Aerobic scope is not maintained at low temperature and is associated with cardiac aerobic capacity in the threespine stickleback *Gasterosteus aculeatus*

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ABSTRACT

Metabolic thermal plasticity is central to the survival of fishes in a changing environment. The eurythermal threespine stickleback *Gasterosteus aculeatus* displays thermal plasticity at the cellular level with an increase in the activity of key metabolic enzymes in response to cold acclimation. However, it is unknown if these changes are sufficient to completely compensate for the depressive effects of cold temperature on whole organismal metabolic rate ($\dot{M}O_2$). We hypothesized that as a cold tolerant, eurythermal fish, absolute aerobic scope (AAS), the difference between maximum metabolic rate (MMR) and standard metabolic rate (SMR), would be maintained in *G. aculeatus* following acclimation to a range of temperatures that span its habitat temperatures. To test this hypothesis, *G. aculeatus* were acclimated to 5, 12, and 20°C for 20 – 32 weeks and SMR, MMR, and aerobic scope (AS) were quantified at each acclimation temperature. The maximal activity of citrate synthase (CS), a marker enzyme of aerobic metabolism, was also quantified in heart ventricles to determine if cardiac aerobic capacity is associated with AS at these temperatures. SMR increased with acclimation temperature and was significantly different among all three temperature groups. MMR was similar between animals at 5 and 12°C and between animals at 12 and 20°C but was 2.6-fold lower in fish at 5°C compared with ones at 20°C, resulting in a lower AAS in fish at 5°C compared with ones at 12 and 20°C. Correlated with a higher AAS in animals acclimated to 12 and 20°C was a larger relative ventricular mass and higher CS activity per 100 g body mass compared with animals at 5°C. Together, our results indicate that despite their eurythermal nature, AS is not maintained at low temperature but is associated with cardiac aerobic metabolic capacity.

Keywords: aerobic metabolism, aerobic scope, metabolic rate, stickleback**,** temperature acclimation

1. INTRODUCTION

Many fish species experience daily and seasonal fluctuations in habitat temperature that impact all levels of biological organization and physiological processes (Gamperl & Farrell, 2004; Jensen *et al.*, 2017; Metzger & Schulte, 2018). In response to an acute increase in temperature, metabolic rate ($\dot{M}O_2$) increases due to the thermodynamic effect of temperature on the catalytic rate of enzymes, and conversely, as temperature decreases, $\dot{M}O_2$ declines. However, many fish species can reversibly modify their phenotype in response to a change in environmental conditions over a period of weeks or months through the process of acclimation. The changes in gene expression and/or the activities of metabolic enzymes that occur during acclimation may result in a decrease (in response to warming) or increase (in response to cooling) in $\dot{M}O_2$ so that thermal performance improves (Guderley, 1990; Johnston & Dunn, 1987). The Q_{10} temperature coefficient describes the thermal sensitivity of a biochemical reaction and can be used to assess the extent of thermal compensation. With an acute increase in temperature, the $\dot{M}O_2$ of fishes typically increases with a Q_{10} between two and three but with thermal acclimation, the Q_{10} typically decreases (Havird *et al.*, 2020) and may reach a value of one if there is complete thermal compensation (Farrell, 2016). A review of standard metabolic rate (SMR) in teleosts found that in response to temperature acclimation, Q_{10}s within a species range between 0.45 and 3.41 with a median value of 2.4 (Clarke & Johnston, 1999). Overall, eurythermal fishes display a greater degree of thermal plasticity in $\dot{M}O_2$ and aerobic scope (AS) compared with stenothermal species and are therefore considered more resilient to climate warming (Healy & Schulte, 2012).

For several fish species, the extent of thermal compensation is greater in SMR than maximum metabolic rate (MMR), resulting in a decline in absolute aerobic scope (AAS; the difference between MMR and SMR) at temperatures outside of the temperature optimum (T_{opt}), which may negatively impact fitness (Ekström *et al.*, 2016; Steinhausen *et al.*, 2008). Because the heart drives convective oxygen delivery, cardiac performance is intrinsically linked to AS, which declines at temperatures above and below T_{opt} (Farrell *et al.*, 2009; Steinhausen *et al.*, 2008). The ability to maintain cardiac rhythmicity and AS across a range of temperatures is likely dependent on the integration of several biochemical and physiological traits, including membrane integrity (Biederman *et al.*, 2021), ion channel function (Haverinen & Vornanen, 2020), and mitochondrial function (Iftikar *et al.*, 2014). Heart rate and cardiac output are often used as metrics of cardiac performance to assess the ability of fishes to maintain AS (Gilbert & Farrell, 2021; Sandblom *et al.*, 2016, 2014), but because aerobic metabolism is essential for maintaining ATP levels and fueling the work of the heart, the activity of marker enzymes of aerobic metabolism such as citrate synthase (CS), may be another useful proxy for cardiac performance and AS.

