

Environmental variation associated with overwintering elicits marked metabolic plasticity in a temperate salmonid, *Salvelinus fontinalis*

Ella K. Middleton¹, Matthew J. H. Gilbert^{1,*}, Thomas Landry², Simon G. Lamarre², Ben Speers-Roesch¹

¹Department of Biological Sciences, University of New Brunswick, Saint John, Canada

²Département de Biologie, Université de Moncton, Moncton, Canada

* Author for correspondence: mgilbert5@alaska.edu

Keywords: Brook char, thermal acclimation, energy expenditure, oxygen uptake, protein synthesis, food deprivation, winter

Summary statement

Food deprivation and frigid temperatures, common winter challenges, elicit tissue-specific energy conservation mechanisms and thermal compensation in brook char. Despite lower resting metabolism during starvation, aerobic metabolic capacity was maintained.

ABSTRACT

Poleward winters commonly expose animals, including fish, to frigid temperatures and low food availability. Fishes that remain active over winter must therefore balance trade-offs between conserving energy and maintaining physiological performance in the cold, yet the extent and underlying mechanisms of these trade-offs are not well understood. We investigated the metabolic plasticity of brook char (*Salvelinus fontinalis*), a temperate salmonid, from the biochemical to whole-animal level in response to cold and food deprivation. Acute cooling (1°C day⁻¹) from 14°C to 2°C had no effect on food consumption but reduced activity by 77%. We then assessed metabolic performance and demand over 90 days with exposure to warm (8°C) or cold winter (2°C) temperatures while fed or starved. Resting metabolic rate (RMR) decreased substantially during initial cooling from 8°C to 2°C ($Q_{10}=4.2-4.5$) but brook char exhibited

remarkable thermal compensation during acclimation ($Q_{10}=1.4 - 1.6$). Conversely, RMR was substantially lower (40-48%) in starved fish, conserving energy. Thus, the absolute magnitude of thermal plasticity may be masked or modified under food restriction. This reduction in RMR was associated with atrophy and decreases in *in vivo* protein synthesis rates, primarily in non-essential tissues. Remarkably, food deprivation had no effect on maximum oxygen uptake rates and thus aerobic capacity, supporting the notion that metabolic capacity can be decoupled from RMR in certain contexts. Overall, our study highlights the multi-faceted energetic flexibility of *Salvelinus* spp. that likely contributes to their success in harsh and variable environments and may be emblematic of winter-active fishes more broadly.

INTRODUCTION

Temperate winters are commonly characterized by frigid temperatures and low food availability, which pose distinct energetic challenges for ectotherms (Fernandes and McMeans, 2019; Shuter et al., 2012). The impact of food deprivation on survival and reproductive success depends on existing endogenous energy stores, the ability to acquire exogenous energy despite reduced availability, and the rate of energy expenditure (Hurst, 2007; Secor and Carey, 2016). Frigid temperatures can exacerbate or alleviate winter energy limitations. Specifically, cold can constrain physiological functionality, in part, by slowing biochemical reaction rates (Schulte, 2015), which can impair feeding and digestion (Fernandes and McMeans, 2019). However, this same passive slowing effect markedly reduces energy expenditure with whole-animal metabolic rate generally decreasing by >50% for every 10°C of cooling ($Q_{10}\sim 2-3$; Clarke, 2017; Clarke & Johnston, 1999).

In response to winter cold some fish become dormant or lethargic, feeding minimally if at all (Reeve et al., 2022), while others including most salmonids remain relatively active (Auer et al., 2020; Blanchfield et al., 2009; Cunjak and Power, 1987; Fraser et al., 1993). Many winter-active fishes upregulate metabolic processes to partially compensate for constraints imposed by the cold (i.e., thermal compensation via acclimation). However, this compensation raises whole-animal resting metabolic rate thereby counteracting beneficial energy savings of cooling (Guderley, 1990; Norin and Speers-Roesch, 2021). While intuitive, the extent of this trade-off is poorly understood and the combined energetic effects of food deprivation and cold have rarely

been examined in fishes, despite being common co-occurring winter challenges (Huey and Buckley, 2022).

Metabolic flexibility could allow fishes to balance their energy expenditure over winter in a context-dependent manner (Auer et al., 2016b; O'Connor et al., 2000; Reeve et al., 2022; Speers-Roesch et al., 2018; Van Leeuwen et al., 2012). For instance, when faced with food deprivation, fish could conserve energy by blunting metabolic thermal compensation. A lack of compensation of resting metabolic rate has been previously observed in winter-dormant and lethargic fishes (Healy et al., 2017; Reeve et al., 2022), but a mechanistic basis is unclear. Alternatively, metabolic thermal compensation could be retained but additional energy conservation mechanisms could be employed; beyond reducing activity, fish could actively downregulate specific costly physiological functions (e.g., protein synthesis, mitochondrial abundance; [Salin et al., 2018](#)) in non-critical tissues (e.g., digestive system) thereby reducing whole-animal energy expenditure while maintaining the functionality of critical tissues. Indeed, a primary way that other fasting ectotherms and hibernating mammals reduce energetic demands in food-poor environments is through such targeted reductions in resting metabolic costs (Auer et al., 2016b; Naya et al., 2009; Tøien et al., 2011; Van Leeuwen et al., 2012). However, this downregulation could be accompanied by a reduction in maximum performance (e.g., digestive capacity or maximum metabolic rate, MMR) (Bennett and Ruben, 1979); higher resting metabolic rates may be associated with greater maximal metabolic performance, as it costs energy to maintain the machinery supporting elevated metabolic performance (Auer et al., 2015; Guderley, 2004; Monternier et al., 2014) ([but see: Auer et al., 2016; Barceló et al., 2017; Koteja, 1987; Salin et al., 2016; Zeng et al., 2018](#)). In contrast, in more food-rich environments the maintenance and thermal compensation of digestive and aerobic capacity may justify any additional cost (i.e., elevated resting metabolic rate). Still, we have limited insight into how winter-active fishes balance the trade-offs between the potential need for energy conservation with the cost of maintaining an active, high-capacity phenotype over winter. This knowledge gap is of particular concern as winter conditions are rapidly changing which may impact the efficacy of fishes' overwintering strategies (Morash et al., 2021).

To address this knowledge gap, we studied brook char, a temperate salmonid native to north-eastern North America (MacCrimmon and Campbell, 1969). Brook char, like most salmonids, can remain active and feeding in the winter (Cunjak and Power, 1987; Cunjak et al.,

1987) and can experience food limitation (Spares et al., 2014), making them ideal to study energetic trade-offs associated with overwintering. First, to determine the effect of cooling on voluntary feeding and activity, which are key energetic traits, brook char were gradually cooled from 14°C to 2°C while being fed daily and monitored continuously. In our second experiment, we exposed brook char to two ecologically relevant winter water temperatures (8°C and 2°C) for up to 90 days while either fed or starved. To understand the metabolic and energetic impacts of these conditions we assessed resting and maximum oxygen uptake, tissue-specific energy demands (*in vivo* protein synthesis rate and organ sizes) and endogenous energy storage. Resting metabolic rate was assessed at four timepoints over 90 days to reveal the temporal dynamics of plasticity in response to cold and food deprivation, a valuable approach to study acclimation that is rarely employed (Havird et al., 2020; Somero, 2015). Given the well-documented environmental plasticity of *Salvelinus* fishes (Armstrong and Bond, 2013; Dutil, 1986; Gilbert and Farrell, 2021; Hutchings, 1996; Muir et al., 2016), we hypothesized that these winter-active fish possess the metabolic flexibility to balance energetic trade-offs of combined variation in temperature and food availability. Accordingly, we predicted that brook char would thermally compensate for prolonged exposure to 2°C resulting in an increase in resting metabolic rate over time, but to a greater extent in fed fish. Furthermore, we predicted that brook char would reduce resting metabolic rate in response to starvation, conserving energy but compromising maximum aerobic performance. Finally, we expected reductions in resting metabolic rate to be largely driven by reductions in energy demand in tissues and processes associated with growth and digestion.

METHODS

All experiments were approved by the Animal Care Committee of the University of New Brunswick (UNB), Saint John, following the Canadian Council on Animal Care standards (UNB 2020-4S-03).

Experiment 1: The effect of cooling on activity and food consumption of brook char

Experimental animals

Juvenile (<1 year) brook char were obtained from a captive breeding population at UNB Fredericton, New Brunswick, Canada in October 2020. They were held at UNB Saint John in 400 L circular fibreglass holding tanks supplied with flow-through dechlorinated freshwater (~15°C) and fed dry pellets (1.8mm Gemma, Skretting, St. Andrews, NB, Canada) to satiety three times per week. During holding and both experiments, fish were always under a winter photoperiod (10L:14D) typical for temperate latitudes; a simulated sunrise and sunset (30 min each) were included in the lighting period to minimize potential effects of sudden lighting changes on behaviour (Ryu et al., 2020).

Brook char used for Experiment 1 (n=15, length=82.9 ± 5.8 mm, mass=5.30 ± 0.84 g, mean ± S.D.) were transferred to three 90 L glass aquaria (5 fish per aquarium) two weeks before the start of the experiment. These tanks were supplied with recirculated temperature-controlled filtered dechlorinated freshwater (14°C ± 0.5°C) and fed dry pellets once per day to satiety.

Experimental design

To quantify the behavioural responses of brook char to cooling we measured spontaneous activity and food consumption during an acute cooling period from 14°C to 2°C (1°C day⁻¹), and a subsequent acclimation period of two weeks at 2°C ± 0.5°C.

Activity was measured as described in Reeve et al. (2022) with minor modifications. Briefly, the housing system consisted of a clear outer acrylic aquarium containing 15 clear plastic arenas (20.2x15.6x9.7 cm), each housing a single fish (n=15); each arena received recirculating, filtered, aerated and temperature-controlled freshwater from the external bath. The system was illuminated from below by one near-infrared lamp (940 nm) and fish were continuously video recorded from above with two infrared-sensitive cameras (640x480p, 10-15 fps, IDS Imaging, Obersulm, Germany). Fish were allowed 36 hours at 14°C to adjust to the arenas with food withheld before the recording of activity and food consumption began. After this adjustment period, the fish were exposed to 14°C for an additional 24 hours, following

which the system was cooled by 1°C each morning until it reached 2°C. Cooling lasted ~30 minutes, during which time feeding counts were performed. Fish were fed a standard ration of dry pellets (0.5% of body mass) each morning and any remaining pellets were counted the following morning to estimate daily food consumption.

Experiment 2: The effects of prolonged starvation at 8°C or 2°C on brook char energetics and metabolism

Experimental animals

The second experiment was conducted with brook char that were obtained in 2018 from the same breeding population as in Experiment 1. These fish were reared under the same holding conditions as Experiment 1 but were fed larger pellets (23 kJ g⁻¹; 40A 4.0 mm BioTrout, Bio-Oregon, Washington State, USA) prior to the start of the experiment because they were larger. Two weeks before beginning Experiment 2, 60 brook char (length=214.3 ± 19.15 mm, mass=137.78 ± 38.36 g, mean ± S.D.) were distributed equally among four recirculating freshwater systems at 8°C ± 0.5°C which served as the treatment systems (see below) once the experiment started. During the two weeks before Experiment 2 began, all fish were fed dry pellets once per day (~0.5% average body mass ration per aquarium).

Experimental design

Experiment 2 lasted for a total of 96 days and had a full-factorial design. Each of the four treatment systems consisted of a 180 L sump with bio- and solids filters, and three 90 L glass aquaria that each housed five fish and contained a cylindrical PVC shelter. The sump was supplied with a constant drip of dechlorinated freshwater (not strong enough to alter water temperatures) to further maintain water quality. Fish in the four acclimation systems were exposed to either 2°C or 8°C (i.e., two systems per temperature) and were either fed daily (~0.5% body mass ration) or starved (no feed). The temperatures were chosen based on data for winter water temperature throughout the natural range of brook char (Brook Trout Atlas; Government of Canada, 2023; MacCrimmon and Campbell, 1969; USGS). Given that their range

extends to high latitudes and elevations, exposure to stable cold temperatures including 2°C can last for many months. The feeding treatments were applied to aquaria within each treatment system such that fed and starved fish were housed next to each other within the same system at a given temperature. For the fed group, a relatively small ration of 0.5% body mass was chosen to ensure the entirety of the ration was eaten even at 2°C, which standardized food intake between the groups (i.e. fish at 2°C and 8°C ate the same amount). Further, the ration was ecologically relevant in a winter context given that overwintering brook char would often not have regular access to large meals but was still above maintenance as fed fish at 2°C and 8°C exhibited positive growth (Table 1).

Fish assigned to 2°C treatments were cooled from 8°C to 2°C over ~10 hours (i.e. acutely) immediately prior to the 48h respirometry measurements that were used to assess the standard rate of oxygen consumption ($\dot{M}O_{2\text{standard}}$) as a proxy for standard metabolic rate (see below). We could only perform respirometry on 7 fish at the same time and so the start of the 2°C treatments had to be staggered to allow time for respirometry measurements. As such, 7 fish from a given housing system were transferred to the respirometers at 8°C and then cooled prior to the start of the measurements. The remaining fish in that housing system were then cooled immediately in the housing system, prior to being transferred to the respirometer. This practical approach meant that the first group of fish in the respirometers could be transferred back to the housing tanks which were now at 2°C, and the next group placed in the respirometers already at 2°C while ensuring all fish tested at 2°C had the same thermal experience prior to the estimation of $\dot{M}O_{2\text{standard}}$. ‘Day 0’ for fish assigned to the 2 and 8°C was considered as 24h after fish had first been in the respirometers at their acclimation temperature. In all cases (fed and starved), food was withheld for a total of 72 hours before the point considered ‘day 0’ to ensure fish were in a post absorptive state during the first estimation of $\dot{M}O_{2\text{standard}}$. Food was then withdrawn for the remainder of the experimental acclimation period for fish in the starved treatment whereas the daily ration was resumed for those in the fed treatment. Note, on the 35th day of the experiment, a chiller malfunctioned on one of the two 2°C systems and water temperature increased to a maximum of 11.7°C overnight. The following morning temperature was lowered gradually back to 2°C over the course of ~12 hours with no short-term mortality.

Oxygen consumption rate ($\dot{M}O_2$) analysis and spontaneous activity

Aerobic metabolic rates were estimated using the measurements of oxygen consumption rates ($\dot{M}O_2$; $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) obtained through automated intermittent-closed oxygen respirometry (Svendsen et al. 2016). We used 6.1 L (34x23x8.7 cm) plastic respirometers (LOCK & LOCK, Seoul, South Korea). Each respirometer was fit with a recirculating pump (Eheim 300) for constant mixing, and the recirculating loops were fit with individual temperature-compensated optodes (FireSting, PyroScience, Aachen, Germany) to measure dissolved oxygen (DO). DO in an empty respirometer was recorded simultaneously to record background changes in DO that were then subtracted from the fish' recordings. 'Day 0' was defined as described above and subsequent timepoints for respirometry were considered relative to this starting point. Fish were weighed and measured after each respirometry trial and standard length and body mass measurements were used to calculate Fulton's condition factor (see data analysis section). Following the first respirometry trial, fish were tagged (Elastomer implant, Northwest Marine Technology Inc., Anacortes, WA, USA) to allow for subsequent identification. At each subsequent measurement food was withheld from fed fish for 72 hours prior to the period from which standard metabolic rate (SMR) was estimated (i.e., 48 hours before the start of respirometry measurements) to allow gut evacuation (Chabot et al., 2016a; Chabot et al., 2016b; Cutts et al., 2002).

SMR, the basal maintenance cost of living (Chabot et al., 2016a; Norin and Speers-Roesch, 2021), was estimated ($\dot{M}O_{2\text{standard}}$) in each fish at four time points during the experiment (0, 30, 60 and 90 days). The flush period was two minutes, and the closed period was 10 min at 8°C and 22 min at 2°C. $\dot{M}O_2$ was measured from the slope of the decline in DO content (mg L^{-1}) during each closed period, which were extracted using an automated template in Microsoft Excel (v16.62, Microsoft Corp., 2018). At 2°C and 8°C, the first four minutes of each closed period were excluded from the slope calculation. Within the remaining closed period, we calculated $\dot{M}O_2$ using one six-minute slope at 8°C and two six-minute slopes at 2°C resulting in $\dot{M}O_2$ measurements every 12 minutes on average at both temperatures despite differing flush cycle lengths. Fish remained in the respirometers for ~48 hours for each $\dot{M}O_{2\text{standard}}$ trial, with the initial 24 hours being considered an adjustment period and the lowest 20th percentile of all $\dot{M}O_2$ measurements in the final 24 hours was taken as $\dot{M}O_{2\text{standard}}$ (Chabot et al., 2016b). $\dot{M}O_{2\text{standard}}$

estimates were generated using several methods to assess whether any differences among groups were a result of changes in body mass, fat content or activity. Regardless of which method was used to estimate $\dot{M}O_{2\text{standard}}$, the values, patterns of results, and Q_{10} 's (see supporting information) were similar, and our conclusions were identical, indicating that the 20th percentile method was robust for this study.

