmonids (Deane and Woo, 2009). Nutritional restriction often leads to liver GH resistance, a condition in which the downregulation of GH receptor results in decreased plasma IGF-I despite elevated plasma GH (Gray et al., 1992; Perez-Sanchez et al., 1994; Pierce et al., 2005). Indeed, it is possible that the observed effects of cortisol on the GH/IGF-I axis may be a secondary response to reduced food intake.

Cortisol modulates aspects of metabolism such as increasing plasma glucose levels via gluconeogenesis in the liver (Vanderboon et al., 1991; Wendelaar Bonga, 1997; Mommsen et al., 1999). In the current study, we found that long-term exposure to high temperature caused increased plasma cortisol without accompanying increases in plasma glucose. However, recent work from our laboratory demonstrated increased plasma glucose levels above 21.2°C in brook trout exposed to acute temperature increases (Chadwick et al., 2015). Vanlandeghem et al. (2010) observed an increase in plasma glucose in largemouth bass 1 h after heat shock, but not after 6 h. It is possible that glucose responds to acute, but not chronic, temperature elevations in brook trout and that we missed the signal due to the timing of our sampling. Increases in cortisol may in fact induce increased glucose production, which is matched by increased glucose utilization, resulting in no net increase in plasma glucose. It is also possible that fish at elevated temperatures simply depleted their hepatic glycogen stores as a result of this chronic thermal stressor. Indeed, we observed a 55% decrease in hepatosomatic index after 24 days at 24°C compared with 16°C controls.

In the current study, elevated temperature also induced a cellular stress response. Gill HSP70 levels increased with temperature and were 11- and 56-fold higher at 22°C and 24°C, respectively, than they were at 16°C. There was relatively little gill HSP70 at 20°C, but at 22°C expression had been induced, suggesting a threshold for induction of the HSP70 response of between 20°C and 22°C. Acute (hours) temperature exposures indicate a threshold for HSP70 induction of 20.7°C in brook trout (Chadwick et al., 2015). Interestingly, the threshold for the heat shock response is similar to both the upper limits for growth as well as to their upper ecological limit (Wehrly et al., 2007). Acute exposure to elevated temperature has been shown to increase red blood cell HSP70 abundance above control levels in brook trout at 25°C, but not at lower temperatures (Lund et al., 2003); however, they found increased HSP70 mRNA at 22°C in a variety of tissues. It is likely that the assay used in their study was not as sensitive as ours due to the use of an antibody that recognized the constitutive and inducible isoforms of HSP70, but there are also likely to be tissue-specific differences in HSP expression patterns. In Atlantic salmon, a close relative with greater thermal tolerance than brook trout, the threshold for induction of HSP70 and HSP30 was found to be between 22°C and 25°C (Lund et al., 2002). Acute exposure to 25°C or 26°C resulted in increased hepatic HSP70 levels in rainbow trout and Chinook salmon, but these studies were not designed to determine temperature thresholds for induction (Mesa et al., 2002; Rendell et al., 2006). In addition to these laboratory studies, field-based studies have observed a positive relationship between water temperature and HSP levels in brook trout, rainbow trout and Atlantic salmon (Lund et al., 2002; Werner et al., 2005; Feldhaus et al., 2010; Chadwick et al., 2015).

Here, we showed decreased growth at temperatures high enough to induce gill HSP70. The HSP response is not without energetic costs and may therefore impact growth. It has been suggested that the synthesis of HSPs consumes an inordinate amount of cellular or organismal nutrient stores and could occupy enough of the transcriptional and translational machinery within the cell to hinder other essential biochemical pathways, including those associated with growth (Feder and Hofmann, 1999). If this is true then one might expect to see an attenuated HSP response in individuals subjected to nutritional restriction. In a number of species, starved fish exhibit increased HSP levels under non-stressful temperature conditions (Cara et al., 2005; Yengkokpam et al., 2008; Piccinetti et al., 2012), but when given a temperature increase the HSP response in starved fish is less than that of fed fish (Deng et al., 2009; Piccinetti et al., 2012; Han et al., 2012). Viant et al. (2003) reported a correlation between HSP induction and reduced metabolic condition in juvenile steelhead trout. Currently, the metabolic cost of mounting an HSP response represents a gap in our understanding of the cellular stress response.