The thermal plasticity of cardiac metabolism varies among different fish species. An increase in relative ventricular mass (RVM), mitochondrial density, and the maximal activity of aerobically poised enzymes are typical acclimatory responses to cold temperature that increase cardiac performance in the cold, yet there are several exceptions likely attributable to differences in winter activity level (Guderley, 1990). For example, in the cold active striped bass *Morone saxatilis* (Walbaum 1792), RVM is higher in 5°C cold-acclimated fish compared with ones at 25°C, but cardiac mitochondrial density and citrate synthase (CS) activity do not change in response to cold acclimation (Rodnick & Sidell, 1997). The Atlantic killifish *Fundulus heteroclitus* (Linneaus 1766) displays a remarkable tolerance for extreme environmental conditions but is inactive in the winter. In this species, RVM and CS activity remain unchanged with thermal acclimation and mitochondrial function is lower in hearts of 5°C acclimated fish compared with fish at 15°C (Chung *et al.*, 2017). In contrast, hearts of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) display a canonical cold acclimatory response with increases in RVM and cardiac CS activity (Patey & Driedzic, 1997). Assessing the thermal plasticity of cardiac aerobic metabolism may provide insight to cardiac performance, AS, and organismal thermal tolerance.

The threespine stickleback *Gasterosteus aculeatus* (Linnaeus 1758), is a convenient model organism for studying thermal plasticity in AS and cardiac metabolism, as this small teleost is widely distributed across the Northern Hemisphere in thermally variable marine, brackish, and freshwater environments (Wootton, 1984). Several studies have characterized increases in the maximal rate of metabolic enzymes in response to temperature acclimation in several tissues of *G. aculeatus*, including liver, oxidative, and glycolytic muscles (Guderley *et al.*, 2001; Orczewska *et al.*, 2010; Vézina & Guderley, 1991), suggesting there is likely thermal plasticity in $\dot{M}O_2$ as well, although this has not been investigated, nor has the thermal plasticity of cardiac metabolism in *G. aculeatus*, which is central to thermal tolerance (Ekström *et al.*, 2017; Farrell *et al.*, 2009). In the nine-spined stickleback, *Pungitius pungitius* (Linnaeus 1758), the degree of thermal compensation in AS varies among freshwater and marine populations with the greatest degree of thermal compensation in AS occurring in fishes inhabiting cold, freshwater (Bruneaux *et al.*, 2014). We sought to determine if AS is maintained in *G. aculeatus* following acclimation to temperatures that span the temperature range of their natural habitat (5, 12, and 20°C) and whether cardiac CS activity is associated with AS. Since *G. aculeatus* is a highly eurythermal species that displays robust thermal plasticity at the cellular level, we hypothesized that AS would display a high degree of thermal compensation in an Alaskan freshwater population of *G. aculeatus* and that the activity of cardiac CS per g fish would be associated with AS.

2. METHODS

2.1 *Gasterosteus aculeatus* capture and acclimation

Gasterosteus aculeatus were collected in September 2019 from Kashwitna Lake, Alaska (61.835°N, 150.079°W) using minnow traps. The water temperature was 17°C at the time of collection. Although the annual temperature range for Kashwitna Lake is unknown, the temperature of other nearby lakes in central Alaska ranges between 4°C and 21°C (Alaska Department of Environmental Conservation, 2004). Animals (n=184) were transported to the University of Alaska Fairbanks (UAF) where 18 to 22 animals were maintained in each of 20- or 29- gal recirculating tanks, respectively (9 tanks total). Tanks were filled with deionized water supplemented with 0.35 ppt Instant Ocean at 12ºC for 22 weeks. Animals in a subset of tanks (6) were then acclimated to 5 or 20ºC by changing the temperature at a rate of 3 – 4ºC per day. For acclimation to 20ºC, the water was heated with submersible heaters and then tanks were moved into a 20ºC room to maintain tank temperature. For acclimation to 5 ºC, animals from 3 tanks were transferred into a single 60-gal insulated tank equipped with an inline chiller (Aqua Logic Delta Star, San Diego, CA) and water temperature was decreased as described above. All animals were held on a 10 h light, 14 h dark cycle and fed blood worms twice daily.