One individual was removed from $\dot{M}O_{2\text{standard}}$ analysis at 90 days due to a blockage in the recirculating loop that did not allow for accurate oxygen readings, resulting in a sample size of 13 instead of 14 for starved fish at 8°C in the final time point (90 days). The sample sizes for $\dot{M}O_{2\text{standard}}$ of fish starved at 2°C increased from 10 at time 0 and 30 days to 11 at 60 and 90 days. This increase was a result of three mortalities between day 0 and 60 that were entirely removed from the dataset, followed by the addition of a spare individual into respirometry measurements. This spare individual was added to help offset the loss in sample size and was already housed in the appropriate treatment tank but had not previously undergone respirometry. In total, seven mortalities occurred (fed at 2°C: 4; starved at 2°C: 3) throughout the experiment and these fish were removed from all data analysis.

In addition to $\dot{M}O_{2\text{standard}}$, the average $\dot{M}O_2$ ($\dot{M}O_{2\text{average}}$) was taken over the final 24 hours of each respirometry trial, which incorporated variation in $\dot{M}O_2$ that occurred largely because of spontaneous activity of fish in the respirometers (Fig S1). Spontaneous activity of the fish was measured during respirometry trials using near-infrared-sensitive cameras positioned above the respirometers (720x1080p, 30 fps, with two attached illuminators, Zosi Technology Ltd., China; and 720x1280p, 30 fps; Model: ELP-USB3MP01H-RL36, Elpccv, Ailipu Technology Co., Ltd., China). Average and median activity was calculated at each time point (Fig. S2b,c).

Lastly, the maximum ability to uptake oxygen in support of aerobic metabolism was estimated as $\dot{M}O_{2\text{max}}$ once, at 90 days, immediately following the final estimate of $\dot{M}O_{2\text{standard}}$. Fish were manually chased for five minutes; a standardized measurement period chosen based on studies of $\dot{M}O_{2\text{max}}$ in relation to exercise mode and duration (Little et al., 2020; Zhang et al., 2020). Both previous studies demonstrated that the duration of the sampling window used to extract $\dot{M}O_{2\text{max}}$ was a more important consideration than the duration of the chase protocol itself. Additionally, field and laboratory studies on a closely related species, the Arctic char, confirmed that fish were at or near exhaustion by five minutes at cold temperatures (Gilbert, 2020; Gilbert et al., 2020). Following the chase, fish were immediately sealed in the respirometers and the

flush pumps remained off until the DO fell by $\sim 2 \text{ mg L}^{-1}$ ($>80\%$ air saturation); flush pumps were then turned on to allow DO to rise $\sim 0.5 \text{ mg L}^{-1}$ and then shut off again to fall by $\sim 0.5 \text{ mg L}^{-1}$ at which time the respirometers were returned to their regular automated flush cycles. If DO levels fell below $\sim 80\%$ air saturation, respirometers were again manually flushed to increase the DO in the chamber. This manual intermittent procedure maximized the duration of oxygen consumption recordings immediately following chasing to improve the accuracy of $\dot{M}O_{2\text{max}}$ estimates. Fish were then left to recover within the respirometers for at least 12 hours before being returned to their respective acclimation systems. $\dot{M}O_{2\text{max}}$ was taken as the steepest DO slope over 90 seconds which was detected using an iterative algorithm applied over all DO recordings following the chase event as previously described (Zhang et al., 2018). The absolute aerobic scope (AAS) of each fish was calculated as the difference between its $\dot{M}O_{2\text{standard}}$ and $\dot{M}O_{2\text{max}}$.

Tissue collection and tissue energetics analyses

Fish were chemically euthanized (300 mg L^{-1} MS-222 and 600 mg L^{-1} sodium bicarbonate, Sigma-Aldrich, MO, USA) three days following the final respirometry measurements. Blood was immediately extracted via caudal puncture and tested in duplicate for glucose (Accu-Chek, Roche Diabetes Care, Basel, Switzerland) and hematocrit (centrifuged 5 min at 5000 g ; Sorvall Legend 17 Microcentrifuge, ThermoScientific, MA, USA). Body fat (%) was measured using a fish fat meter (FFM-692, Distell, Fauldhouse, UK). Tissue samples (ventricle, liver, gut and white muscle) were dissected, sexed (30 females, 20 males; see data file), weighed and immediately frozen in liquid nitrogen. Tissue and plasma samples were stored at -80°C until analysis (4-16 mo. for tissues, ~ 19 mo. for plasma). The remaining carcasses were frozen at -20°C , ground (Paderno Meat Grinder, Padinox Inc., ON, Canada), aliquoted and freeze dried (FreeZone 12 L, Labconco Corp., MO, USA) for 48 hours (-50°C and 0.12 mBar) followed by further homogenization (three 10 sec pulses; Model 043-5781-8, Master Chef Elite Grinder, Trileaf Distribution, ON, Canada). The ventricle, liver, gut and white muscle samples were manually ground on liquid nitrogen with mortar and pestle. All samples were stored at -80°C until further analysis. Plasma was analyzed for triglyceride (TG) content using a commercial assay kit (10010303, Cayman Chemical, MI, USA).

In vivo protein synthesis rates in the ventricle, liver, gut and white muscle were estimated using the flooding dose technique (Cassidy et al., 2016; Lamarre et al., 2015). Briefly, four hours prior to euthanization, fish were given a 1 mL per 100 g body mass intraperitoneal injection of 150 mM of phenylalanine solution, which contained 50% deuterium labeled phenylalanine ([D5]-phenylalanine, 98%, Cambridge Isotope Laboratories, Inc., MA, USA). Prior to sampling tissues, the abdominal cavity of the fish was rinsed with 0.8% saline solution to wash off any unabsorbed tracer. The fractional rate of protein synthesis (K_s ; % day⁻¹) was then assessed as described in Lamarre et al. (2015).

Bomb calorimetry (6765 Combination Calorimeter, Parr Instrument Company, IL, USA) was used to determine the carcass energy density (kJ g dry mass⁻¹) as per the manufacturer's protocols (Brett et al., 1969).

A commercial assay kit (ab65336, Abcam Inc., ON, Canada) was used to quantify the TG contents of the liver, gut and white muscle.

The Bradford assay was used to quantify protein content in the liver, gut, white muscle and carcass. Briefly, dry (carcass; 0.0125 ± 0.0007 g) or wet (liver, gut and white muscle; 0.05 ± 0.005 g) tissue was added to 500 μ L of homogenization buffer (50 mM TRIS, 1mM EDTA, 3.5% NaCl, pH = 7.9 ± 0.05) and homogenized with a motorized micro pestle (2 x 30 sec pulses; Cole-Parmer, Argos Technologies, QC, Canada) followed by sonication (2 x 10 sec pulses; Q55 Sonicator, QSonica, Newtown, CT, USA). Then, 750 μ L of homogenization buffer was added to each 1.5 mL tube and samples were vortexed then centrifuged (10 min at 5000 g and 4°C; 5424R, Eppendorf AG, Germany). The supernatant was then diluted further (1:4 for gut, 1:8 for carcass, white muscle and liver), vortexed and assessed for protein content using Bradford reagent (B6919, Sigma, Sigma-Aldrich, ON, Canada) on a microplate reader at 595 nm (Epoch2 Microplate Reader, BioTek Instruments, Winooski, VT, USA).

Data Analysis

Spontaneous activity analysis

The daily spontaneous activity of brook char in Experiment 1 was measured over a ~24 hour video recording at each temperature or day at 2°C using an automated tracking software

(ToxTrac, v2.84; Rodriguez et al., 2018). The first two hours (7-9 am) of the recordings on each new day were removed from analysis to reduce the effect of disturbance from feeding that occurred within this time. ImageJ (v1.52a, National Institutes of Health, USA; Schneider et al., 2012) was used to obtain the pixel-to-distance calibration required to make accurate distance moved calculations. Spontaneous activity in Experiment 1 is expressed as the average speed over 24 hours at each temperature or each day at 2°C. Average speed was calculated as the total distance moved by the fish in the recorded period divided by the corresponding duration and standardized to the length of each fish (average body lengths moved per minute, BL min⁻¹).

The spontaneous activity of brook char in respirometers in Experiment 2 was measured during the second ~24 hours of each 48-hour respirometry measurement period (i.e., during the 24 hours when $\dot{M}O_{2\text{standard}}$ and $\dot{M}O_{2\text{average}}$ were estimated) using automated tracking software (Ethovision XT v.16, Noldus Information Technology BV, Netherlands). Activity level was quantified as the percentage of pixel change over 10 samples per second of video and averaged to one activity measurement for every $\dot{M}O_2$ measurement. The percentage of pixel change was chosen as the unit of activity measurement because fish were confined in respirometers so they occasionally had bouts of intense activity that did not result in a large distance travelled. Due to an instance of a brief camera and infrared lighting failure while recording during the 60-day measurement, a total of 120 minutes of video was removed from the analysis. We also removed outright the activity measurements of eight individuals for one measurement day due to excessive surface water movement from a misplaced air stone. Thus, the resulting sample sizes for fed at 8°C, starved at 8°C, fed at 2°C and starved at 2°C were 10, 12, 9, and 11 at time 0; 14, 14, 9 and 11 at time 1 (30 days) and 14, 14, 10 and 11 at time 2 (60 days) and time 3 (90 days), respectively.

Calculating lean body mass

Lean body mass was calculated after 90 days using the body fat measurement (as a proportion) and the following equation:

$$\text{Lean Mass} = \text{body mass} - (\text{body mass} * \text{body fat})$$

Calculating Fulton's condition factor

Fulton's condition factor (K) was calculated using the following equation:

$$K = \frac{\text{Mass (g)}}{\text{Standard Length (mm)}^3} \times 10^5$$

Calculating Q_{10} 's

The Q_{10} values characterizing the thermal sensitivity of various rates were calculated using the following equation:

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

Where R_1 was the rate for a biological process at the lower temperature, T_1 , and R_2 is the rate at the higher temperature, T_2 .

Calculating somatic indices

One-way linear models or generalized linear models (LM or GLM; see below) were constructed for each organ mass or length and were used to calculate a size corrected organ mass for all treatment groups. The model residuals from the body mass or length term for each individual were added to the model estimated value at the average mass (0.152 kg) or length (225 mm; intestine length only) to obtain a size adjusted organ mass or length for each individual. The adjusted organ masses were then divided by the average body mass or length and multiplied by 100 to generate the somatic index as a percentage. Adjusting organ mass to a common body mass in this way compensates for any deviation from a 1:1 mass or length scaling relationship, which is an assumption made when calculating somatic indices. Additionally, to examine how the heart, the central circulatory organ, and digestive organs changed relative to one another among treatments we also calculated the gut:heart ratio by dividing total gut mass by ventricular mass. The gut:heart ratio can be useful to explore relative changes in organ sizes because it should be more independent of changes in body composition (e.g., fat accumulation or increased water content) than traditional somatic indices.

Statistics

Statistical analysis was done using R (Core Team, 2014) in R Studio or Prism v.9 (GraphPad Software, San Diego, USA), with data visualization completed using Prism v.9 (GraphPad Software, San Diego, USA). Statistical significance was accepted at $p < 0.05$. All values are presented as mean \pm standard error (S.E.M.), unless otherwise noted.

Experiment 1: Statistics for behavioural responses to cooling

Generalized linear mixed effects models (GLMM) were used to evaluate the effect of cooling on the spontaneous activity (family=Gamma, link=log) and food consumption (package “glmmTMB” (Brooks et al., 2017); family=beta, link=logit) of brook char. The beta distribution does not recognize values of 0 or 1, so food consumption data was transformed using the following equation: $y' = (y \cdot (n-1) + 0.5) / n$; where y is food consumption as a proportion and n is the sample size (Smithson and Verkuilen, 2006). The data were split into acute cooling (14°C to 2°C) and acclimating (two weeks at 2°C) phases. During acute cooling, spontaneous activity and food consumption were modelled as a function of temperature with fish ID included as a random factor to account for repeated measures. During the two-week acclimation at 2°C, spontaneous activity and food consumption, were modelled as a function of acclimation duration with fish ID as a random factor. Type II Wald chi-square tests were then performed on all models using the “car” package (Fox et al., 2007) and post hoc multiple comparisons were performed using the “emmeans” package (Lenth et al., 2019) with Holm adjustments.

Experiment 2: Statistics for metabolic performance and energetics

Data for Experiment 2 were analyzed using linear models (LM) or linear mixed effects models if there were repeated measures (LMM), unless parametric assumptions were not met, in which case the corresponding generalized model (GLM or GLMM ; family=Gamma, link=log) was used. Each metric was modeled as a function of temperature, feeding status and time (if applicable) with lean body mass (90 day measurements only), body mass or body length as a covariate when applicable. Fish ID was included as a random factor for models with repeated

measures (see Supplemental Information for additional details and sample sizes). In each case, type II Wald chi-square tests were performed on all models using the “car” package, followed by supervised post hoc comparisons of estimated marginal means (EMM) with a Holm post adjustment to account for multiple comparisons using the “emmeans” package when significant effects or interactions were found.

The allometric scaling of $\dot{M}O_2$ with body mass was accounted for in data presentation by adjusting $\dot{M}O_{2\text{standard}}$ and $\dot{M}O_{2\text{average}}$ to the average body mass. To do this, EMM at the average mass were presented in place of means, or the residuals of the mass- $\dot{M}O_2$ term in each model were added to the predicted $\dot{M}O_2$ value from the model at the average mass (0.146 kg) to generated individual adjusted values for boxplots. Mass-specific values were then generated by dividing these adjusted $\dot{M}O_2$ value by the average mass (Gilbert, 2020). For the 90-day metabolic performance metrics, $\dot{M}O_2$ was adjusted to the average lean body mass (0.154 kg) of the fish. This lean-mass adjustment was only done at 90 days, because it was the only time point at which we measured body fat.

RESULTS

Experiment 1: Characterizing the effect of cooling on spontaneous activity and daily food consumption of brook char

Daily food consumption remained high (>90%) during cooling (14°C to 2°C) ($X^2_{12,15}=7.55$, $p>0.05$) and only minor day-to-day variation occurred over the two weeks at 2°C ($X^2_{14,15}=58.55$, $p<0.0001$; Fig. 1). Spontaneous activity decreased by 77% ($Q_{10}(14^\circ\text{C}-2^\circ\text{C}) = 3.4$; Table 2) with cooling from 14°C to 2°C ($X^2_{12,15}=859.43$, $p<0.0001$; Fig. 1). Spontaneous activity was then maintained or slightly increased during the two weeks at 2°C (+10% from day 1 to day 10; $X^2_{14,15}=42.78$, $p<0.0001$; Fig. 1).

Experiment 2: The effects of prolonged starvation at 8°C or 2°C on brook char energetics and performance

Growth metrics

Body length, body mass, and Fulton's condition factor (K) increased over the 90 day acclimation period in fed fish similarly at both temperatures but length remained unchanged and mass and K decreased in starved fish (Table 1, Table S2).

Aerobic metabolism

$\dot{M}O_{2\text{standard}}$ markedly decreased following initial acute cooling from 8°C to 2°C in both feeding treatments (Holm post hoc, $p < 0.0001$; Fig. 2, Table S2), resulting in high Q_{10} values (4.2-4.5; Table 2). The effect of temperature and feeding treatment on $\dot{M}O_{2\text{standard}}$ varied over the experiment resulting in significant two-way interactions of time with temperature and feeding treatment (Wald chi-square test, $p < 0.01$ for both; Table S2). Specifically, $\dot{M}O_{2\text{standard}}$ at 8°C remained constant in fed fish over time, but decreased by 44% in starved fish (Fig. 2). At 2°C, $\dot{M}O_{2\text{standard}}$ in fed fish increased by 71% with acclimation (Fig. 2), but only by 17% in starved fish, with this increase occurring between 30 and 90 days (Holm post hoc, $p < 0.0001$) (Fig. 2). Consequently, $\dot{M}O_{2\text{standard}}$ of starved fish at both temperatures was significantly lower than in fed fish after 90 days (-48% at 8°C and -40% at 2°C) (Fig. 2 and 3a). The difference in $\dot{M}O_{2\text{standard}}$ between 8°C and 2°C decreased over time in both fed and starved fish ($Q_{10}=1.4-2.8$; Table 2, Fig. 2 and 3a); after 90 days $\dot{M}O_{2\text{standard}}$ for fed fish was significantly lower at 2°C than 8°C (Holm post hoc, $p < 0.001$), but not for starved fish (Holm post hoc, $p > 0.05$). $\dot{M}O_{2\text{average}}$ showed a similar pattern to $\dot{M}O_{2\text{standard}}$, with significant interactions between temperature and time as well as feeding status and time (Wald chi-square test, $p < 0.01$ for both; Fig. S2a, Table S2). A minor exception is that, at 90 days, $\dot{M}O_{2\text{average}}$ was similar in fish fed at 2°C and starved at 8°C (Holm post hoc, $p > 0.05$).