Our findings of decreased growth with increased temperature oscillation are similar to those reported in Lahontan cutthroat trout where growth declined with increasing magnitude of daily oscillation around a mean of 18°C (Meeuwig et al., 2004). In their study, mass growth rate was 24% and 52% lower in the 6°C and 12°C daily oscillation treatments, respectively, than in 18°C controls. A study in rainbow trout used several constant temperature treatments each with a corresponding 4°C daily oscillation treatment around the same mean temperature (Hokanson et al., 1977) and found that specific growth rate was similar across most of the constant and oscillating treatments but at the highest mean temperature (22°C), specific growth rate in the oscillating treatment (2.12 g day^{-1}) was substantially lower than in the constant control (3.94 g day^{-1}) (Hokanson et al., 1977). This is in agreement with work in coho and Atlantic salmon that utilized relatively low peak temperatures of 17°C and 20°C, respectively (Shrimpton et al., 2007; Thomas et al., 1986) and found no difference in growth between oscillating and constant temperature treatments. We propose that oscillating temperatures that involve daily exposure to stressful temperatures (e.g. those that induce an endocrine and/or cellular stress response) will have negative effects on growth rate, whereas those that are below the threshold for inducing stress will have little or no effect on growth. In streams containing brook trout in northeastern USA, daily variations in temperature can be as high as 6°C (J.G.C. and S.D.M., unpublished observations). Therefore, the temperature variations in these laboratory studies, including the current study, feature temperature oscillations that are ecologically relevant. The consistency of these findings give validity to the idea that daily temperature variations that are elevated beyond the thermal optimum result in reduced growth. It seems likely that these effects of daily temperature on growth will also occur in fish in the wild. Thus, predictive models of the impact of climate change on fish (and other ectotherms) will be improved by the inclusion of daily variations in temperature and their impact on growth and metabolism.

We were surprised that we did not observe an endocrine stress response in our oscillating temperature experiment, especially in light of the clear HSP70 response. Elevated plasma cortisol was clearly shown in individuals exposed to the chronically elevated temperatures of Experiment 1, and we have observed elevated plasma cortisol and glucose levels in wild brook trout sampled at sites with elevated temperatures (Chadwick et al., 2015). This is in addition to a literature that has shown elevations in plasma cortisol and glucose in response to elevated temperatures in other salmonids (Quigley and Hinch, 2006; Steinhausen et al., 2008). In a separate experiment from their growth study, Thomas et al. (1986) observed elevated plasma cortisol in individuals exposed to a daily oscillation

Journal of Experimental Biology

of 6.5–20°C when compared with more moderate daily oscillations and constant temperature controls. However, redband trout subjected to 8°C oscillations of 8–16°C or 18–26°C did not exhibit elevated plasma cortisol levels (Cassinelli and Moffitt, 2010). It is also possible that there was an endocrine stress response induced during our growth study and that we missed the signal due to the timing of our sampling. In Atlantic salmon, plasma cortisol and glucose have been shown to return to baseline levels within hours following an acute crowding stressor (Carey and McCormick, 1998). In our growth study, we sampled fish in the morning when water temperatures had been below 21°C for at least 9 h, potentially giving them time to recover from any thermal stress experienced during the previous day. However, in our acute exposures we sampled after 1 h at peak temperatures and still did not observe elevated plasma cortisol or glucose, suggesting that these oscillating temperature treatments were not severe or long enough to induce an endocrine stress response. It is possible that plasma cortisol will only be elevated (or detectably different) when temperatures are high enough to induce severe and long-term reductions in food consumption and growth. It should also be noted that there are diurnal (circadian) rhythms in plasma cortisol in fish that are independent of temperature (Audet and Claireaux, 1992) that may have affected our ability to detect temperature impacts on cortisol.