Respirometry trials were conducted after animals were acclimated for 20 – 25 weeks (42 – 47 weeks after capture) for 5°C animals and 21 – 32 weeks (43 – 54 weeks after capture) for 20°C animals. Respirometry trials began 44 – 60 weeks after capture for the 12°C animals. Animals were euthanized for enzyme assays after 12 weeks of acclimation to 5 and 20°C and after 34 weeks at 12°C by immersion in liquid nitrogen and then stored at -80ºC until use. All procedures were approved by the UAF Institutional Animal Care Committee (1253621-22).

Fulton's condition factor was calculated as:

$$K = bm/{L_s}^3 \times 100$$

where bm = wet body mass of the fish (to the nearest 0.1 g) and L_s = standard length (to the nearest 0.1 cm).

Relative ventricular mass (RVM) was calculated for the animals used in the CS assays as:

$$RVM = hm/bm \times 100$$

where hm = heart mass (to the nearest 0.1 mg).

2.2 Respirometry

$\dot{M}O_2$ was measured using a 170 mL glass Loligo swim tunnel respirometer (Loligo Systems, Viborg, Denmark) with toxic-free polyvinyl chloride tubing (chamber volume = 184 mL, tube volume = 1.24 mL). The ratio of body mass to total respirometer volume (chamber volume + tube volume) was an average of 1:103 with only one animal smaller than the recommended 1:50 to 1:150 range for swimming respirometers (Svendsen *et al.*, 2016). The respirometer was shrouded in black plastic to minimize disturbance and placed in an environmental chamber held at the acclimation temperature of the fish. Water temperature, measured once per second, was maintained at the acclimation temperature by circulating water through a stainless-steel chilling coil submerged in a cooler filled with ice water. Dissolved oxygen level (DO) was measured once per second with a dipping probe mini oxygen sensor connected to Witrox 1 oxygen meter (Loligo Systems, Viborg, Denmark) that was placed within the inner chamber of the respirometer. For each fish, the oxygen sensor was calibrated at the acclimation temperature to 100% air saturation using vigorously aerated water in the respirometer and to 0% air saturation using 2% sodium sulfite. The respirometer was drained and rinsed with deionized water at the conclusion of each experiment and sanitized with a dilute bleach solution after every fourth trial to minimize background microbial respiration rates.

A pilot study determined that SMR was equivalent between *G. aculeatus* fasted for 36 or 60 h regardless of acclimation temperature (not reported), so animals were fasted for 36 h. Standard length (cm) and body mass (g) were measured and then animals were placed into the respirometer and allowed to recover from the stress of handling for 1 h. Water velocity was increased up to a maximum of 50 cm s^{-1} to habituate the fish to swimming in the tunnel and to estimate maximum swimming velocity (V_{max}). The velocity was then reduced to 0.6 ± 0.1 cm s^{-1} (mean ± sd for all trials) for the remainder of the trial to circulate water while allowing the fish to maintain its position without swimming. After at least 6 h, DO was measured overnight for 10 h to quantify SMR using intermittent flow respirometry with the following settings: flush for 7 min, wait for 1 min, and measure for 28, 15, or 12 min at 5, 12, and 20ºC, respectively.

The following day, MMR was measured by increasing velocity by 10% of the fish's V_{max} every 2 min until 40% V_{max} was attained. Thereafter, $\dot{M}O_2$ was measured using intermittent respirometry with the following settings: flush for 2 min, wait for 1 min, and measure for 5 or 7 min. If the fish was not swimming at the onset of the wait or measurement period, it was induced to swim by decreasing the velocity to 0 cm s^{-1} and then immediately increasing it to the target velocity for that cycle. Oxygen consumption was measured at each velocity for two cycles before increasing the velocity by 10% V_{max} until the fish could no longer right itself or was repeatedly unable or unwilling to burst swim. If the fish completed two cycles at V_{max} and was still not fatigued, the fish was induced to swim every time it stopped swimming until it was fatigued for one additional cycle. If the fish became fatigued during a measurement period, the velocity was decreased to 0.6 ± 0.1 cm s^{-1} (mean ± sd for all trials) for the remainder of the period plus one additional cycle following fatigue. MMR trials lasted for 143 ± 50 min (mean ± sd) for each animal. DO was maintained above 71% saturation for all trials (only 0.3% of cycles dropped below 80% saturation) and always returned to 100% saturation during the flush period.