$\dot{M}O_{2\text{max}}$ was higher in fed fish at 8°C than fed or starved fish at 2°C (Holm post hoc, $p < 0.05$; Table 2, Fig. 3b, Table S4). $\dot{M}O_{2\text{max}}$ was similar among starved fish at the two

temperatures and fed fish at 2°C (Holm post hoc, $p>0.05$; Fig. 3b). AAS exhibited the same pattern, except the AAS of fed fish at 8°C was only significantly higher than fed fish at 2°C (Holm post hoc, $p<0.01$; Table 2, Fig. 3c, Table S4).

Median activity

Median activity of fish in the respirometers was very close to zero in all treatments at each time point supporting the use of the 20th percentile approach to estimate $\dot{M}O_{2\text{standard}}$ as the fish were largely inactive (Fig. S2c).

In vivo protein synthesis rates

The K_s varied among treatment groups in an organ-specific manner, but decreased with temperature in all cases (Q_{10} range: 1.2-2.2; Wald chi-square test, $p<0.05$ for all; Table 2, Tables S5 and S6). Ventricle K_s decreased with starvation, however, this difference was only significant at 2°C (-25% at 2°C; Wald chi-square test, $p=0.004$; Fig. 4a, Table S4). Liver K_s was unaffected by feeding status (Wald chi-square test, $p>0.05$; Fig. 4b, Table S3). In contrast, gut K_s was markedly lower in starved fish (-42% at 8°C and -56% at 2°C; Holm post hoc, $p<0.05$; Fig. 4c, Table S4) as was muscle K_s (-36% at 8°C and -32% at 2°C; Fig. 4d, Table S3). K_s was positively correlated with $\dot{M}O_{2\text{standard}}$ in the white muscle, gut and ventricle (Fig. 4). White muscle had the strongest correlation to $\dot{M}O_{2\text{standard}}$ ($R^2=0.329$, $p<0.0001$; Fig. 4h), followed by the gut ($R^2=0.205$, $p<0.01$; Fig. 4g), then the ventricle ($R^2=0.116$, $p<0.05$; Fig. 4e), while the correlation was not significant for liver K_s ($R^2=0.007$, $p>0.05$; Fig. 4f).

Carcass composition

Moisture content (% body mass) was, on average, 5% higher in starved fish than fed fish (Holm post hoc, $p<0.0001$; Table S1, Table S4). Body fat was 25-27% lower in starved fish than fed fish (Holm post hoc, $p<0.01$; Table S1, Table S4). Total relative carcass protein did not differ

between treatment groups (Holm post hoc, $p>0.05$; Table S1, Table S3). Energy density was 9-13% lower in starved fish than fed fish (Holm post hoc, $p<0.01$; Table S1, Table S4).

Organ sizes and somatic indices

After accounting for differences in body size, the mass of the ventricle was only different between fed fish at 8°C and starved at 2°C (Holm post hoc, $p<0.01$; Fig. 5a, Table S4). Liver mass was significantly smaller in starved fish compared to fed fish (Holm post hoc, $p<0.01$; Fig. 5b, Table S4). The mass of the stomach did not differ among treatment groups (Holm post hoc, $p>0.05$; Fig. 5c, Table S3). Pylorus masses and total gut masses were significantly different between all treatment groups, with fed fish at 2°C having the greatest masses (Holm post hoc, $p<0.05$ and $p<0.01$, respectively), and starved fish at 2°C having the lowest masses (Holm post hoc, $p<0.05$ and $p<0.01$, respectively) (Fig. 5d,g, Table S3). Intestine mass was lower at 8°C than 2°C but only in starved fish resulting in significant interaction between temperature and feeding status (Table S3). Additionally, intestine mass was 34% (2°C) and 48% (8°C) smaller in starved fish than fed fish (Wald chi-square test, $p<0.001$); Fig. 5e, Table S3). Intestine length and the gut:heart ratio were significantly lower in starved fish than fed fish (Holm post hoc, $p<0.01$ and $p<0.0001$, respectively) (Fig. 5f,h, Table S4).

Organ triglyceride and protein content

The TG and protein contents varied among treatment groups in an organ-specific manner. Liver TG content was unaffected by temperature and feeding status (Wald chi-square test, $p>0.05$ for both; Fig. 6a, Table S3). Gut TG content was 58% (2°C) and 49% (8°C) lower in starved fish than fed fish (Holm post hoc, $p<0.0001$; Fig. 6b, Table S4). White muscle TG content was also lower in starved fish, albeit to a lesser extent (32% at 2°C and 18% at 8°C; Holm post hoc, $p<0.001$; Fig. 6c, Table S3). Total relative liver TG (i.e., how much of the body mass is made up of liver TG) was significantly lower in starved fish at 8°C than fed fish at 2°C (Holm post hoc, $p<0.01$; Fig. 6d, Table S3). Total relative gut TG was 73-79% lower in starved fish than fed fish at both temperatures (Holm post hoc, $p<0.0001$) and higher at 8°C than at 2°C,

but only in fed fish, resulting in a significant interaction between temperature and feeding treatment (Holm post hoc, $p < 0.05$; Fig. 6e, Table S4).

Liver and white muscle protein contents did not differ between treatment groups (Wald chi-square test, $p > 0.05$ and Holm post hoc, $p > 0.05$, respectively; Fig. 6f,h, Table S4). However, total relative liver protein was significantly lower in starved fish at 8°C (Holm post hoc, $p < 0.01$) and significantly higher in fed fish at 2°C (Holm post hoc, $p < 0.001$) than all other treatment groups (Fig. 6i, Table S4). Gut protein content was significantly higher in fish starved at 8°C than all other groups (Holm post hoc, $p < 0.001$), though total relative gut protein did not differ between treatment groups (Holm post hoc, $p > 0.05$) (Fig. 6g,j, Table S4).

Blood parameters

Plasma glucose was similar among treatment groups except it was lower in starved fish at 8°C compared to those fed at 2°C (Holm post hoc, $p < 0.05$; Tables S1 and S6). Hematocrit did not differ among treatment groups (Holm post hoc, $p > 0.05$; Table S1 and S6). Plasma TG content at 2°C was 56% lower in starved than fed fish (Holm post hoc, $p < 0.05$; Table S1 and S6).

DISCUSSION

When temperatures drop and food becomes scarce over winter, winter-active species (e.g. many salmonids) can face trade-offs between energy conservation and the maintenance of metabolic performance (Auer et al., 2020). Our findings support the hypothesis that winter-active fishes employ behavioural and physiological flexibility in response to cold and food scarcity that allows them to conserve energy while also protecting metabolic performance. Most notably, we found that brook char can adjust their metabolic floor ($\dot{M}O_{2\text{standard}}$) in a food- and temperature-dependent manner to balance the competing needs for energy conservation and thermal compensation. Essentially, brook char greatly depressed $\dot{M}O_{2\text{standard}}$ to conserve energy in the absence of food, underwent marked thermal compensation by increasing $\dot{M}O_{2\text{standard}}$ during cold acclimation, and did both simultaneously when these challenges occurred together, all with no

apparent trade-off in $\dot{M}O_{2\max}$ or AAS. Our assertion that selective metabolic depression and thermal compensation occur simultaneously is supported by two key findings: first, the reduction in $\dot{M}O_{2\text{standard}}$ observed during food deprivation at 8°C was not apparent at 2°C, even though both groups had similar tissue-level starvation responses (reductions in K_s and the atrophy of non-essential tissues) which should depress $\dot{M}O_{2\text{standard}}$. Second, the final thermal sensitivity quotients between starved fish at 8°C and 2°C were low and similar to that of fed fish, suggesting that significant thermal compensation had occurred in brook char in the absence of food despite the final $\dot{M}O_{2\text{standard}}$ still being much lower in starved than fed fish at 2°C. Our findings from the biochemical to whole-animal level indicate that brook char are well suited to cope with variation in winter conditions, show that the apparent extent of metabolic thermal compensation can be food dependent, and provide additional evidence for common metabolic overwintering mechanisms among vertebrates adapted to extreme seasonality.

Activity, feeding and growth in the cold

Brook char, like other salmonids, often remain active and feeding throughout the winter in the wild (Cunjak and Power, 1987; Cunjak et al., 1987; Fraser et al., 1993) and laboratory (Helland et al., 2011). Similarly, we observed continued feeding, growth and increased body condition even during prolonged holding at 2°C. Activity did decline with cooling at a rate suggesting a primarily passive physicochemical effect ($Q_{10(14^\circ\text{C}-2^\circ\text{C})}=3.4$) (Reeve et al., 2022). Despite this decline, brook char activity at 2°C remained elevated compared with winter-dormant or -lethargic species (Reeve et al., 2022), in which active behavioural responses such as sheltering contribute to more pronounced inactivity. Nonetheless, the observed activity reductions in brook char would greatly reduce routine energy demands, supporting the notion that activity decrement is a common energy conservation mechanism among overwintering fishes (Reeve et al., 2022).

Plasticity of resting metabolic demands

When ectotherms are cooled, the passive slowing of resting metabolic demands via Arrhenius effects is generally associated with $Q_{10}=2-3$ (Clarke and Johnston, 1999). Following a

rapid cooling (6°C over ~10 hours) to 2°C, brook char $\dot{M}O_{2\text{standard}}$ markedly declined with high Q_{10} 's (4.2-4.5). A high Q_{10} (e.g., >3.5) for $\dot{M}O_{2\text{standard}}$ during cooling can indicate an active metabolic rate depression (Geiser, 2016; Reeve et al., 2022; Staples, 2016). However, the high thermal sensitivity seen here is more parsimonious with an acute metabolic constraint, as $\dot{M}O_{2\text{standard}}$ greatly increased during subsequent cold acclimation, resulting in typical Arrhenius Q_{10} 's of 2-3 by 30 days and ~1.6 by 60 day. Thus, we found strong metabolic thermal compensation, not depression. The thermal compensation of $\dot{M}O_{2\text{standard}}$ in starved fish appeared less pronounced, likely because thermal compensation was occurring alongside processes that reduce $\dot{M}O_{2\text{standard}}$ during food deprivation. Thermal compensation during cold acclimation has been demonstrated at sub-organismal levels in many fishes (Dean, 1969; Guderley, 1990; Hochachka and Hayes, 1962; Orczewska et al., 2010), although more rarely at the whole-animal level (Peterson and Anderson, 1969). For example, oxygen uptake of brook char muscle homogenates over doubled following cold acclimation (4°C for at least two weeks), though Q_{10} 's remained relatively high (>3) (Hochachka and Hayes, 1962). At the whole-animal level, the Q_{10} for $\dot{M}O_{2\text{standard}}$ from 18°C to 6°C fell from 3.2 in Atlantic salmon acclimated to 18°C to 2.4 following one month of acclimation to 6°C (Peterson and Anderson, 1969). We demonstrated a much more pronounced compensation, with post-acclimation Q_{10} 's (~1.5) that are among the lowest for resting metabolism in teleosts (e.g., rainbow trout (*Oncorhynchus mykiss*): $Q_{10(5^{\circ}\text{C}-26^{\circ}\text{C})}=2.02$, lake char: $Q_{10(9.1^{\circ}\text{C}-22.1^{\circ}\text{C})}=2.74$; Bokma, 2004; Watanabe and Payne, 2023; White et al., 2006). This remarkable compensation during cold exposure has not been previously documented in brook char, likely because studies rarely include acute and acclimated exposures and may not use sufficient acclimation durations (Havird et al., 2020). Indeed, our results demonstrate that common acclimation times of three to four weeks do not capture the full extent of cold acclimation in brook char. The pronounced cold acclimation responses we found here may be more common among winter-active species (e.g., acclimated lumpfish (*Cyclopterus lumpus*), $Q_{10(3^{\circ}\text{C}-15^{\circ}\text{C})}=1.7$, or Arctic grayling (*Thymallus arcticus*), $Q_{10(4^{\circ}\text{C}-12^{\circ}\text{C})}=1.9$; Bokma, 2004; Hvas et al., 2018) than current literature suggests (Abe et al., 2019).

In contrast to thermal compensation in the cold, resting metabolic demands are commonly reduced in animals faced with food deprivation, including in fishes (Auer et al., 2016b; Hvas et al., 2020; O'Connor et al., 2000; Van Leeuwen et al., 2012). For example, following 10 days of starvation at 14°C, juvenile coho salmon (*Oncorhynchus kisutch*)

downregulated their $\dot{M}O_{2\text{standard}}$ by 26-29% (Van Leeuwen et al., 2012). Here, brook char showed a similar initial reduction and by 90 days of starvation, $\dot{M}O_{2\text{standard}}$ was >40% lower in starved fish. This reduction was accompanied by a modest loss of body mass, but substantial reductions in body fat and energy density, alongside an increase in moisture content, which are all typical vertebrate responses to starvation (Brett et al., 1969; Cassidy et al., 2016; Jobling et al., 1998a; Navarro and Gutierrez, 1995; Secor and Carey, 2016). This pattern was primarily driven by a depletion of lipid stores, specifically TG in the white muscle and gut, with little to no reduction in protein content. Thus, even after 90 days of food absence brook char likely remained in the second phase of starvation (lipid use and protein sparing) (Bar, 2014; Bar and Volkoff, 2012; Navarro and Gutierrez, 1995; Secor and Carey, 2016), as seen in Atlantic salmon (Foda, 1975). While TG levels were approaching zero in some individuals, the lack of significant change in protein content suggests that brook char could have survived longer without food, albeit in a critical phase of starvation (phase three: protein catabolism). Thus, the observed depression of $\dot{M}O_{2\text{standard}}$ during starvation and corresponding energy savings (Van Leeuwen et al., 2012) was likely essential to the starvation tolerance seen here.

Energy conservation through tissue selective atrophy and depression of protein synthesis

Animals that experience energy deficits commonly reduce their resting metabolic rate by downregulating energy demands in non-critical energy sinks such as the maintenance of non-essential tissues or protein synthesis (Cassidy et al., 2016; Dabrowski and Guderley, 2003; Fedorov et al., 2009; Méndez and Wieser, 1993; Tøien et al., 2011). Gut atrophy (i.e. changes in mass or length), in particular, is a common adaptive energy conservation response to starvation, given the gut's high metabolism and its disuse when not feeding. Gut mass in starved brook char decreased substantially at both 2°C and 8°C, similar to other *Salvelinus* spp. (e.g., Arctic char, Dolly Varden (*Salvelinus malma*)) in response to starvation (Armstrong and Bond, 2013; Jobling et al., 1998b; Jørgensen et al., 1997). Interestingly, unlike in Atlantic salmon and Dolly Varden, which endure long periods of starvation associated with anadromy (Armstrong and Bond, 2013; Martin et al., 1993), we did not observe a decrease in brook char stomach mass. Brook char will feed opportunistically in winter (Cunjak and Power, 1987), so maintaining the stomach may be worth the additional cost (Cunjak et al., 1987). The gut atrophy observed in starved brook char

corresponded with a large reduction in gut K_s , similar to that seen in rainbow trout following only six days of food deprivation (McMillan and Houlihan, 1989). The substantial combined atrophy and reduction of K_s indicate that the gut is a major site of energy savings during starvation. While this downregulation obviously limits digestive capacity, these systems may be able to recover quickly, given that resting metabolic rates of Atlantic salmon returned to control levels following just one week of feeding (Hvas et al., 2020) and that compensatory growth follow food deprivation is a common (Cassidy et al., 2018; Davis and Gaylord, 2011).

The liver is a major site of protein metabolism and a site of glycogen and lipid loss during energy restriction (Armstrong and Bond, 2013; Dabrowski and Guderley, 2003)(Lahnsteiner, 2022). We found that livers were smaller in starved fish at both 2°C and 8°C, which has energetic implications when considering liver K_s . We found no change in liver K_s following 90 days of starvation in brook char. However, livers were smaller in starved fish relative to their body mass with no change in protein content, so total relative liver K_s would have been lower in starved fish. The response of liver K_s to starvation in salmonids varies among studies, possibly as a result of differences in the ration size for fed fish (McMillan and Houlihan, 1992).

While white muscle K_s values are much lower than liver or gut, white muscle makes up ~50-60% of the body mass. Therefore, its overall contribution to whole-animal energy expenditure is significant (Milligan, 1996). Studies in Arctic char and rainbow trout have reported reductions in white muscle K_s ranging from 72-76% with starvation (Cassidy et al., 2016; Loughna & Goldspink, 1984; McMillan & Houlihan, 1989; Smith, 1981). Here, brook char reduced white muscle K_s by 31-35%. In contrast, ventricular K_s did not change with starvation in Arctic char and rainbow trout (Cassidy et al., 2016; McMillan and Houlihan, 1989), and we saw minor (at 2°C) or no (8°C) significant change in brook char. The lack of, or relatively minor reduction, in ventricular K_s in our study agrees with the absence of ventricular atrophy and is consistent with the heart's essential function and relatively slow cell turnover rates (Bergmann et al., 2009; Secor and Carey, 2016).