In addition to thermal impacts on brook trout growth and stress physiology we also explored the influence of elevated temperature on osmoregulation. Gill NKA activity (50%) and abundance (80%) decreased with temperature and were lowest at 24°C. The greater impact on abundance than activity suggests that the activity per unit molecule NKA is greater at elevated temperatures. Despite this, there were no differences in plasma Cl^- levels in our temperature treatments, and no effect of oscillating temperature on gill NKA activity. Elevated temperature has been shown to reduce the length of the smolt window, as characterized by decreased gill NKA activity and seawater tolerance, in anadromous salmonids (McCormick et al., 1996, 1999), although this may be more related to temperature impacts on development than it is to temperature effects on osmoregulation per se. Similarly, elevated temperature also reduces survival and gill NKA activity in sockeye salmon during their spawning migration (Crossin et al., 2008). An inverse relationship between temperature and gill NKA activity has been observed in cod (Staurnes et al., 1994), halibut (Jonassen et al., 1999) and pupfish (Stuenkel and Hillyard, 1980), but not in turbot (Burel et al., 1996). It is plausible that changes in enzyme kinetics or alterations in behavior at elevated temperatures lessen the demand for both gill NKA activity and abundance, although more exploration in these areas is clearly needed.

Understanding the impact of temperature on animal performance has been a goal of environmental physiology for decades (Fry, 1971). The threat of climate change and introduction of the oxygen- and capacity-limited thermal tolerance (OCLTT; Portner, 2010) have focused renewed interest on the relationship between animal physiology and their distributions in nature. In spite of this interest, there has been relatively little work in fish that characterizes the relationship between elevated temperature and decreasing food consumption and growth. Our results indicate that the decrease in growth with elevated temperature is relatively gradual and not a sharp decrease that characterizes many hypothetical performance curves. This distinction is important, as it shapes our understanding of the response to temperature and the mechanisms behind it. Such a gradual decrease may not be consistent with the catastrophic loss of protein function, which has been suggested to be involved with declining performance at high temperature (Portner, 2010). One possible explanation for this more gradual decrease in food consumption and growth rate is that animals are protecting themselves from a dramatic decrease in aerobic scope that would occur after a large meal. The metabolic costs of digesting a meal can be as high as basal metabolic rate and even higher at elevated temperature (Brett, 1979), and thus could limit aerobic scope. Maintaining a safety margin of aerobic scope may be crucial for predator avoidance and thus important to survival. This hypothesis is consistent with the 'voluntary' nature of lower food consumption at higher temperature. Digestive inefficiencies and greater costs of digestion may play a synergistic role in this scenario.

Here, we demonstrated reduced growth in juvenile brook trout held at constantly elevated temperatures. These treatments also induced the cellular and endocrine stress responses at thresholds similar to those for significant decreases in growth. Growth is an important aspect of life history that affects the reproductive capacity of an individual. In salmonids, a clear relationship between body size and fecundity has been demonstrated (Thorpe et al., 1984), and an inverse relationship between temperature and reproduction has been described in wild brook trout (Robinson et al., 2010). Furthermore, reduced body size may increase an individual's vulnerability to predation and decrease its ability to establish territory and exploit food resources. Taken together, the impact of elevated temperature on growth in brook trout individuals may provide a mechanism by which populations are limited by elevated temperature. We fully acknowledge that the response of any species to a changing environment is dynamic and that changes in growth rate represent only one of many potential responses. Elevated temperatures may impact behavior, feeding and predator avoidance (along with a host of other aspects of physiology), as well as affecting most other species with which brook trout interact. Nonetheless, our results present evidence of a sublethal pathway through which elevated temperatures will affect animal distribution.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.G.C., S.D.M.; Methodology: J.G.C., S.D.M.; Validation: J.G.C., S.D.M.; Formal analysis: J.G.C.; Resources: S.D.M.; Data curation: J.G.C.; Writing - original draft: J.G.C.; Writing - review & editing: J.G.C., S.D.M.; Supervision: S.D.M.; Project administration: S.D.M.; Funding acquisition: J.G.C., S.D.M.