Background microbial respiration rates were measured once or twice before and after each trial with the following settings: flush for 5 min, wait for 1 min, and measure for 30 min. Replicates (when available) were averaged and background respiration rates interpolated using a linear correction (package: FishResp, function: correct.meas, method = "linear"). Regions of the data (or an entire measurement period if necessary) for which DO levels increased were removed prior to calculating background respiration rates. For some trials, DO levels always increased and for these trials, background rates of respiration were set to zero. Background respiration rates were always less than 7% of the SMR estimate.

The slope of a linear model fit to the background corrected DO values for each measurement period (package: FishResp, function: extract.slope, method = “all”) was used to estimate metabolic rate (package: FishResp, function: calculate.MR). All SMR and MMR estimates accounted for the body mass, density, and volume of the fish, as well as total volume of the respirometer using the following equation:

$$mass\dot{M}O_2 = [(\Delta[O_2]/\Delta t) \times (V - D \times bm)] \times bm^{-1}$$

where $\Delta[O_2]/\Delta t$ = slope (mg O_2 L^{-1} h^{-1}), V = total volume of respirometer (L), D = density of animal (1 kg L^{-1} used for all animals), and bm = body mass (kg) (Morozov *et al.*, 2019). Absolute aerobic scope (AAS) was calculated as MMR - SMR. Factorial aerobic scope (FAS) was calculated as MMR SMR^{-1}.

Between 16 and 30 estimates of SMR were obtained for each animal. To prevent underestimating SMR by erroneously removing cycles with low r^2 values, raw DO values were first smoothed to improve r^2, as suggested in (Chabot *et al.*, 2021). Briefly, raw DO values were subdivided into bins that were 5% the length of the measurement cycle (i.e., 36, 45, or 84 s for 5, 12, or 20°C, respectively), producing 20 bins per cycle. The median value was retained for each bin and a linear model was fit to the 20 median DO values and used to estimate SMR for each cycle. Cycles were omitted if $r^2 < 0.95$. Smoothing the data significantly increased the minimum and mean r^2 values ($P < 0.001$) and reduced the percent of cycles removed overall from 18.6% to 1.8%.

The final SMR estimate for each animal was calculated using the mean of the lowest normal distribution (MLND) and the 20% quantile methods described in Chabot *et al*. (2016b) and Reemeyer and Rees (2019). Both methods estimate SMR based on the slope of the best fit line for the background-corrected data, but the MLND method is recommended when the coefficient of variation of MLND (CV_{MLND}) ≥ 5.4, whereas the 20% quantile (in which SMR is placed above the lowest 20% of values) is used when $CV_{MLND} \leq 5.4$ (Chabot *et al.*, 2016b; Reemeyer & Rees, 2019).

Traditionally, MMR is estimated from the slope of a line fit to all measured DO values (Rummer *et al.*, 2016). However, slope may actually vary throughout the measurement period as the fish becomes fatigued and/or as a result of inconsistent swimming effort during the measurement period (Zhang *et al.*, 2019). Zhang *et al*., (2019) proposed using an iterative algorithm to account for changes in $\dot{M}O_2$ during the measurement period, which we attempted to use; however, the CV and sd were high and equivalent for the 17 sampling windows examined between 0.5 and 5 min. Therefore, we calculated MMR by fitting DO to a linear model to estimate the slope of the line and the entire (5 or 7 min) measurement period yielding the highest estimate was designated as MMR (hereafter referred to as “traditional MMR” Norin & Clark, 2016; Rummer *et al.*, 2016). We also employed a second approach (hereafter referred to as “truncated MMR”), in which each measurement period was edited to retain only the steepest declines in DO as long as there was a minimum of 100 s (33% of length of the shortest measurement period) of data remaining (range = 100 – 420 s, mean ± sd = 261 ± 106 s) and processed as described above and as described by Rummer *et al*. (2016) for measuring MMR_{Chase} and MMR_{Circle}.