We focused on organ-specific changes in K_s and mass because they influence whole-animal metabolism and growth (Armstrong and Bond, 2013; Cassidy et al., 2016; Houlihan et al., 1995; Jørgensen et al., 1997; Loughna and Goldspink, 1984; Zaldúa and Naya, 2014). Protein synthesis alone may account for 20-50% of resting metabolic demands in growing fish (Carter

and Houlihan, 2001). Indeed, protein synthesis of the white muscle, and to a lesser extent of the gut and ventricle, were positively correlated with $\dot{M}O_{2\text{standard}}$ in brook char. While protein degradation rates were not estimated here, they should be investigated further as the balance between synthesis and degradation would influence the extent of atrophy observed. Protein degradation rates are reported to increase in the intestine, liver and white muscle with starvation in fishes (Cassidy et al., 2016; Houlihan et al., 1986; Krogdahl and Bakke-McKellep, 2005; Navarro and Gutierrez, 1995; Smith, 1981).

Overall, our results demonstrate that brook char downregulate K_s in an organ-specific manner and possess energy-saving digestive flexibility in response to starvation at 2°C and 8°C. Organ specific K_s did decrease with temperature which would confer additional energy savings in the cold. However, Q_{10} 's were low (1.2 to 2.2) within feeding treatments, suggesting a thermal compensation of K_s during cold acclimation independent of the starvation-induced reductions, consistent with our findings for $\dot{M}O_{2\text{standard}}$. In combination with the processes we identified as helping reduce $\dot{M}O_{2\text{standard}}$, other studies have shown that increased mitochondrial efficiency (i.e., less proton leak) or reductions in mitochondrial abundance (Guderley and St-Pierre, 2002; Salin et al., 2018; Secor and Carey, 2016; Thorall et al., 2023) can contribute greatly to reductions in $\dot{M}O_{2\text{standard}}$ when food is limited.

Conservation of aerobic metabolic performance during starvation

Like other levels of metabolic performance, $\dot{M}O_{2\text{max}}$ often decreases with cooling (Durhack et al., 2021; Mackey et al., 2021), but the responses of $\dot{M}O_{2\text{max}}$ to nutritional status are less well established. If starvation constrained aerobic performance, it could limit capacity for critical tasks like predator avoidance, migration and food acquisition and growth when resources return (Auer et al., 2015). Such a constraint is conceptually possible given the starvation-induced decrease of $\dot{M}O_{2\text{standard}}$ and the traditional view that $\dot{M}O_{2\text{standard}}$ and $\dot{M}O_{2\text{max}}$ are coupled (Bennett & Ruben, 1979). Conservation of $\dot{M}O_{2\text{max}}$ and aerobic performance has been observed in multiple other salmonids with food deprivation ranging from one week to four weeks (Alsop and Wood, 1997; Hvas, 2022; Thorarensen and Farrell, 2006), but these studies did not explicitly assess $\dot{M}O_{2\text{standard}}$ alongside maximal performance. Here, even following a much longer 90-day food deprivation, we explicitly demonstrated that brook char maintained $\dot{M}O_{2\text{max}}$, and thus AAS,

during starvation while simultaneously reducing $\dot{M}O_{2\text{standard}}$. This conservation of aerobic capacity during starvation was associated with the maintenance of plasma glucose, blood-oxygen carrying capacity (inferred from hematocrit) and ventricle mass, with minor or no reduction in ventricular K_s , all of which likely contribute to the maintenance of peak aerobic performance (Gallaughner et al., 1995; Gallaughner et al., 2001; Secor and Carey, 2016). Thus, we show that the responses of $\dot{M}O_{2\text{standard}}$ and $\dot{M}O_{2\text{max}}$ to food availability at winter-relevant temperatures in brook char can become decoupled to maintain aerobic scope. This uncoupling is likely possible because energy savings occurred in processes unrelated to peak exercise induced aerobic performance (e.g., gut size, gut protein synthesis). Our findings in brook char under winter-relevant stressors represents a new environmental context consistent with a growing understanding that maintenance of maximum aerobic performance can be decoupled from resting metabolic rate to varying extents among vertebrates, including other fishes (Armstrong and Bond, 2013; Auer et al., 2016a; Cassidy et al., 2016; Hvas, 2022), amphibians (Naya et al., 2009), reptiles (Secor, 2008), birds (Barceló et al., 2017; Funes et al., 2014) and mammals (Fedorov et al., 2009; Karasov and Douglas, 2013; Lignot, 2012; Secor and Carey, 2016; Tøien et al., 2011). Nonetheless, other limitations imposed by food deprivation and cold on metabolic fuel availability (e.g., reductions of glycogen and lipid reserves) and changes in muscle function could compromise endurance (hours to days) swimming performance and other energetically expensive processes (e.g., reproduction).

Conclusions

Using an integrative examination of energetic responses to prolonged simulated winter conditions, we provide evidence that winter-active fishes, such as brook char, balance the maintenance of a high-capacity phenotype with energy conservation to survive winter and presumably improve subsequent individual fitness. This remarkable plasticity was observed despite fish being from a captive breeding population, and so responses in strains or species (e.g., Arctic char) that more routinely encounter food restriction may be even more pronounced (Turko et al., 2023). Understanding the overwintering physiology of winter-active species is important as ongoing changes in winter conditions, including warming and phenological shifts, could exacerbate energy depletion (Morash et al., 2021), altering the efficacy of current energy

conservation strategies. Brook char showed an impressive ability to assimilate food and compensate for prolonged exposure to frigid temperature, while also displaying strong starvation tolerance (even at a warmer temperature) via an active depression of $\dot{M}O_{2\text{standard}}$. This great bioenergetic flexibility to exploit or tolerate shifting winter food availability and temperatures, exemplifying the robust plasticity of *Salvelinus* fishes that helps explain their success across diverse habitats and may indicate their potential resilience to future winter conditions (Armstrong and Bond, 2013; Dutil, 1986; Gilbert and Farrell, 2021; Hutchings, 1996; MacCrimmon and Campbell, 1969; Muir et al., 2016).

The energetic flexibility of brook char in response to cold and starvation also holds important implications for bioenergetic modeling. Bioenergetics models are commonly used to estimate energy needs and growth of individuals and populations under varying environmental conditions, informing management and conservation (Brownscombe et al., 2017; Brownscombe et al., 2022; Little et al., 2020). However, bioenergetics models currently do not account for potential reductions in resting metabolic demands during periods of restricted feeding. Our findings suggest that the inclusion of variation in metabolism associated with food restriction could markedly improve bioenergetic models.

The present findings also highlight the need for studies, like ours, that combine multiple factors over more ecologically-relevant time courses to properly understand both acute and prolonged responses to environmental change (Havird et al., 2020; Huey and Buckley, 2022). Such experimental designs better capture the temporal dynamics of acclimation, which is particularly important when considering seasonal change that can occur over months rather than weeks. For example, the $\dot{M}O_{2\text{standard}}$ of brook char had only reached a steady state of cold acclimation by 60 days, well beyond typically used acclimation durations (~4 weeks; 30 days). Additionally, multi-factorial designs can reveal unexpected phenotypic outcomes of changing environments, such as an apparent balancing of thermal compensation and starvation-induced reductions in $\dot{M}O_{2\text{standard}}$ of brook char. We recommend that studies on thermal plasticity consider the context of co-varying environmental factors and use experimental time scales most relevant to the animal in the wild.

Acknowledgements

We thank Tillmann Benfey and UNB Fredericton for donating the study fish, two anonymous reviewers for helpful comments, and Kelly Cummings-Martell for animal care support.

Competing Interests

We have no competing interests.

Author contributions

Conceptualization: E.K.M, M.J.H.G., B.S.-R.; Methodology: E.K.M, M.J.H.G., S.G.L., B.S.-R.; Validation: E.K.M, M.J.H.G.; Investigation: E.K.M.; Formal analysis: E.K.M., T.L.; Data curation: E.K.M.; Writing—original draft: E.K.M.; Writing—review & editing: E.K.M, M.J.H.G., S.G.L., B.S.-R.; Supervision: M.J.H.G., B.S.-R.; Funding: E.K.M., M.J.H.G., S.G.L., B.S.-R.

Funding

Grants to B.S.-R.: Discovery Grant (National Science and Engineering Research Council of Canada; NSERC); Young Investigator Award (Harrison McCain Foundation); Start-Up Fund (New Brunswick Innovation Foundation); John Evans Leaders Fund (Canada Foundation for Innovation); University Research Fellowship (University of New Brunswick). Grants and Awards to M.J.H.G: ArcticNet-MEOPAR Postdoctoral Fellowship; Weston Family Fellowship in Northern Science; NSERC Postdoctoral Fellowship; Earth Rangers Science Grant. Awards to E.K.M.: Weston Family Graduate Fellowship in Northern Science; New Brunswick Innovation Foundation STEM Award. Grant to S.G.L.: Discovery Grant (NSERC)

Data availability

<https://figshare.com/s/ec15fb4a979c89aea319>

LITERATURE CITED

- Abe, T. K., Kitagawa, T., Makiguchi, Y. and Sato, K.** (2019). Chum salmon migrating upriver accommodate to environmental temperatures through metabolic compensation. *J. Exp. Biol.* jeb.186189.
- Alsop, D. and Wood, C.** (1997). The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **200**, 2337–2346.
- Armstrong, J. B. and Bond, M. H.** (2013). Phenotype flexibility in wild fish: Dolly Varden regulate assimilative capacity to capitalize on annual pulsed subsidies. *J. Anim. Ecol.* **82**, 966–975.
- Auer, S. K., Salin, K., Rudolf, A. M., Anderson, G. J. and Metcalfe, N. B.** (2015). Flexibility in metabolic rate confers a growth advantage under changing food availability. *J. Anim. Ecol.* **84**, 1405–1411.
- Auer, S. K., Salin, K., Rudolf, A. M., Anderson, G. J. and Metcalfe, N. B.** (2016a). Differential effects of food availability on minimum and maximum rates of metabolism. *Biol. Lett.* **12**, 20160586.
- Auer, S. K., Salin, K., Anderson, G. J. and Metcalfe, N. B.** (2016b). Flexibility in metabolic rate and activity level determines individual variation in overwinter performance. *Oecologia* **182**, 703–712.
- Auer, S. K., Killen, S. S. and Rezende, E. L.** (2017). Resting vs. active: a meta-analysis of the intra- and inter-specific associations between minimum, sustained, and maximum metabolic rates in vertebrates. *Funct. Ecol.* **31**, 1728–1738.
- Auer, S. K., Solowey, J. R., Rajesh, S. and Rezende, E. L.** (2020). Energetic mechanisms for coping with changes in resource availability. *Biol. Lett.* **16**, 20200580.
- Bar, N.** (2014). Physiological and hormonal changes during prolonged starvation in fish. *Can. J. Fish. Aquat. Sci.* **71**, 1447–1458.
- Bar, N. and Volkoff, H.** (2012). Adaptation of the Physiological, Endocrine, and Digestive System Functions to Prolonged Food Deprivation in Fish. In *Comparative Physiology of Fasting, Starvation, and Food Limitation* (ed. McCue, M. D.), pp. 69–89. Berlin, Heidelberg: Springer Berlin Heidelberg.

- Barceló, G., Love, O. P. and Vézina, F.** (2017). Uncoupling Basal and Summit Metabolic Rates in White-Throated Sparrows: Digestive Demand Drives Maintenance Costs, but Changes in Muscle Mass Are Not Needed to Improve Thermogenic Capacity. *Physiol. Biochem. Zool.* **90**, 153–165.
- Bennett, A. F. and Ruben, J. A.** (1979). Endothermy and activity in vertebrates. *Science* **206**, 649–654.
- Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., Zupicich, J., Alkass, K., Buchholz, B. A., Druid, H., et al.** (2009). Evidence for Cardiomyocyte Renewal in Humans. *Science* **324**, 98–102.
- Blanchfield, P. J., Tate, L. S., Plumb, J. M., Acolas, M.-L. and Beaty, K. G.** (2009). Seasonal habitat selection by lake trout (*Salvelinus namaycush*) in a small Canadian shield lake: constraints imposed by winter conditions. *Aquat. Ecol.* **43**, 777–787.
- Bokma, F.** (2004). Evidence against universal metabolic allometry. *Funct. Ecol.* **18**, 184–187.
- Brett, J. R., Shelbourn, J. E. and Shoop, C. T.** (1969). Growth rate and body composition of fingerling sockeye salmon, *Oncorhynchus nerka*, in relation to temperature and ration size. *J. Fish. Board Can.* **26**, 2363–2394.
- Brook Trout Atlas.**
- Brooks, M., Bolker, B., Kristensen, K. and Maechler, M.** (2017). Package ‘glmmTMB.’ *CRAN*.
- Brownscombe, J. W., Lawrence, M. J., Deslauriers, D., Filgueira, R., Boyd, R. J. and Cooke, S. J.** (2022). Applied fish bioenergetics. In *Fish Physiology*, pp. 141–188. Elsevier.
- Carter, C. G. and Houlihan, D. F.** (2001). Protein synthesis. *Fish Physiol.* **20**, 31–75.
- Cassidy, A. A., Saulnier, R. J. and Lamarre, S. G.** (2016). Adjustments of Protein Metabolism in Fasting Arctic Charr, *Salvelinus alpinus*. *PLOS ONE* **11**, e0153364.
- Cassidy, A. A., Blier, P. U., Le François, N. R., Dionne, P., Morin, P. J. and Lamarre, S. G.** (2018). Effects of fasting and refeeding on protein and glucose metabolism in Arctic charr. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **226**, 66–74.
- Chabot, D., McKenzie, D. J. and Craig, J. F.** (2016a). Metabolic rate in fishes: definitions, methods and significance for conservation physiology: editorial. *J. Fish Biol.* **88**, 1–9.
- Chabot, D., Steffensen, J. F. and Farrell, A. P.** (2016b). The determination of standard metabolic rate in fishes: measuring smr in fishes. *J. Fish Biol.* **88**, 81–121.

- Clarke, A.** (2017). *Principles of thermal ecology: Temperature, energy and life*. Oxford University Press.
- Clarke, A. and Johnston, N. M.** (1999). Scaling of metabolic rate with body mass and temperature in teleost fish. *J. Anim. Ecol.* **68**, 893–905.
- Core Team, R.** (2014). R: A language and environment for statistical computing. Vienna, Austria. *R Found. Stat. Comput.*
- Cunjak, R. A. and Power, G.** (1987). The feeding and energetics of stream-resident trout in winter*. *J. Fish Biol.* **31**, 493–511.
- Cunjak, R. A., Curry, A. and Power, G.** (1987). Seasonal Energy Budget of Brook Trout in Streams : *Trans. Am. Fish. Soc.* **116**, 817–828.
- Cutts, C. J., Metcalfe, N. B. and Taylor, A. C.** (2002). Juvenile Atlantic Salmon (*Salmo salar*) with relatively high standard metabolic rates have small metabolic scopes: *Metabolic scope in juvenile salmon. Funct. Ecol.* **16**, 73–78.
- Dabrowski, K. and Guderley, H.** (2003). Intermediary metabolism. In *Fish nutrition*, pp. 309–365. Elsevier.
- Davis, K. B. and Gaylord, T. G.** (2011). Effect of fasting on body composition and responses to stress in sunshine bass. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **158**, 30–36.
- Dean, J. M.** (1969). The metabolism of tissues of thermally acclimated trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* **29**, 185–196.
- Durhack, T. C., Mochacz, N. J., Macnaughton, C. J., Enders, E. C. and Treberg, J. R.** (2021). Life through a wider scope: Brook Trout (*Salvelinus fontinalis*) exhibit similar aerobic scope across a broad temperature range. *J. Therm. Biol.* **99**, 102929.
- Dutil, J.-D.** (1986). Energetic Constraints and Spawning Interval in the Anadromous Arctic Charr (*Salvelinus alpinus*). *Copeia* **1986**, 945.
- Fedorov, V. B., Goropashnaya, A. V., Tøien, Ø., Stewart, N. C., Gracey, A. Y., Chang, C., Qin, S., Perte, G., Quackenbush, J., Showe, L. C., et al.** (2009). Elevated expression of protein biosynthesis genes in liver and muscle of hibernating black bears (*Ursus americanus*). *Physiol. Genomics* **37**, 108–118.
- Fernandes, T. and McMeans, B. C.** (2019). Coping with the cold: energy storage strategies for surviving winter in freshwater fish. *Ecography* **42**, 2037–2052.