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References
Audet, C. and Claireaux, G. (1992). Diel and seasonal changes in resting levels of various blood parameters in brook trout (*Salvelinus fontinalis*). *Can. J. Fish. Aquat. Sci.* **49**, 870-877.
Baldwin, N. S. (1956). Food consumption and growth of brook trout at different temperatures. *Trans. Am. Fish. Soc.* **86**, 323-328.
Bernier, N. J. and Peter, R. E. (2001). The hypothalamic–pituitary–interrenal axis and the control of food intake in teleost fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **129**, 639-644.

Brett, J. R. (1979). Bioenergetics and growth. *Fish Physiol.* **8**, 599-675.
Burel, C., Person-Le Ruyet, J., Gaumet, F., Le Roux, A., Severe, A. and Boeuf, G. (1996). Effects of temperature on growth and metabolism in juvenile turbot. *J. Fish Biol.* **49**, 678-692.
Callaghan, N. I., Tunnah, L., Currie, S. and MacCormack, T. J. (2016). Metabolic adjustments to short-term diurnal temperature fluctuation in the rainbow trout (*Oncorhynchus mykiss*). *Physiol. Biochem. Zool.* **89**, 498-510.
Cara, J. B., Aluru, N., Moyano, F. J. and Vijayan, M. M. (2005). Food-deprivation induces HSP70 and HSP90 protein expression in larval gilthead sea bream and rainbow trout. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **142**, 426-431.
Carey, J. B. and McCormick, S. D. (1998). Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr. *Aquaculture* **168**, 237-253.
Cassinelli, J. D. and Moffitt, C. M. (2010). Comparison of growth and stress in resident redband trout held in laboratory simulations of montane and desert summer temperature cycles. *Trans. Am. Fish. Soc.* **139**, 339-352.
Chadwick, J. G., Nislow, K. H. and McCormick, S. D. (2015). Thermal onset of cellular and endocrine stress responses correspond to ecological limits in brook trout, an iconic cold water fish. *Conserv. Physiol.* **3**, cov017.
Crossin, G. T., Hinch, S. G., Cooke, S. J., Welch, D. W., Patterson, D. A., Jones, S. R. M., Lotto, A. G., Leggatt, R. A., Mathes, M. T., Shrimpton, J. M. et al. (2008). Exposure to high temperature influences the behaviour, physiology, and survival of sockeye salmon during spawning migration. *Can. J. Zool.* **86**, 127-140.
Deane, E. E. Woo, N. Y. S. (2009). Modulation of fish growth hormone levels by salinity, temperature, pollutants and aquaculture related stress: a review. *Rev. Fish Biol. Fish.* **19**, 97-120.
De Boeck, G., Alsop, D. and Wood, C. (2001). Cortisol effects on aerobic and anaerobic metabolism, nitrogen excretion, and whole-body composition in juvenile rainbow trout. *Physiol. Biochem. Zool.* **74**, 858-868.
Deng, D.-F., Wang, C. F., Lee, S., Bai, S. and Hung, S. S. O. (2009). Feeding rates affect heat shock protein levels in liver of larval white sturgeon (*Acipenser transmontanus*). *Aquaculture* **287**, 223-226.
DuBeau, S. F., Pan, F., Tremblay, G. C. and Bradley, T. M. (1998). Thermal shock of salmon *in vivo* induces the heat shock protein HSP 70 and confers protection against osmotic shock. *Aquaculture* **168**, 311-323.
Dwyer, W. P., Piper, R. G. and Smith, C. E. (1983). Brook trout growth efficiency as affected by temperature. *Prog. Fish-Cult.* **45**, 161-163.
Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.
Feldhaus, J. W., Heppell, S. A., Li, H. and Mesa, M. G. (2010). A physiological approach to quantifying thermal habitat quality for Redband Rainbow Trout (*Oncorhynchus mykiss gairdneri*) in the south Fork John Day River, Oregon. *Environ. Biol. Fishes* **87**, 277-290.
Flebbe, P. A., Roghair, L. D. and Bruggink, J. L. (2006). Spatial Modeling to project southern Appalachian trout distribution in a warmer climate. *Trans. Am. Fish. Soc.* **135**, 1371-1382.
Fry, F. E. J. (1951). Some environmental relations of the speckled trout (*Salvelinus fontinalis*). *Proc. N.E.Atlantic Fish. Conf.* **1**, 1-29.
Fry, F. E. J. (1971). The effect of environmental factors on the physiology of fish. In *Fish Physiology Volume VI, Environmental Relations and Behavior* (ed. W. S. Hoar and D. J. Randall), pp. 1-98. New York: Academic Press.
Fry, F. E. J., Hart, S. A. and Walker, K. F. (1946). Lethal temperature relations for a sample of young speckled trout, *Salvelinus fontinalis*. *Univ. Toronto Stud. Biol. Ser.* **54**, 9-35.
Gonçalves, J., Carraça, S., Damasceno-Oliveira, A., Fernández-Durán, B., Diaz, J., Wilson, J. and Coimbra, J. (2006). Effect of reduction in water salinity on osmoregulation and survival of large Atlantic salmon held at high water temperature. *N. Am. J. Aquac.* **68**, 324-329.
Gray, E. S., Kelley, K. M., Law, S., Tsai, R., Young, G. and Bern, H. A. (1992). Regulation of hepatic growth-hormone receptors in coho salmon (Oncorhynchus-Kisutch). *Gen. Comp. Endocrinol.* **88**, 243-252.
Gregory, T. R. and Wood, C. M. (1999). The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. *Physiol. Biochem. Zool.* **72**, 286-295.
Han, D., Huang, S. S. Y., Wang, W.-F., Deng, D.-F. and Hung, S. S. O. (2012). Starvation reduces the heat shock protein responses in white sturgeon larvae. *Environ. Biol. Fishes* **93**, 333-342.
Hokanson, K. E. F., McCormick, J. H., Jones, B. R. and Tucker, J. H. (1973). Thermal requirements for maturation, spawning, and embryo survival of the brook trout, *Salvelinus fontinalis*. *J. Fish. Res. Board Can.* **30**, 975-984.
Hokanson, K. E. F., Kleiner, C. F. and Thorslund, T. W. (1977). Effects of constant temperatures and diel temperature fluctuations on specific growth and mortality rates and yield of juvenile rainbow trout, *Salmo gairdneri*. *J. Fish. Res. Board Can.* **34**, 639-648.
Iwama, G. K., Vijayan, M. M., Forsyth, R. B. and Ackerman, P. A. (1999). Heat shock proteins and physiological stress in fish. *Am. Zool.* **39**, 901-909.
Iwama, G. K., Afonso, L. O. B., Todgham, A., Ackerman, P. and Nakano, K. (2004). Are HSPs suitable for indicating stressed states in fish? *J. Exp. Biol.* **207**, 15-19.
Johnsson, J. I., Jönsson, E. and Björnsson, B. T. (1996). Dominance, nutritional state, and growth hormone levels in rainbow trout (*Oncorhynchus mykiss*). *Horm. Behav.* **30**, 13-21.
Jonassen, T. M., Imsland, A. K. and Stefansson, S. O. (1999). The interaction of temperature and fish size on growth of juvenile halibut. *J. Fish Biol.* **54**, 556-572.
Kajimura, S., Hirano, T., Visitacion, N., Moriyama, S., Aida, K. and Grau, E. G. (2003). Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus*. *J. Endocrinol.* **178**, 91-99.
Lankford, S. E., Adams, T. E., Miller, R. A. and Cech, J. J.Jr (2005). The cost of chronic stress: Impacts of a nonhabituating stress response on metabolic variables and swimming performance in sturgeon. *Physiol. Biochem. Zool.* **78**, 599-609.
Leal, E., Fernández-Durán, B., Guillot, R., Ríos, D. and Cerda-Reverter, J. M. (2011). Stress-induced effects on feeding behavior and growth performance of the sea bass (*Dicentrarchus labrax*): a self-feeding approach. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **181**, 1035-1044.
Lund, S. G., Caissie, D., Cunjak, R. A., Vijayan, M. M. and Tufts, B. L. (2002). The effects of environmental heat stress on heat-shock mRNA and protein expression in Miramichi Atlantic salmon (*Salmo salar*) parr. *Can. J. Fish. Aquat. Sci.* **59**, 1553-1562.
Lund, S. G., Lund, M. E. A. and Tufts, B. L. (2003). Red blood cell HSP 70 mRNA and protein as bioindicators of temperature stress in the brook trout (*Salvelinus fontinalis*). *Can. J. Fish. Aquat. Sci.* **60**, 460-470.
Madison, B. N., Tavakoli, S., Kramer, S. and Bernier, N. J. (2015). Chronic cortisol and the regulation of food intake and the endocrine growth axis in rainbow trout. *J. Endocrinol.* **226**, 103-119.