Foda, A. (1975). *Effects of Starvation on Hatchery-Reared Atlantic Salmon (Salmo salar)*.

Halifax, Nova Scotia: Resource development branch fisheries and marine service department of the environment.

Fox, J., Friendly, G. G., Graves, S., Heiberger, R., Monette, G., Nilsson, H., Ripley, B., Weisberg, S., Fox, M. J. and Suggests, M. (2007). The car package. *R Found. Stat. Comput.* **1109**, 1431.

Fraser, N. H. C., Metcalfe, N. B. and Thorpe, J. E. (1993). Temperature-dependent switch between diurnal and nocturnal foraging in salmon. *Proc. R. Soc. Lond. B Biol. Sci.* **252**, 135–139.

Funes, S. C., Filippa, V. P., Cid, F. D., Mohamed, F., Caviedes-Vidal, E. and Chediack, J. G. (2014). Effect of fasting in the digestive system: Histological study of the small intestine in house sparrows. *Tissue Cell* **46**, 356–362.

Gallaughier, P. E., Thorarensen, H. and Farrell, A. P. (1995). Hematocrit in oxygen transport and swimming in rainbow trout (*Oncorhynchus mykiss*). *Respir. Physiol.* **102**, 279–292.

Gallaughier, P. E., Thorarensen, H., Kiessling, A. and Farrell, A. P. (2001). Effects of high intensity exercise training on cardiovascular function, oxygen uptake, internal oxygen transport and osmotic balance in chinook salmon (*Oncorhynchus tshawytscha*) during critical speed swimming. *J. Exp. Biol.* **204**, 2861–2872.

Geiser, F. (2016). Conserving energy during hibernation. *J. Exp. Biol.* **219**, 2086–2087.

Gilbert, M. J. H. (2020). Thermal limits to the cardiorespiratory performance of Arctic char (*Salvelinus alpinus*) in a rapidly warming north.

Gilbert, M. J. H. and Farrell, A. P. (2021). The thermal acclimation potential of maximum heart rate and cardiac heat tolerance in Arctic char (*Salvelinus alpinus*), a northern cold-water specialist. *J. Therm. Biol.* **95**, 102816.

Gilbert, M. J. H., Harris, L. N., Malley, B. K., Schimnowski, A., Moore, J.-S. and Farrell, A. P. (2020). The thermal limits of cardiorespiratory performance in anadromous Arctic char (*Salvelinus alpinus*): a field-based investigation using a remote mobile laboratory. *Conserv. Physiol.* **8**, coaa036.

Government of Canada (2023). Real-Time Hydrometric Data Map Search.

Guderley, H. (1990). Functional significance of metabolic responses to thermal acclimation in fish muscle. *Am J Physiol* **259**, 8.

- Guderley, H.** (2004). Metabolic responses to low temperature in fish muscle. *Biol. Rev.* **79**, 409–427.
- Guderley, H. and St-Pierre, J.** (2002). Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. *J. Exp. Biol.* **205**, 2237–2249.
- Havird, J. C., Neuwald, J. L., Shah, A. A., Mauro, A., Marshall, C. A. and Ghalambor, C. K.** (2020). Distinguishing between active plasticity due to thermal acclimation and passive plasticity due to Q_{10} effects: Why methodology matters. *Funct. Ecol.* **34**, 1015–1028.
- Healy, T. M., Chung, D. J., Crowther, K. G. and Schulte, P. M.** (2017). Metabolic and regulatory responses involved in cold acclimation in Atlantic killifish, *Fundulus heteroclitus*. *J. Comp. Physiol. B* **187**, 463–475.
- Helland, I. P., Finstad, A. G., Forseth, T., Hesthagen, T. and Ugedal, O.** (2011). Ice-cover effects on competitive interactions between two fish species: Ice-cover and competition. *J. Anim. Ecol.* **80**, 539–547.
- Hochachka, P. W. and Hayes, F. R.** (1962). The effect of temperature acclimation on pathways of glucose metabolism in the trout. *Can. J. Zool.* **40**, 261–270.
- Houlihan, D. F., Carter, C. G. and McCarthy, I. D.** (1995). Protein synthesis in fish. In *Biochemistry and molecular biology of fishes*, pp. 191–220. Elsevier.
- Huey, R. B. and Buckley, L. B.** (2022). Designing a Seasonal Acclimation Study Presents Challenges and Opportunities. *Integr. Org. Biol.* **4**, obac016.
- Hurst, T. P.** (2007). Causes and consequences of winter mortality in fishes. *J. Fish Biol.* **71**, 315–345.
- Hutchings, J. A.** (1996). Adaptive phenotypic plasticity in brook trout, *Salvelinus fontinalis*, life histories. *Écoscience* **3**, 25–32.
- Hvas, M.** (2022). Swimming energetics of Atlantic salmon in relation to extended fasting at different temperatures. *Conserv. Physiol.* **10**, coac037.
- Hvas, M., Stien, L. H. and Oppedal, F.** (2020). The metabolic rate response to feed withdrawal in Atlantic salmon post-smolts. *Aquaculture* **529**, 735690.
- Jobling, M., Koskela, J. and Savolainen, R.** (1998a). Influence of dietary fat level and increased adiposity on growth and fat deposition in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquac. Res.* **29**, 601–607.

- Jobling, M., Johansen, S. J. S., Foshaug, H., Burkow, I. C. and Jørgensen, E. H.** (1998b). Lipid dynamics in anadromous Arctic charr, *Salvelinus alpinus* (L.): seasonal variations in lipid storage depots and lipid class composition. *Fish Physiol Biochem* **18**, 225–240.
- Jørgensen, E. H., Johansen, S. J. S. and Jobling, M.** (1997). Seasonal patterns of growth, lipid deposition and lipid depletion in anadromous Arctic charr. *J. Fish Biol.* **51**, 312–326.
- Karasov, W. H. and Douglas, A. E.** (2013). Comparative Digestive Physiology. In *Comprehensive Physiology* (ed. Terjung, R.), pp. 741–783. Wiley.
- Koteja, P.** (1987). On the relation between basal and maximum metabolic rate in mammals. *Comp. Biochem. Physiol. A* **87**, 205–208.
- Krogdahl, Å. and Bakke-McKellep, A. M.** (2005). Fasting and refeeding cause rapid changes in intestinal tissue mass and digestive enzyme capacities of Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **141**, 450–460.
- Lahnsteiner, F.** (2022). Seasonal differences in thermal stress susceptibility of diploid and triploid brook trout, *S. fontinalis* (Teleostei, Pisces). *J. Fish Biol.* jfb.15118.
- Lamarre, S. G., Saulnier, R. J., Blier, P. U. and Driedzic, W. R.** (2015). A rapid and convenient method for measuring the fractional rate of protein synthesis in ectothermic animal tissues using a stable isotope tracer. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **182**, 1–5.
- Lenth, R., Singmann, H., Love, J., Buerkner, P. and Herve, M.** (2019). Package ‘emmeans.’
- Lignot, J.-H.** (2012). Changes in Form and Function of the Gastrointestinal Tract During Starvation: From Pythons to Rats. *Comp. Physiol. Fasting Starvation Food Limit.* 217–236.
- Little, A. G., Dressler, T., Kraskura, K., Hardison, E., Hendriks, B., Prystay, T., Farrell, A. P., Cooke, S. J., Patterson, D. A., Hinch, S. G., et al.** (2020). Maxed Out: Optimizing Accuracy, Precision, and Power for Field Measures of Maximum Metabolic Rate in Fishes. *Physiol. Biochem. Zool.* **93**, 243–254.
- Loughna, P. T. and Goldspink, G.** (1984). The effects of starvation upon protein turnover in red and white myotomal muscle of rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* **25**, 223–230.
- MacCrimmon, H. R. and Campbell, J. S.** (1969). World Distribution of Brook Trout, *Salvelinus fontinalis*. *J Fish Res Board Can* **26**, 27.

- Mackey, T. E., Hasler, C. T., Durhack, T., Jeffrey, J. D., Macnaughton, C. J., Ta, K., Enders, E. C. and Jeffries, K. M.** (2021). Molecular and physiological responses predict acclimation limits in juvenile brook trout (*Salvelinus fontinalis*). *J. Exp. Biol.* **224**, jeb241885.
- Martin, N. B., Houlihan, D. F., Talbot, C. and Palmer, R. M.** (1993). Protein metabolism during sexual maturation in female Atlantic salmon (*Salmo salar* L). *Fish Physiol. Biochem.* **12**, 131–141.
- McMillan, D. N. and Houlihan, D. F.** (1989). Short-term responses of protein synthesis to re-feeding in rainbow trout. *Aquaculture* **79**, 37–46.
- McMillan, D. N. and Houlihan, D. F.** (1992). Protein synthesis in trout liver is stimulated by both feeding and fasting. *Fish Physiol. Biochem.* **10**, 23–34.
- Méndez, G. and Wieser, W.** (1993). Metabolic responses to food deprivation and refeeding in juveniles of *Rutilus rutilus* (Teleostei: Cyprinidae). *Environ. Biol. Fishes* **36**, 73–81.
- Milligan, C. L.** (1996). Metabolic recovery from exhaustive exercise in rainbow trout. *Comp. Biochem. Physiol. A Physiol.* **113**, 51–60.
- Monternier, P.-A., Marmillot, V., Rouanet, J.-L. and Roussel, D.** (2014). Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks. *J. Exp. Biol.* jeb.104505.
- Morash, A.J., Speers-Roesch, B., Andrew, S. and Currie S.** (2021) The physiological ups and downs of thermal variability in temperate freshwater ecosystems. *J. Fish Biol.* **98**, 1524–1535.
- Muir, A. M., Hansen, M. J., Bronte, C. R. and Krueger, C. C.** (2016). If Arctic charr *Salvelinus alpinus* is ‘the most diverse vertebrate’, what is the lake charr *Salvelinus namaycush*? *Fish Fish.* **17**, 1194–1207.
- Navarro, I. and Gutierrez, J.** (1995). Fasting and starvation. In *Biochemistry and molecular biology of fishes*, pp. 393–434. Elsevier.
- Naya, D. E., Veloso, C., Sabat, P. and Bozinovic, F.** (2009). The effect of short- and long-term fasting on digestive and metabolic flexibility in the Andean toad, *Bufo spinulosus*. *J. Exp. Biol.* **212**, 2167–2175.
- Norin, T. and Speers-Roesch, B.** (2021). Metabolism. In *The physiology of fishes* (ed. Currie, S.) and Evans, D. H.), pp. 129–142. CRC Press.
- O’Connor, K. I., Taylor, A. C. and Metcalfe, N. B.** (2000). The stability of standard metabolic rate during a period of food deprivation in juvenile Atlantic salmon. *J. Fish Biol.* **57**, 41–51.

- Orczewska, J. I., Hartleben, G. and O'Brien, K. M.** (2010). The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **299**, R352–R364.
- Peterson, R. H. and Anderson, J. M.** (1969). Influence of temperature change on spontaneous locomotor activity and oxygen consumption of Atlantic salmon, *Salmo salar*, acclimated to two temperatures. *J. Fish. Board Can.* **26**, 93–109.
- Reeve, C., Rowsey, L. E. and Speers-Roesch, B.** (2022). Inactivity and the passive slowing effect of cold on resting metabolism as the primary drivers of energy savings in overwintering fishes. *J. Exp. Biol.* **225**, jeb243407.
- Robinson, J. S. and Mead, J. F.** (1973). Lipid Absorption and Deposition in Rainbow Trout (*Salmo gairdnerii*). *Can. J. Biochem.* **51**, 1050–1058.
- Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T., Andersson, P. L. and Andersson, M.** (2018). ToxTrac: a fast and robust software for tracking organisms. *Methods Ecol. Evol.* **9**, 460–464.
- Ryu, H. S., Song, J. A., Park, H.-S., Choi, Y. J. and Choi, C. Y.** (2020). Physiological and oxidative stress response of goldfish *Carassius auratus* induced by a light dimming system. *Fish Physiol. Biochem.* **46**, 585–595.
- Salin, K., Auer, S. K., Rudolf, A. M., Anderson, G. J., Selman, C. and Metcalfe, N. B.** (2016). Variation in Metabolic Rate among Individuals Is Related to Tissue-Specific Differences in Mitochondrial Leak Respiration. *Physiol. Biochem. Zool.* **89**, 511–523.
- Salin, K., Villasevil, E. M., Anderson, G. J., Auer, S. K., Selman, C., Hartley, R. C., Mullen, W., Chinopoulos, C. and Metcalfe, N. B.** (2018). Decreased mitochondrial metabolic requirements in fasting animals carry an oxidative cost. *Funct. Ecol.* **32**, 2149–2157.
- Schneider, C. A., Rasband, W. S. and Eliceiri, K. W.** (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675.
- Schulte, P. M.** (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856–1866.
- Secor, S. M.** (2008). Digestive physiology of the Burmese python: broad regulation of integrated performance. *J. Exp. Biol.* **211**, 3767–3774.

Secor, S. M. and Carey, H. V. (2016). Integrative Physiology of Fasting. In *Comprehensive Physiology* (ed. Terjung, R.), pp. 773–825. Wiley.

Shuter, B. J., Finstad, A. G., Helland, I. P., Zweimüller, I. and Hölker, F. (2012). The role of winter phenology in shaping the ecology of freshwater fish and their sensitivities to climate change. *Aquat. Sci.* **74**, 637–657.

Smith, M. A. K. (1981). Estimation of growth potential by measurement of tissue protein synthetic rates in feeding and fasting rainbow trout, *Salmo gairdnerii* Richardson. *J. Fish Biol.* **19**, 213–220.

Smithson, M. and Verkuilen, J. (2006). A better lemon squeezer? Maximum-likelihood regression with beta-distributed dependent variables. *Psychol. Methods* **11**, 54–71.

Somero, G. (2015). Temporal patterning of thermal acclimation: from behavior to membrane biophysics. *J. Exp. Biol.* **218**, 500–500.

Spares, A. D., Dadswell, M. J., MacMillan, J., Madden, R., O'Dor, R. K. and Stokesbury, M. J. W. (2014). To fast or feed: an alternative life history for anadromous brook trout *Salvelinus fontinalis* overwintering within a harbour: winter feeding of *salvelinus fontinalis*. *J. Fish Biol.* **85**, 621–644.

Speers-Roesch, B., Norin, T. and Driedzic, W. R. (2018). The benefit of being still: energy savings during winter dormancy in fish come from inactivity and the cold, not from metabolic rate depression. *Proc. R. Soc. B Biol. Sci.* **285**, 20181593.

Staples, J. F. (2016). Metabolic Flexibility: Hibernation, Torpor, and Estivation. In *Comprehensive Physiology* (ed. Terjung, R.), pp. 737–771. Wiley.

Svendsen, M. B. S., Bushnell, P. G. and Steffensen, J. F. (2016). Design and setup of intermittent-flow respirometry system for aquatic organisms: how to set up an aquatic respirometry system. *J. Fish Biol.* **88**, 26–50.

Thoral, E., Roussel, D., Gasset, E., Dutto, G., Queiros, Q., McKenzie, D. J., Bourdeix, J.-H., Metral, L., Saraux, C. and Teulier, L. (2023). Temperature-dependent metabolic consequences of food deprivation in the European sardine. *J. Exp. Biol.*

Thorarensen, H. and Farrell, A. P. (2006). Postprandial Intestinal Blood Flow, Metabolic Rates, and Exercise in Chinook Salmon (*Oncorhynchus tshawytscha*). *Physiol. Biochem. Zool.* **79**, 688–694.

- Tøien, Ø., Blake, J., Edgar, D. M., Grahn, D. A., Heller, H. C. and Barnes, B. M.** (2011). Hibernation in Black Bears: Independence of Metabolic Suppression from Body Temperature. *Science* **331**, 906–909.
- Turko, A. J., Firth, B. L., Craig, P. M., Eliason, E. J., Raby, G. D. and Borowiec, B. G.** (2023). Physiological differences between wild and captive animals: a century-old dilemma. *J. Exp. Biol.* **226**, jeb246037
- USGS National Water Dashboard.
- Van Leeuwen, T. E., Rosenfeld, J. S. and Richards, J. G.** (2012). Effects of food ration on SMR: influence of food consumption on individual variation in metabolic rate in juvenile coho salmon (*Onchorhynchus kisutch*): Food ration effects on variation in SMR. *J. Anim. Ecol.* **81**, 395–402.
- Watanabe, Y. Y. and Payne, N. L.** (2023). Thermal sensitivity of metabolic rate mirrors biogeographic differences between teleosts and elasmobranchs. *Nat. Commun.* **14**, 2054.
- White, C. R., Phillips, N. F. and Seymour, R. S.** (2006). The scaling and temperature dependence of vertebrate metabolism. *Biol. Lett.* **2**, 125–127.
- Zaldúa, N. and Naya, D. E.** (2014). Digestive flexibility during fasting in fish: A review. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **169**, 7–14.
- Zeng, L.-Q., Fu, C. and Fu, S.-J.** (2018). The effects of temperature and food availability on growth, flexibility in metabolic rates and their relationships in juvenile common carp. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **217**, 26–34.
- Zhang, Y., Claireaux, G., Takle, H., Jørgensen, S. M. and Farrell, A. P.** (2018). A three-phase excess post-exercise oxygen consumption in Atlantic salmon *Salmo salar* and its response to exercise training: Three-phase EPOC in *S. SALAR*. *J. Fish Biol.* **92**, 1385–1403.
- Zhang, Y., Gilbert, M. J. H. and Farrell, A. P.** (2020). Measuring maximum oxygen uptake with an incremental swimming test and by chasing rainbow trout to exhaustion inside a respirometry chamber yields the same results. *J. Fish Biol.* **97**, 28–38.