McCormick, S. D. (1993). Methods for nonlethal gill biopsy and measurement of Na+, K+ -ATPase activity. *Can. J. Fish. Aquat. Sci.* **50**, 656-658.
McCormick, J. H., Jones, B. R. and Hokanson, K. E. F. (1972). Effects of temperature on growth and survival of young brook trout, *Salvelinus fontinalis*. *J. Fish. Res. Board Can.* **29**, 1107-1112.
McCormick, S. D., Shrimpton, J. M. and Zydlewski, J. D. (1996). Temperature effects on osmoregulatory physiology of juvenile anadromous fish. In *Global Warming: Implications for Freshwater and Marine Fish*. (ed. C. M. Wood and D. G. McDonald), pp. 279-301. Cambridge: Cambridge University Press.
McCormick, S. D., Cunjak, R. A., Dempson, B., O'Dea, M. F. and Carey, J. B. (1999). Temperature-related loss of smolt characteristics in Atlantic salmon (*Salmo salar*) in the wild. *Can. J. Fish. Aquat. Sci.* **56**, 1649-1667.
McCormick, S. D., Regish, A. M. and Christensen, A. K. (2009). Distinct freshwater and seawater isoforms of Na+/K+-ATPase in gill chloride cells of Atlantic salmon. *J. Exp. Biol.* **212**, 3994-4001.
McMahon, T. E., Zale, A. V., Barrows, F. T., Selong, J. H. and Danehy, R. J. (2007). Temperature and competition between bull trout and brook trout: A test of the elevation refuge hypothesis. *Trans. Am. Fish. Soc.* **136**, 1313-1326.
Meeuwig, M. H., Dunham, J. B., Hayes, J. P. and Vinyard, G. L. (2004). Effects of constant and cyclical thermal regimes on growth and feeding of juvenile cutthroat trout of variable sizes. *Ecol. Freshw. Fish.* **13**, 208-216.
Meisner, J. D. (1990). Potential loss of thermal habitat for brook trout, due to climatic warming, in 2 southern ontario streams. *Trans. Am. Fish. Soc.* **119**, 282-291.
Meka, J. M. and McCormick, S. D. (2005). Physiological response of wild rainbow trout to angling: impact of angling duration, fish size, body condition, and temperature. *Fish. Res.* **72**, 311-322.
Mesa, M. G., Weiland, L. K. and Wagner, P. (2002). Effects of acute thermal stress on the survival, predator avoidance, and physiology of juvenile fall chinook salmon. *Northwest. Sci.* **76**, 118-128.
Mommsen, T. P., Vijayan, M. M. and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* **9**, 211-268.
Morgan, J. D. and Iwama, G. K. (1996). Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. *Fish Physiol. Biochem.* **15**, 385-394.
O'Connor, C. M., Gilmour, K. M., Arlinghaus, R., Matsumura, S., Suski, C. D., Philipp, D. P. and Cooke, S. J. (2011). The consequences of short-term cortisol elevation on individual physiology and growth rate in wild largemouth bass (*Micropterus salmoides*). *Can. J. Fish. Aquat. Sci.* **68**, 693-705.
Pankhurst, N. W., King, H. R. and Ludke, S. L. (2008a). Relationship between stress, feeding and plasma ghrelin levels in rainbow trout, *Oncorhynchus mykiss*. *Mar. Freshwater Behav. Physiol.* **41**, 53-64.
Pankhurst, N. W., Ludke, S. L., King, H. R. and Peter, R. E. (2008b). The relationship between acute stress, food intake, endocrine status and life history stage in juvenile farmed Atlantic salmon, *Salmo salar*. *Aquaculture* **275**, 311-318.
Pelis, R. M. and McCormick, S. D. (2001). Effects of growth hormone and cortisol on Na+-K+-2Cl(−) cotransporter localization and abundance in the gills of Atlantic salmon. *Gen. Comp. Endocrinol.* **124**, 134-143.
Perez-Sanchez, J., Marti-Palanca, H. and Le Bail, P.-Y. (1994). Homologous growth-hormone (Gh) binding in gilthead sea bream (*Sparus-Aurata*) - effect of fasting and refeeding on hepatic Gh-binding and plasma somatomedin-like immunoreactivity. *J. Fish Biol.* **44**, 287-301.
Peterson, B. C. and Small, B. C. (2005). Effects of exogenous cortisol on the GH/IGF-I/IGFBP network in channel catfish. *Domest. Anim. Endocrinol.* **28**, 391-404.

Piccinetti, C. C., Ricci, L. A., Tokle, N., Radaelli, G., Pascoli, F., Cossignani, L., Palermo, F., Mosconi, G., Nozzi, V., Raccanello, F. et al. (2012). Malnutrition may affect common sole (*Solea solea* L.) growth, pigmentation and stress response: molecular, biochemical and histological implications. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **161**, 361-371.

Pierce, A. L., Shimizu, M., Beckman, B. R., Baker, D. M. and Dickhoff, W. W. (2005). Time course of the GH/IGF axis response to fasting and increased ration in chinook salmon (*Oncorhynchus tshawytscha*). *Gen. Comp. Endocrinol.* **140**, 192-202.

Portner, H.-O. (2010). Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* **213**, 881-893.

Portner, H. O. and Farrell, A. P. (2008). Ecology, physiology and climate change. *Science* **322**, 690-692.

Portner, H. O., Farrell, A. P., Knust, R., Lannig, G., Mark, F. C. and Storch, D. (2009). Adapting to climate change response. *Science* **323**, 876-877.

Quigley, J. T. and Hinch, S. G. (2006). Effects of rapid experimental temperature increases on acute physiological stress and behaviour of stream dwelling juvenile chinook salmon. *J. Therm. Biol.* **31**, 429-441.

Rendell, J. L., Fowler, S., Cockshutt, A. and Currie, S. (2006). Development-dependent differences in intracellular localization of stress proteins (HSPs) in rainbow trout, *Oncorhynchus mykiss*, following heat shock. *Comp. Biochem. Physiol. D Genomics Proteomics* **1**, 238-252.

Robinson, J. M., Josephson, D. C., Weidel, B. C. and Kraft, C. E. (2010). Influence of variable interannual summer water temperatures on brook trout growth, consumption, reproduction, and mortality in an unstratified adirondack lake. *Trans. Am. Fish. Soc.* **139**, 685-699.

Shrimpton, J. M., Zydlewski, J. D. and Heath, J. W. (2007). Effect of daily oscillation in temperature and increased suspended sediment on growth and smolting in juvenile chinook salmon, *Oncorhynchus tshawytscha*. *Aquaculture* **273**, 269-276.

Smith, T. R., Tremblay, G. C. and Bradley, T. M. (1999). Characterization of the heat shock protein response of Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **20**, 279-292.