Figures and Tables

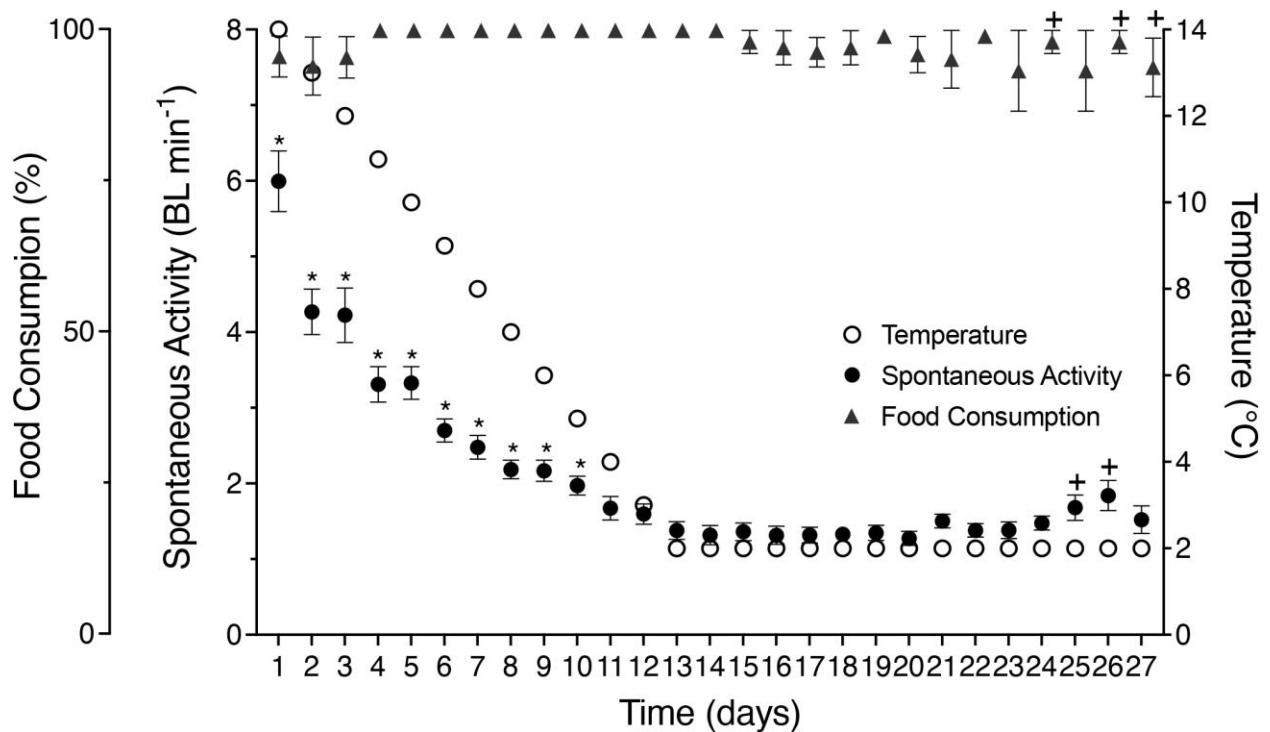


Fig. 1. Food consumption (A; closed triangles) and spontaneous activity (B; closed circles) of juvenile brook char (Experiment 1) during acute cooling ($1^{\circ}\text{C day}^{-1}$; open circles) and subsequent holding at 2°C for two weeks. Food consumption is the percentage of the daily ration (0.5% body mass). Data are means \pm S.E.M. ($n=15$). Asterisks (*) and crosses (+) indicate significant differences relative to the first day at 2°C (day 13) for the period before or after that day, respectively (GLMMs and Type II Wald chi-square tests).

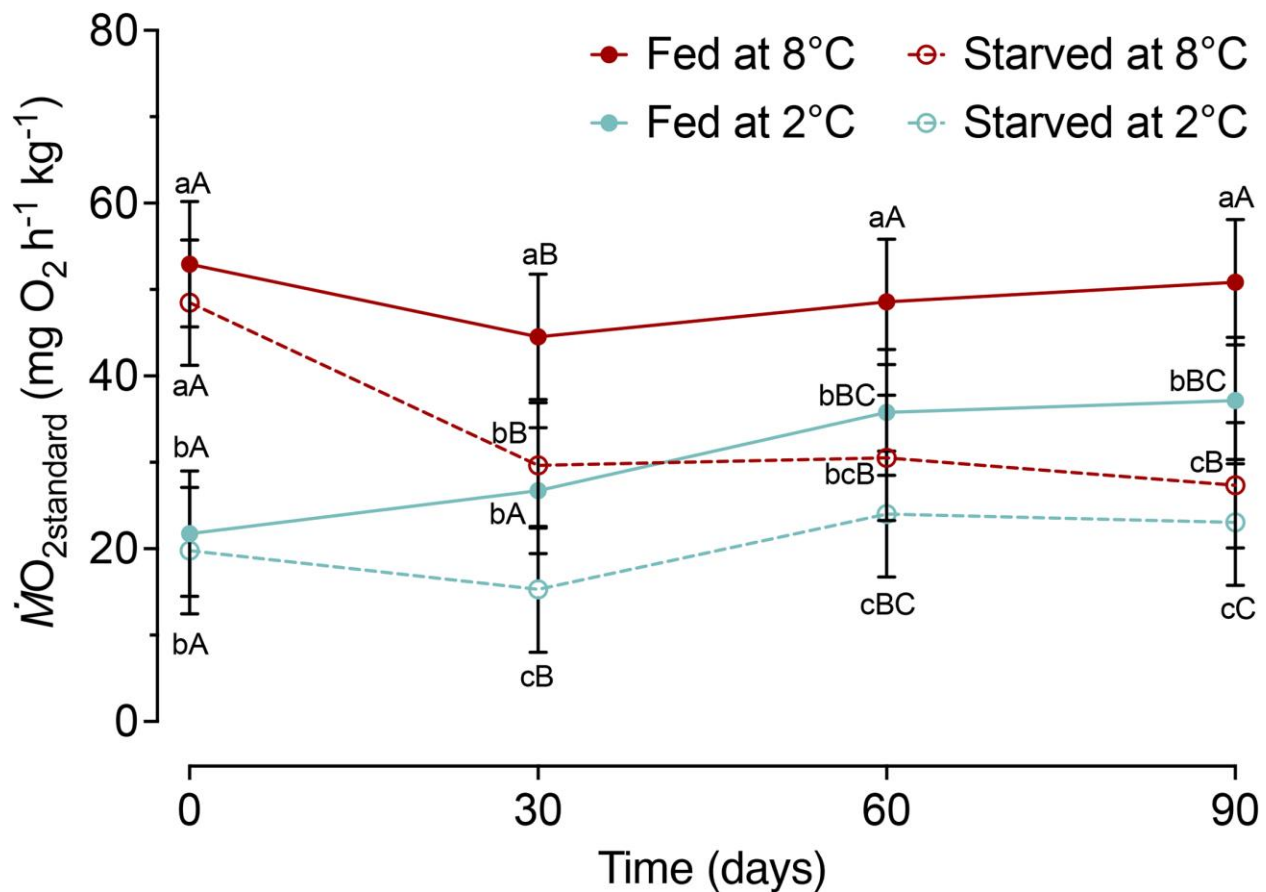


Fig. 2. The standard rate of oxygen consumption ($\dot{M}O_{2\text{standard}}$) of adult brook char over 90 days of acclimation to 2°C (teal) or held at 8°C (red; control) while fed daily (closed circles with solid line) or starved (open circles with dotted line) (Exp. 2). Treatments started on day 0; for cold acclimation fish were acutely cooled (over ~10 hours) from 8°C to 2°C. Data are estimated marginal means (\pm S.E.M., $n=10-14$; see methods for exact sample sizes) generated from a GLMM and adjusted to an average mass of 0.146 kg. Different lowercase letters represent significant differences between treatments within a time point and different uppercase letters represent differences between time points within a treatment group (GLMMs and Type II Wald chi-square tests).

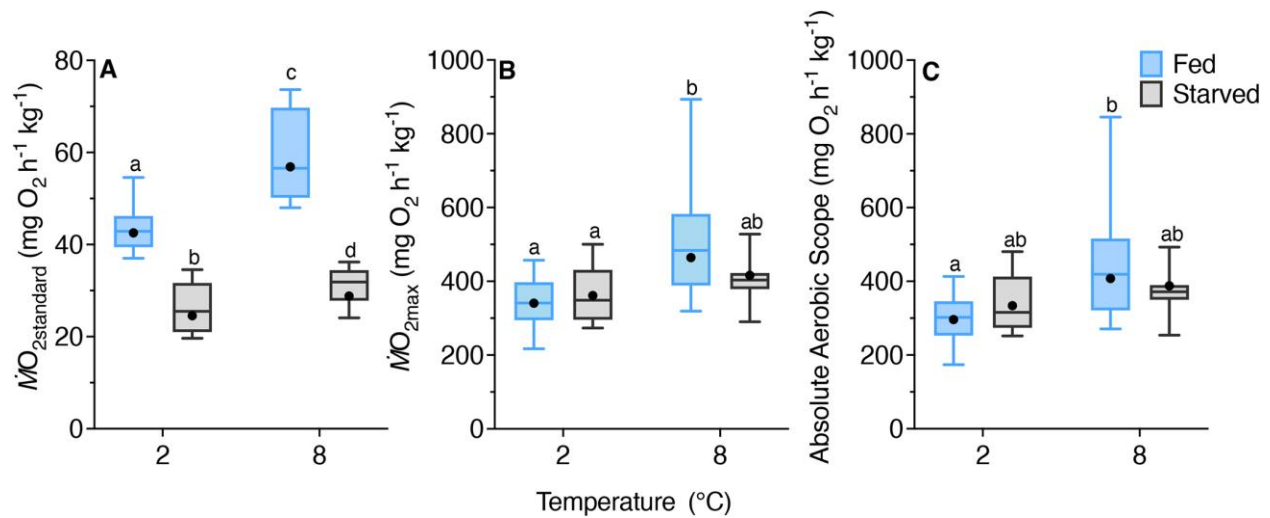


Fig. 3. Aerobic metabolic performance of adult brook char following 90 days at 2°C or 8°C while either fed or starved. Data are shown for standard and maximum oxygen consumption ($\dot{M}\text{O}_{2\text{standard}}$, A; $\dot{M}\text{O}_{2\text{max}}$, B) and for absolute aerobic scope (C). Data are adjusted to an average lean body mass of 0.154 kg (see methods). The boxplots represent the median and interquartile range with whiskers indicating the 5-95% confidence interval; the filled black circle represents the estimated marginal mean ($n=10-14$; see methods for exact sample sizes) from the corresponding GLM. Different letters indicate significant differences between treatment groups (GLMs and Type II Wald chi-square tests).

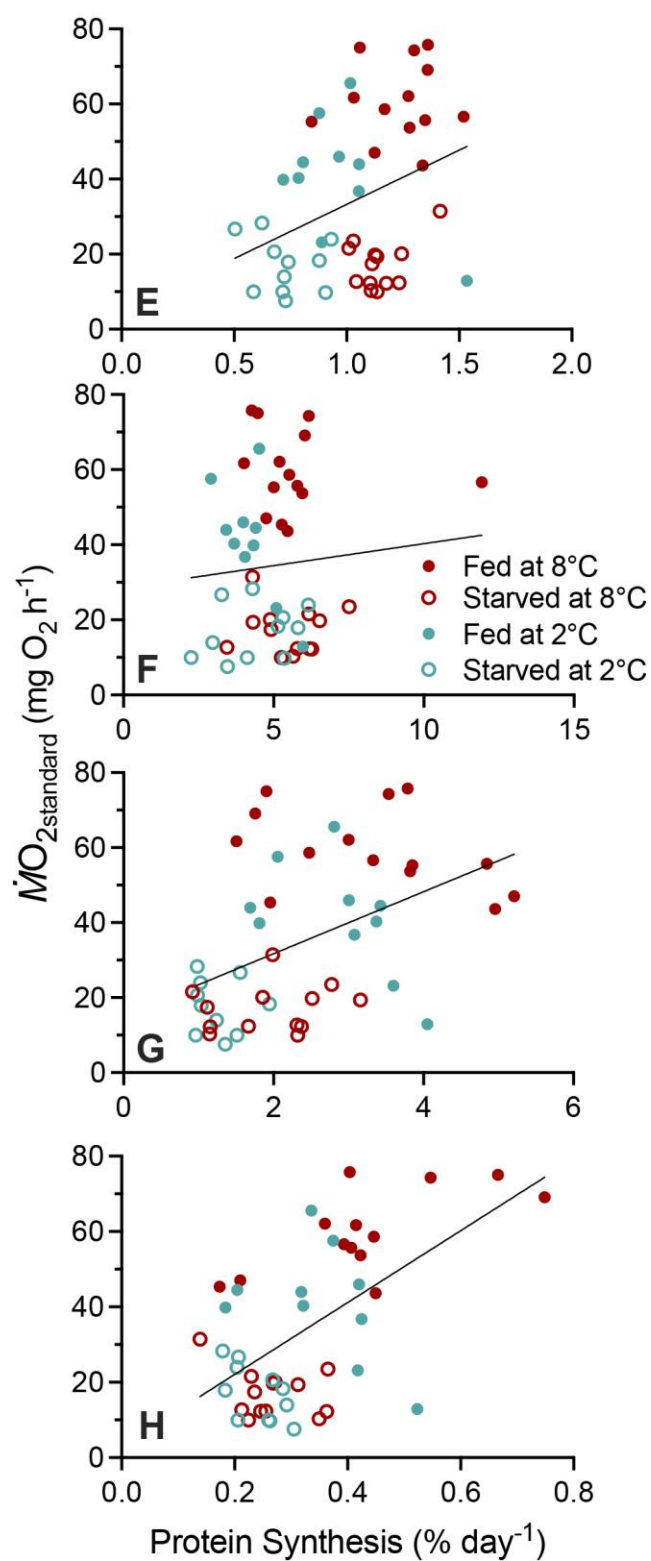
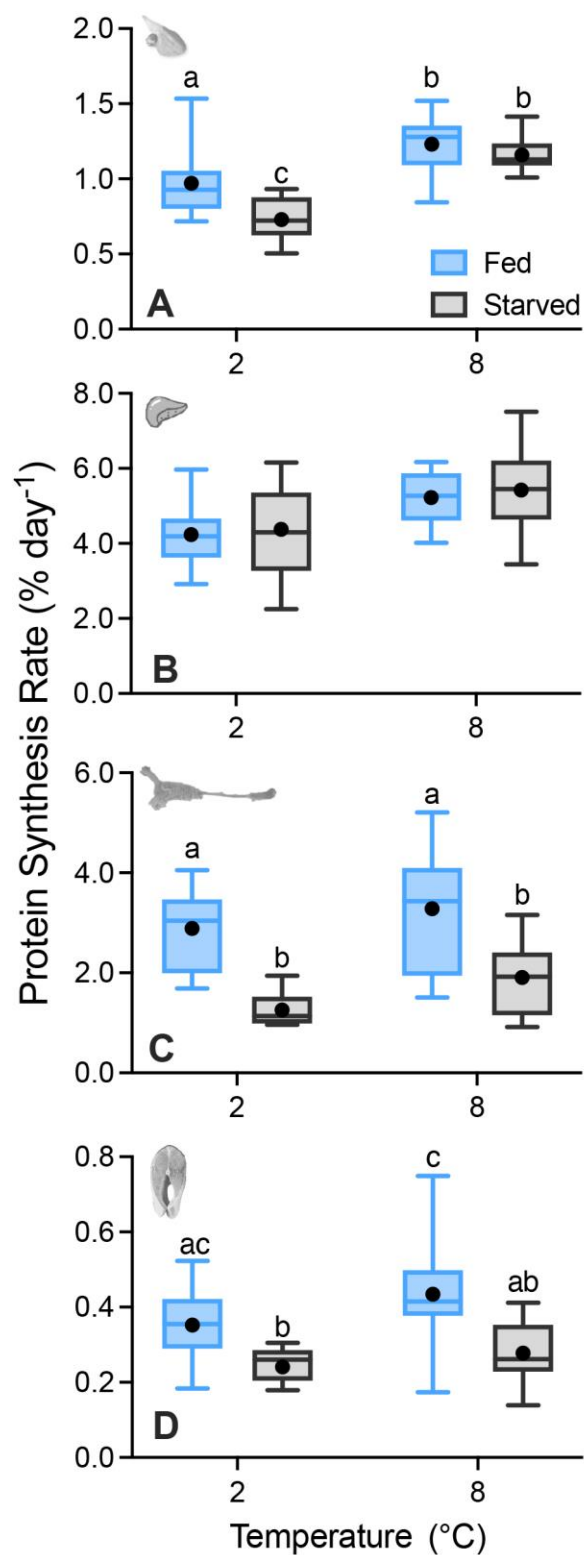


Fig. 4. Fractional rates of *in vivo* protein synthesis in brook char ventricle (A), liver (B), gut (C) and white muscle (D) and their respective linear regression with $\dot{M}O_{2\text{standard}}$ (E-H) following 90 days at 2°C (teal) or 8°C (red) while either fed daily (blue; closed circles) or starved (grey; open circles). Boxplots represent the median and interquartile range with whiskers indicating the 5-95% confidence interval; the filled black circle represents the mean (n=10-14; see methods for exact sample sizes). Different letters represent significant differences between treatment groups (GLM or LM and Type II Wald chi-square tests). Linear regressions are shown for the ventricle (E; $p < 0.05$, $R^2 = 0.116$), liver (F; $p > 0.05$, $R^2 = 0.007$), gut (G; $p < 0.01$, $R^2 = 0.205$), white muscle (H; $p < 0.0001$, $R^2 = 0.329$).