Sokolova, I. M. (2013). Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. *Integr. Comp. Biol.* **53**, 597-608.

Speare, D. J., Macnair, N. and Hammell, K. L. (1995). Demonstration of tank effect on growth of juvenile rainbow trout (*Oncorhynchus mykiss*) during an *ad libitum* feeding trial. *Am. J. Vet. Res.* **56**, 1372-1379.

Staurnes, M., Rainuzzo, J. R., Sigholt, T. and Jørgensen, L. (1994). Acclimation of Atlantic cod (Gadus-Morhua) to cold-water - stress-response, osmoregulation, gill lipid-composition and gill Na-K-Atpase activity. *Comp. Biochem. Physiol. A Physiol.* **109**, 413-421.

Steinhausen, M. F., Sandblom, E., Eliason, E. J., Verhille, C. and Farrell, A. P. (2008). The effect of acute temperature increases on the cardiorespiratory performance of resting and swimming sockeye salmon (*Oncorhynchus nerka*). *J. Exp. Biol.* **211**, 3915-3926.

Stitt, B. C., Burness, G., Burgomaster, K. A., Currie, S., McDermid, J. L. and Wilson, C. C. (2014). Intraspecific variation in thermal tolerance and acclimation capacity in brook trout (*Salvelinus fontinalis*): physiological implications for climate change. *Physiol. Biochem. Zool.* **87**, 15-29.

Stuenkel, E. L. and Hillyard, S. D. (1980). Effects of temperature and salinity on Gill Na+-K+ ATPase activity in the pupfish, *Cyprinodon salinus*. *Comp. Biochem. Physiol. A Physiol.* **67**, 179-182.

Thomas, R. E., Gharrett, J. A., Carls, M. G., Rice, S. D., Moles, A. and Korn, S. (1986). Effects of fluctuating temperature on mortality, stress, and energy reserves of juvenile coho salmon. *Trans. Am. Fish. Soc.* **115**, 52-59.

Thorpe, J. E., Miles, M. S. and Keay, D. S. (1984). Developmental rate, fecundity and egg size in Atlantic salmon, *Salmo salar* L. *Aquaculture* **43**, 289-305.

Vanderboon, J., Vandenthillart, G. E. E. J. and Addink, A. D. F. (1991). The effects of cortisol administration on intermediary metabolism in teleost fish. *Comp. Biochem. Physiol. A Physiol.* **100**, 47-53.

Vanlandeghem, M. M., Wahl, D. H. and Suski, C. D. (2010). Physiological responses of largemouth bass to acute temperature and oxygen stressors. *Fish. Manag. Ecol.* **17**, 414-425.

Viant, M. R., Werner, I., Rosenblum, E. S., Gantner, A. S., Tjeerdema, R. S. and Johnson, M. L. (2003). Correlation between heat-shock protein induction and reduced metabolic condition in juvenile steelhead trout (*Oncorhynchus mykiss*) chronically exposed to elevated temperature. *Fish Physiol. Biochem.* **29**, 159-171.

Wehrly, K. E., Wang, L. Z. and Mitro, M. (2007). Field-based estimates of thermal tolerance limits for trout: Incorporating exposure time and temperature fluctuation. *Trans. Am. Fish. Soc.* **136**, 365-374.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* **77**, 591-625.

Werner, I., Smith, T. B., Feliciano, J. and Johnson, M. L. (2005). Heat shock proteins in juvenile steelhead reflect thermal conditions in the Navarro River watershed, California. *Trans. Am. Fish. Soc.* **134**, 399-410.

Wikelski, M. and Cooke, S. J. (2006). Conservation physiology. *Trends Ecol. Evol.* **21**, 38-46.

Yengkokpam, S., Pal, A. K., Sahu, N. P., Jain, K. K., Dalvi, R., Misra, S. and Debnath, D. (2008). Metabolic modulation in *Labeo rohita* fingerlings during starvation: HSP70 expression and oxygen consumption. *Aquaculture* **285**, 234-237.