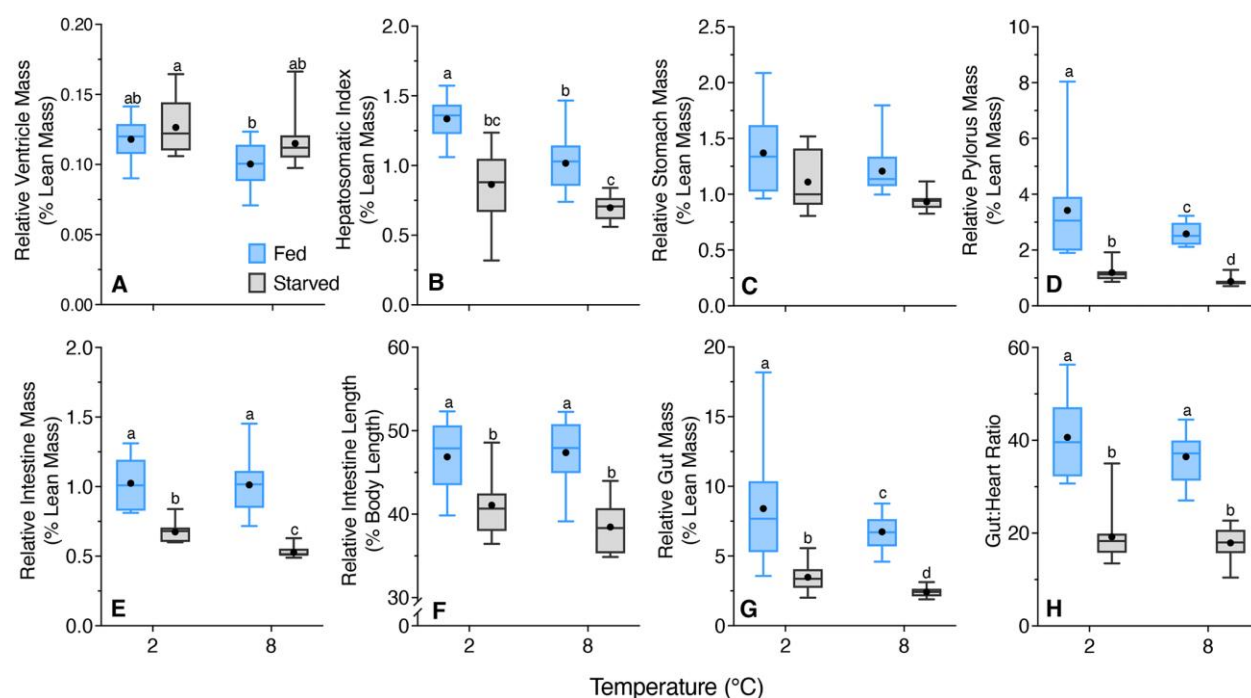


Fig. 5. Brook char organ-somatic indices following 90 days at 2°C or 8°C while either fed daily or starved. Data for the ventricle (A), liver (B), stomach (C), pylorus (D), intestine (E&F), total gut (G) and gut:heart ratio (H) are shown as boxplots representing the median and interquartile range with whiskers indicating the 5-95% confidence interval; the filled black circle represents the mean (n=10-15; see methods for exact sample sizes). Different letters represent significant differences between treatment groups within a panel (GLM or LM and Type II Wald chi-square tests).

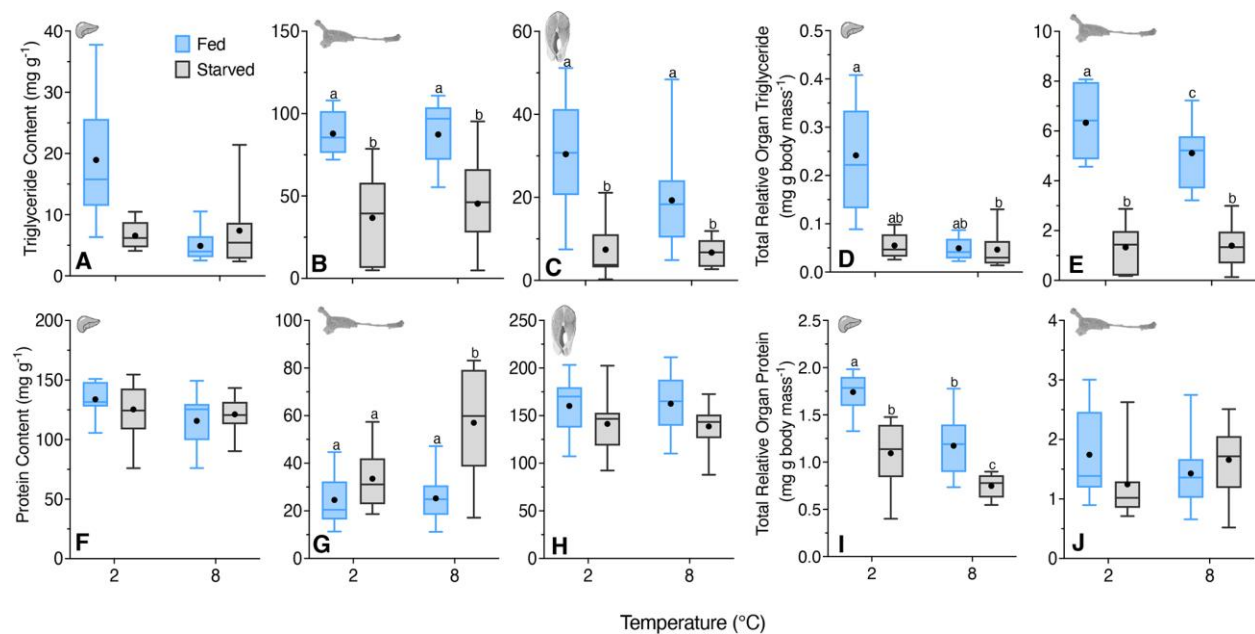


Fig. 6. Organ-specific triglyceride (TG) and protein content in brook char following 90 days of daily feeding or starvation at 2°C or 8°C. The TG and protein contents are presented for each organ (A-C, F-H, respectively) and the total relative organ TG and protein (D-E, I-J, respectively). Total relative organ tissue TG and protein were calculated as the total TG or protein (TG or protein concentration multiplied by organ mass) divided by lean body mass. Data for the liver (A,D,F,I), gut (B,E,G,J) and white muscle (C,H) are shown as boxplots representing the median and interquartile range with whiskers indicating the 5-95% confidence interval; the filled black circle represents the mean ($n=10-15$; see methods for exact sample sizes). Different letters represent significant differences between treatment groups (GLM or LM and Type II Wald chi-square tests).

Table 1. Length, mass, body condition and specific growth rates of adult brook char (Experiment 2) that were fed (0.5% body mass ration) or starved at 8°C or 2°C for 90 days.

Treatment	Day	Length (mm)	Mass (g)	Fulton's Condition Factor (K)	Specific Growth Rate (% mass day ⁻¹)
Fed at 8°C (n=14)	0	211.6 ± 15.9 ^a	128.0 ± 25.6 ^a	1.34 ± 0.14 ^a	
	30	220.4 ± 15.3 ^b	143.9 ± 27.7 ^b	1.33 ± 0.13 ^a	0.132 ± 0.04
	60	226.6 ± 15.7 ^c	165.3 ± 32.5 ^c	1.45 ± 0.16 ^b	0.154 ± 0.12
	90	234.9 ± 13.6 ^d	187.1 ± 30.2 ^{dA}	1.43 ± 0.13 ^{bA}	0.146 ± 0.10
Starved at 8°C (n=15, expect for SGR where n=14)	0	210.9 ± 14.2 ^a	133.1 ± 26.1 ^a	1.41 ± 0.14 ^a	
	30	212.8 ± 14.5 ^a	124.6 ± 24.0 ^b	1.28 ± 0.16 ^b	-0.073 ± 0.02
	60	211.9 ± 14.2 ^a	121.2 ± 23.6 ^{bc}	1.26 ± 0.14 ^b	-0.031 ± 0.01
	90	211.8 ± 14.2 ^a	118.7 ± 23.1 ^{cB}	1.23 ± 0.14 ^{bA}	-0.024 ± 0.01
Fed at 2°C (n=10)	0	206.6 ± 21.9 ^a	126.6 ± 43.1 ^a	1.38 ± 0.11 ^a	
	30	212.7 ± 21.6 ^b	142.4 ± 50.3 ^b	1.42 ± 0.11 ^a	0.127 ± 0.05
	60	220.1 ± 24.8 ^c	163.7 ± 58.0 ^c	1.47 ± 0.09 ^b	0.152 ± 0.05
	90	226.6 ± 24.5 ^d	177.9 ± 63.8 ^{dAB}	1.46 ± 0.10 ^{bA}	0.092 ± 0.03
Starved at 2°C (n=11)	0	227.1 ± 22.2 ^a	160.2 ± 45.3 ^a	1.34 ± 0.12 ^a	
	30	229.5 ± 21.6 ^a	153.9 ± 43.3 ^{ab}	1.25 ± 0.09 ^b	-0.046 ± 0.06
	60	228.9 ± 22.2 ^a	149.0 ± 43.4 ^{bc}	1.21 ± 0.09 ^b	-0.037 ± 0.02
	90	229.4 ± 22.4 ^a	146.1 ± 42.5 ^{cAB}	1.18 ± 0.06 ^{bB}	-0.022 ± 0.01

Data are means ± S.D. Significant differences between time points within treatment groups are indicated by different lowercase letters. Significant differences between treatment groups at 90 days are indicated by different uppercase letters.

Table 2. Thermal sensitivity quotients (Q_{10}) of spontaneous activity (Experiment 1) and metabolic rates, aerobic scope and protein synthesis rates (Experiment 2) of brook char. Q_{10} were calculated using the mean values at each temperature.

Trait	Temperature Interval	Time Point (days)	Fed	Starved
Spontaneous Activity (BL min ⁻¹ ; Exp. 1)	14°C– 2°C	1 – 13	3.4	N/A
	8°C – 2°C	7 – 13	2.7	N/A
$\dot{M}O_{2\text{standard}}$ (mgO ₂ h ⁻¹ kg ⁻¹ ; Exp. 2)	8°C – 2°C	0	4.5	4.2
		30	2.2	2.8
		60	1.6	1.5
		90	1.6	1.4
$\dot{M}O_{2\text{average}}$ (mgO ₂ h ⁻¹ kg ⁻¹ ; Exp. 2)	8°C – 2°C	0	5.5	5.4
		30	2.8	1.9
		60	1.7	1.8
		90	1.4	1.6
$\dot{M}O_{2\text{max}}$ (mgO ₂ h ⁻¹ kg ⁻¹ ; Exp. 2)	8°C – 2°C	90	1.8	1.2
Absolute Aerobic Scope (mgO ₂ h ⁻¹ kg ⁻¹ ; Exp. 2)	8°C – 2°C	90	1.8	1.2
Fractional Protein Synthesis Rates (K _s ; % day ⁻¹ ; Exp. 2)	8°C – 2°C	Liver 90	1.6	1.5
		Gut 90	1.2	2.0
		Muscle 90	1.4	1.3
		Ventricle 90	1.5	2.0

Exp., Experiment

$\dot{M}O_{2\text{standard}}$, Standard oxygen consumption

$\dot{M}O_{2\text{average}}$, Average oxygen consumption

$\dot{M}O_{2\text{max}}$, Maximum oxygen consumption

Supplemental Methods

Calculating average and median activity

The average and median activity of each individual at each of the four time points was calculated by taking the mean or the median of all spontaneous activity measurements over a given 24-hour measurement period. Average activity was heavily skewed by brief bouts of high activity that had a transient effect on $\dot{M}O_2$. Therefore, median activity was also examined to better understand more typical activity levels. Median activity can indicate if the fish are mostly inactive while in the respirometers, which is helpful for ensuring that $\dot{M}O_{2\text{standard}}$ estimates are actually reflecting $\dot{M}O_2$ at zero activity. We did not explore or interpret the average activity values as a representation of routine voluntary activity like in Experiment 1 as fish in Experiment 2 were confined in the respirometers and were not fed during these measurements.

Validation of $\dot{M}O_{2\text{standard}}$ estimates

We measured spontaneous activity simultaneously with $\dot{M}O_2$, a method that allows us to account for any variation in activity between treatments (Reeve et al., 2022; Speers-Roesch et al., 2018). Fish at warmer temperatures and fish that are fed may inherently have higher activity levels which could result in an overestimate of $\dot{M}O_{2\text{standard}}$ using traditional methods, which could then lead to the identification of a greater difference in resting metabolic demands between treatment groups than what actually exists (Speers-Roesch et al., 2018). Thus, we validated the $\dot{M}O_{2\text{standard}}$ estimates obtained through a traditional lowest approach (20th percentile of all $\dot{M}O_2$ values) (Chabot et al., 2016b) using two other methods that involved estimating $\dot{M}O_2$ when activity was zero. First, we conducted linear regressions using Prism v.9 (GraphPad Statistical Software, San Diego, USA) for each treatment group with each individual's $\dot{M}O_2$ estimates as a

function of the corresponding spontaneous activity estimates over each 24-hour measurement period at each of the four time points (Figure S1). This regression provided a y-intercept \pm S.E.M. for the relationship between $\dot{M}O_2$ and activity which was considered as the $\dot{M}O_2$ at zero activity for each treatment group (Figure S1). Second, we generated an estimated marginal means of $\log(\dot{M}O_2)$ at zero activity using generalized linear mixed effects models (family=Gamma, link=log) in R Studio (Core Team, 2014) with $\dot{M}O_2$ ($\text{mgO}_2 \text{ h}^{-1}$) as a function of spontaneous activity, temperature and treatment, with body mass included as a covariate and fish ID as a random factor. We took the antilog of the estimated marginal means of $\dot{M}O_{2\text{standard}}$ and its standard error to obtain an estimate of $\dot{M}O_{2\text{standard}}$ for comparison. We also compared these values to those calculated by the commonly used method of adjusting $\dot{M}O_{2\text{standard}}$ to each individual's body mass (or lean mass when available).

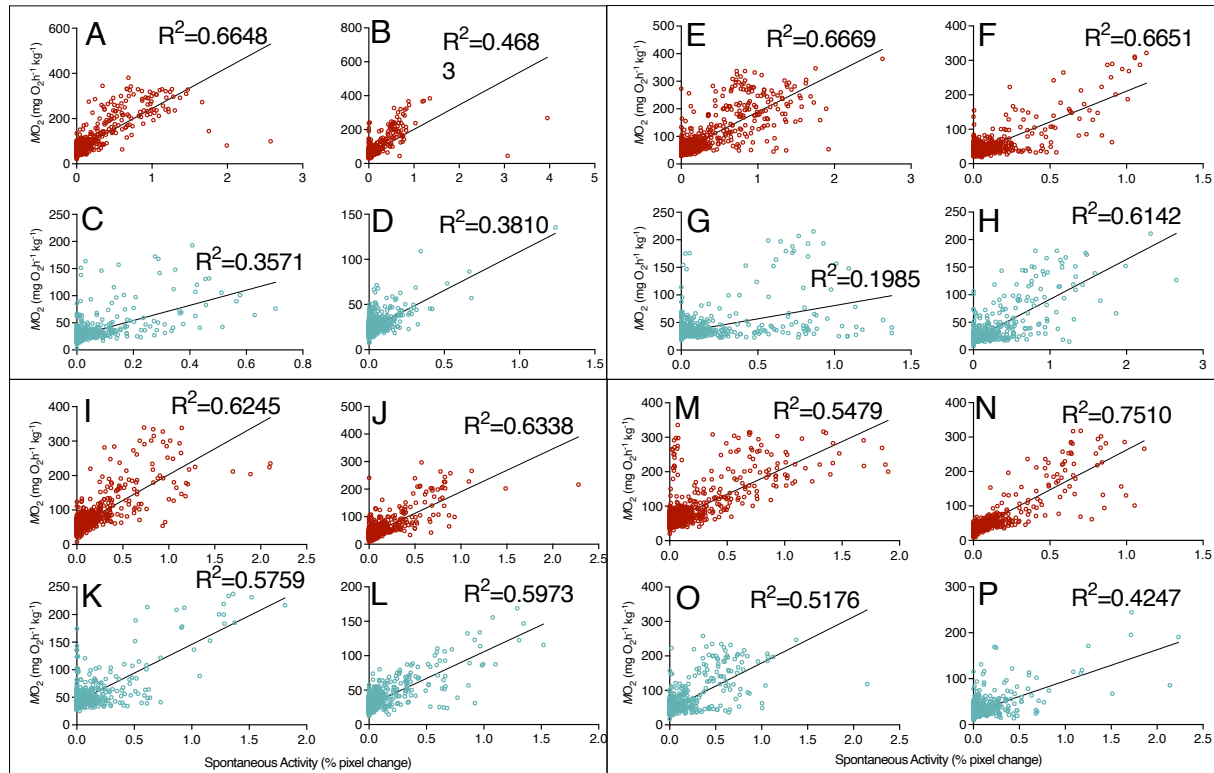


Fig. S1. Linear regressions between spontaneous activity and $\dot{M}O_2$ at the initial (day 0; top left quadrant), 30-day (top right quadrant), 60-day (bottom left quadrant) and 90-day (bottom right quadrant) measurements (Experiment 2). Data are shown for fed (A,C,E,G,I,K,M,O) and starved (B,D,F,H,J,L,N,P) fish at 2°C (blue circles; C,D,G,H,K,L,O,P) and 8°C (red circles; A,B,E,F,I,J,M,N). Linear regressions were performed using Prism v.9 (GraphPad Statistical Software, San Diego, USA) with the $\dot{M}O_2$ measurements of each individual related to the corresponding spontaneous activity estimate over a 24-hour measurement period to obtain a y-intercept which was used as an estimate of $\dot{M}O_{2\text{standard}}$ at 0 activity for each treatment group. Note that axes are on different scales because of the range in absolute values among treatments.

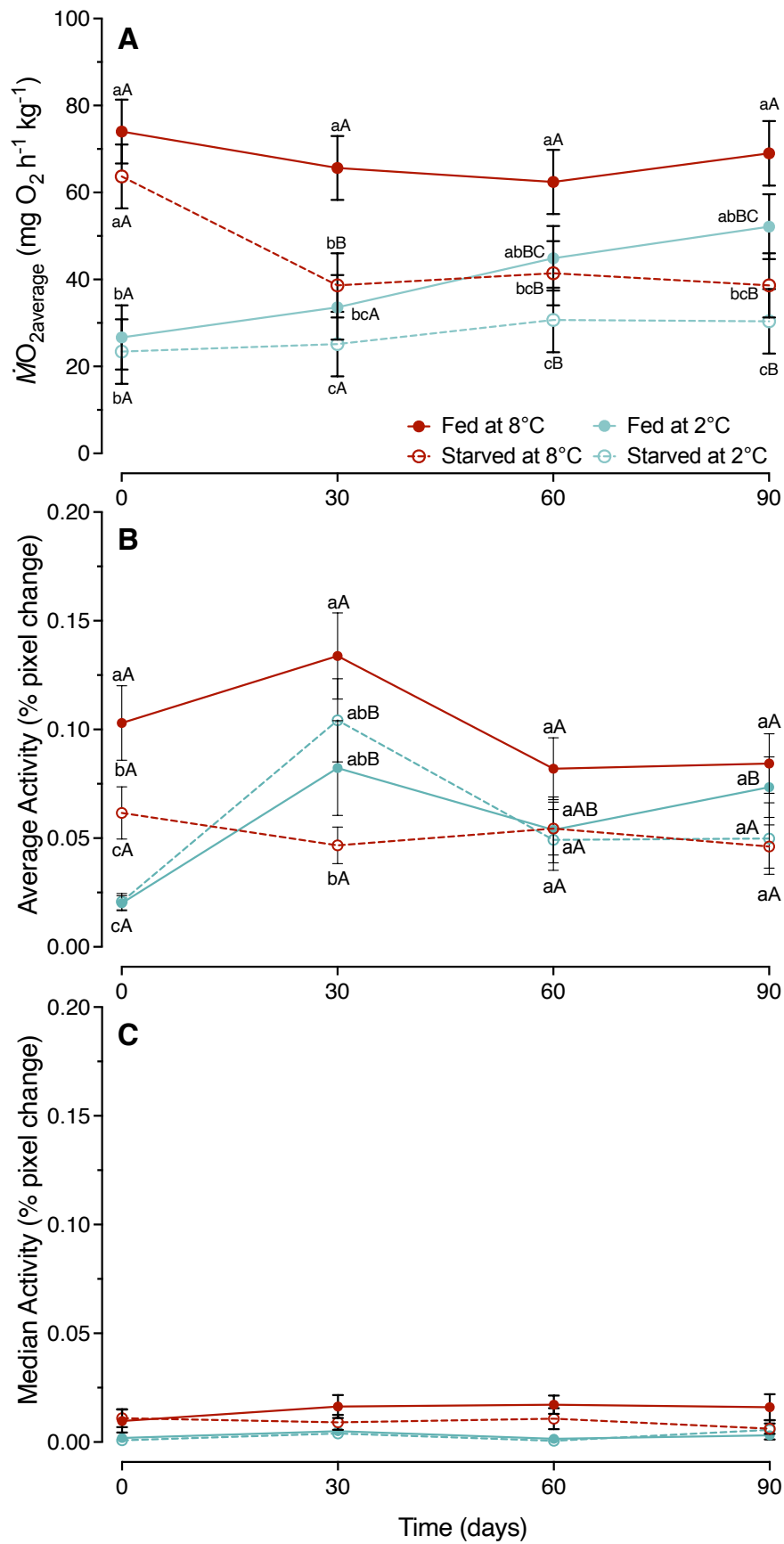


Fig. S2. Average oxygen consumption ($\dot{M}O_{2\text{average}}$, A), average activity (B) and median activity (C) of adult brook char (Experiment 2) exposed to either 2°C (blue) or 8°C (red) while fed (closed circles and solid line) or starved (open circles and dotted line) over 90 days. Fish were either maintained at 8°C or acutely cooled from 8°C to 2°C (day 0) and subsequent cold acclimated for 90 days. Activity data was taken from a 24-hour measurement period, in which oxygen consumption ($\dot{M}O_2$) was measured simultaneously. Different lowercase letters represent significant differences between the treatment groups within each time point and different uppercase letters represent significant differences across time points within a treatment group (GLMM and Type II Wald chi-square test; Table S2). Median activity was lower at 2°C than at 8°C however, no specific pairwise comparisons were significant (LMM and Type II Wald chi-square test; Table S2).

Table S1. Blood parameters and carcass composition of adult brook char (Experiment 2) measured after 90 days of exposure to 2°C or 8°C while either fed (0.5% body mass ration) or starved. Glucose and triglyceride were measured in plasma.

	Fed at 8°C n=14 (n=10 for [triglyceride])	Starved at 8°C n=15 (n=14 for hematocrit, n=10 for [triglyceride])	Fed at 2°C n=10	Starved at 2°C n=11 (n=10 for [triglyceride])
Glucose	4.09 ± 0.11 ^{ab}	3.63 ± 0.11 ^a	4.28 ± 0.22 ^b	3.79 ± 0.18 ^{ab}
Hematocrit (%)	35.47 ± 0.84 ^a	32.52 ± 0.92 ^a	33.89 ± 1.65 ^a	31.65 ± 1.01 ^a
Triglyceride (mg dL⁻¹)	234.79 ± 30.91 ^{ab}	212.11 ± 23.28 ^{ab}	269.64 ± 41.45 ^a	119.79 ± 23.82 ^b
Moisture Content (%)	72.38 ± 0.20 ^a	76.30 ± 0.35 ^b	72.46 ± 0.48 ^a	76.68 ± 0.58 ^b
Body Fat (%)	3.46 ± 0.08 ^a	2.53 ± 0.10 ^b	3.44 ± 0.15 ^a	2.59 ± 0.19 ^b
Total Relative Carcass Protein (mg g body mass⁻¹)	192.86 ± 23.34 ^a	253.00 ± 30.48 ^a	249.36 ± 45.18 ^a	272.04 ± 40.50 ^a
Energy Density (kJ/g)	24.83 ± 0.38 ^a	22.65 ± 0.20 ^b	26.10 ± 0.46 ^a	22.68 ± 0.61 ^b

Data are means ± S.E.M. Significant differences between treatment groups are indicated by different letters.

Table S2. (See next page) Summary of statistical outputs obtained from generalized linear mixed effects models (GLMM; family=Gamma, link=log) or linear mixed effects models (LMM; median activity) examining the effects of temperature (2°C or 8°C), prolonged starvation, time under treatment, and their interactions on energetic and morphological variables of adult brook char (Experiment 2). These analyses correspond with the data shown in Table 1 (main text), Figure S2 and Figure 2 (main text). Significance ($p < 0.05$) was assessed using type II Wald chi-square tests and is indicated in bold.

	Temperature (df=1)		Feeding Status (df=1)		Time (df=3)		Mass (df=1)		Temperature: Treatment (df=1)		Temperature: Time (df=3)		Treatment: Time (df=3)		3-way Interaction (df = 3)	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Length	0.5	0.47	0.1	0.81	327. 6	<0.01			2.9	0.09	1.2	0.76	291.2	<0.01	2.9	0.42
Body Mass	0.4	0.54	1.4	0.24	545. 3	<0.01			1.6	0.21	4.9	0.18	2005. 5	<0.01	12.9	0.01
Body Condition	0.02	0.89	8.8	<0.01	44.6	<0.01			1.2	0.27	6.7	0.08	229.6	<0.01	1.3	0.74
Average Activity	5.1	0.02	13.3	<0.01	23.3	<0.01			4.3	0.04	41.4	<0.01	3.1	0.37	5.8	0.12
Median Activity (LMM)	11.0	<0.01	1.5	0.22	0.6	0.90			1.0	0.32	2.4	0.50	1.5	0.69	2.8	0.43
$\dot{M}O_{2\text{standard}}$	132.1	<0.01	49.4	<0.01	100. 1	<0.01	123.9	<0.01	0.01	0.91	149	<0.01	51.6	<0.01	7.7	0.05
$\dot{M}O_{2\text{average}}$	95.1	<0.01	29.2	<0.01	31.0	<0.01	67.9	<0.01	0.3	0.56	114.4	<0.01	25.5	<0.01	2.9	0.42

Table S3. Summary of statistical outputs obtained from generalized linear models (GLM; family=Gamma, link=log) examining the effects of temperature (2°C or 8°C), prolonged starvation (90 days) and their interactions on energetic and morphological variables of adult brook char (Experiment 2). These analyses correspond with the data shown in Figures 4, 6, 7 and 8 in the main text. Significance ($p < 0.05$) was assessed using type II Wald chi-square tests and is indicated in bold.

	Temperature (df=1)		Feeding Status (df=1)		Lean Mass or Mass (df=1)		Temperature: Treatment (df=1)	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Stomach Mass	4.41	0.036	2.43	0.119	101.63	<0.001	0.05	0.819
Pylorus Mass	14.18	<0.001	131.79	<0.001	69.47	<0.001	0.145	0.704
Intestine Mass	19.52	<0.001	103.74	<0.001	145.45	<0.001	15.12	<0.001
Relative Gut Mass	19.22	<0.001	127.29	<0.001	136.46	<0.001	0.05	0.816
Liver TG Content	3.08	0.079	0.55	0.458			0.06	0.799
Total Relative Liver TG	8.28	0.004	4.79	0.029	1.77	0.183	0.01	0.936
White Muscle TG Content	2.44	0.118	46.23	<0.001			1.03	0.209
Total Relative Carcass Protein	2.63	0.105	1.59	0.208	21.12	<0.001	0.04	0.833
Liver K_s	10.77	0.001	0.04	0.846			0.29	0.593
White Muscle K_s	4.23	0.040	25.36	<0.001			0.16	0.685

TG, Triglyceride

K_s , Protein synthesis rate

Table S4. Summary of statistical outputs obtained from linear models (LM) examining the effects of temperature (2°C or 8°C), prolonged starvation (90 days) and their interactions on energetic and morphological variables of adult brook char (Experiment 2). These analyses correspond with the data shown in Figures 3, 4, 6, 7 and 8 in the main text. Significance ($p < 0.05$) was assessed using type II Wald chi-square tests and is indicated in bold.

	Temperature			Feeding Status			Lean Mass or log(Mass)			Temperature × Feeding Status		
	F	df	p	F	df	p	F	df	p	F	df	p
Ventricle Mass	9.29	1,45	0.004	4.53	1,45	0.039	155.33	1,45	<0.001	0.49	1,45	0.486
Liver Mass	20.49	1,45	<0.001	39.12	1,45	<0.001	95.59	1,45	<0.001	2.36	1,45	0.132
Intestine Length	0.86	1,45	0.360	45.34	1,45	<0.001	26.53	1,45	<0.001	1.76	1,45	0.192
Gut:Heart Ratio	2.49	1,45	0.122	87.86	1,45	<0.001	2.42	1,45	0.127	0.79	1,45	0.379
Spleen Mass	4.19	1,45	0.047	5.75	1,45	0.021	47.78	1,45	<0.001	0.72	1,45	0.382
Body Fat	0.03	1,46	0.868	48.41	1,46	<0.001				0.11	1,46	0.737
Moisture Content	0.35	1,46	0.555	104.09	1,46	<0.001				0.15	1,46	0.704
Energy Density	2.27	1,46	0.139	43.99	1,46	<0.001				2.28	1,46	0.138
Gut TG Content	0.788	1,46	0.379	62.21	1,46	<0.001				0.309	1,46	0.581
Total Relative Gut TG Content	2.77	1,45	0.103	117.13	1,45	<0.001	1.14	1,45	0.292	4.87	1,45	0.032
Liver Protein Content	3.83	1,45	0.057	0.01	1,45	0.925				1.63	1,45	0.209
Total Relative Liver Protein	37.75	1,44	<0.001	43.40	1,44	<0.001	1.19	1,44	0.281	1.46	1,44	0.234
Gut Protein Content	9.46	1,45	0.004	29.24	1,45	<0.001				7.03	1,45	0.011
Total Relative Gut Protein	0.61	1,44	0.438	0.86	1,44	0.358	6.45	1,44	0.015	6.91	1,44	0.012
White Muscle Protein Content	0.001	1,46	0.975	7.36	1,46	0.009				0.091	1,46	0.764
$\dot{M}O_{2\text{standard}}$	19.37	1,43	<0.001	107.69	1,43	<0.001	75.26	1,43	<0.001	1.57	1,43	0.218
$\dot{M}O_{2\text{max}}$	14.71	1,43	<0.001	0.09	1,43	0.764	46.22	1,43	<0.001	1.81	1,43	0.186
Absolute Aerobic Scope	12.18	1,43	0.001	0.25	1,43	0.620	36.69	1,43	<0.001	1.60	1,43	0.212
Gut K_s	4.23	1,44	0.046	35.38	1,44	<0.001				0.26	1,44	0.613
Ventricle K_s	51.96	1,44	<0.001	9.22	1,44	0.004				3.05	1,44	0.088
Glucose	1.39	1,46	0.245	10.19	1,46	0.003				0.01	1,46	0.930
Hematocrit	1.23	1,45	0.273	5.92	1,45	0.019				0.102	1,45	0.751
Plasma Triglyceride	0.74	1,36	0.397	6.63	1,36	0.014				3.60	1,36	0.066

TG, Triglyceride

$\dot{M}O_{2\text{standard}}$, Standard oxygen consumption

$\dot{M}O_{2\text{max}}$, Maximum oxygen consumption

K_s , Protein synthesis rate