2.3 Enzymology

Tissues were homogenized on ice (10% w/v) in 40 mM HEPES, 1 mM EDTA, and 2 mM $MgCl_2$, pH of 7.8 at 5°C with a Tenbroek ground glass homogenizer. The maximal activity of citrate synthase (CS; EC 2.3.3.1) was measured at 20 ± 0.5°C using a modified protocol described by Srere *et al*. (1963). The final reaction mixture contained 0.25 mmol l^{-1} 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 0.40 mmol l^{-1} acetyl coenzyme A (CoA), 0.5 mmol l^{-1} oxaloacetate, 75 mmol l^{-1} Tris-HCl, pH 8.2. Background activity was measured for 5 min in the absence of the initiating substrate oxaloacetate. The progress of the reaction was monitored by following the reduction of DTNB at 412 nm for 5 min following the addition of oxaloacetate using a SpectraMax Plus384 plate reader (Molecular Devices, Sunnyvale, CA) at 20°C. All measurements (n = 5) were made in triplicate and activity was expressed as µmol product min^{-1} g^{-1} wet mass. Homogenates from hearts of the 5 and 12°C temperature groups were then each pooled to obtain a sufficient amount of material and CS activity was measured at the acclimation temperature using a Perkin Elmer Lambda 40 spectrophotometer (Perkin-Elmer, Waltham, MA, USA) equipped with a refrigerated, circulating water bath. The Q_{10} was calculated for CS activity between 20 and 12°C (for the 12°C animals) and between 20 and 5°C (for the 5°C animals) using the average activity of CS measured at 20°C and CS activity of the pooled sample measured at the acclimation temperature. The Q_{10} was then applied to CS activity measurements made at 20°C in 5 and 12°C acclimated individuals to calculate CS activity for each individual at their acclimation temperature for animals held at 12 and 5°C.

2.4 Statistical analyses

Respirometry data were collected using AutoResp version 2.3.0 (Loligo Systems, Viborg, Denmark) and processed using RStudio version 1.3.1093 (R Studio Team, 2016) operating with R version 4.0.3 (R Core Team, 2018). Code was modified from the "FishResp" package version 1.0.3 (Morozov *et al.*, 2019) to import respirometry data into R and correct raw data for background $\dot{M}O_2$. Statistical analyses were conducted using Prism v.8 (Graphpad Software Inc., San Diego, CA) and R version 4.0.3 (R Core Team, 2018). Graphs were produced using Prism v.8.

All datasets were analyzed for outliers using the Robust Regression and Outlier Removal method (ROUT), for normality with the Shapiro-Wilk test, and for equal variances with the Brown-Forsythe test. One SMR estimate was identified as an outlier and was removed from all analyses (along with the associated AAS and FAS estimates for that fish). Final sample sizes for analyses of SMR (including potential correlations between SMR and acclimation time), AAS, and FAS were n = 6, 6, and 5 for 5, 12, and 20°C, respectively, whereas n = 6 was used for all temperature groups for measurements of physical characteristics and MMR. No outliers were identified for the animals used for the CS assays, so n = 5 for all temperature groups for analyses regarding physical characteristics, CS activity, and RVM.

Respirometry trials were completed over a span of 17 weeks. A simple linear regression was used to determine if SMR or MMR varied over the time span of the experiments. Significant differences among animals acclimated to different temperatures were determined using an ANOVA followed by a Tukey's Honest Significant Difference test when data were normally distributed. For data that were not normally distributed, a Kruskal-Wallis test followed by a

Dunn's multiple comparisons test was used. An unpaired t-test or a Mann-Whitney test (for data that were normally or not normally distributed, respectively) was conducted to determine if there were differences in physical characteristics between animals used for respirometry trials versus those used for the CS assays within each temperature group. A paired t-test test was used to determine if MMR estimates differed when calculated using the traditional or truncated method (sin-transformed data were used for the 5°C acclimation group to satisfy normality requirements). An analysis of covariance (ANCOVA) followed by a Tukey's Honest Significant Difference test was used to identify significant differences in CS activity among the acclimation groups with body mass as a covariate. Significance was accepted when $P \leq 0.05$.

Q_{10} was calculated as:

$$Q_{10} = (R_2/R_1)^{10/(T_2 - T_1)}$$

where R_1 and R_2 are the mean measurements from all animals at temperature T_1 or T_2, respectively.

3. RESULTS

3.1 Physical characteristics of *G. aculeatus*

Regardless of acclimation temperature, *G. aculeatus* used for measuring AS were similar in length (ANOVA: $F = 0.24$; $df = 2, 15$; $P = 0.79$) and mass (ANOVA: $F = 2.51$; $df = 2, 15$; $P = 0.11$) among all temperature groups, but the condition factor was 1.2-fold higher in animals at 5°C compared with ones at 20°C (Kruskal-Wallis: $H = 9.24$, $df = 3, 18$, $P < 0.01$; Table 1).

Among temperature groups, *G. aculeatus* used for measuring the maximal activity of CS were similar in length (ANOVA: $F = 0.92$; $df = 2, 12$; $P = 0.42$), but not body mass (ANOVA: $F = 7.00$; $df = 2, 12$; $P < 0.05$) (Table 1). Body mass of the 5°C animals was 1.4-fold larger than animals at 12 and 20°C (Table 1). Furthermore, RVM was 1.7- or 1.8-fold larger in animals at 12 and 20°C, respectively, compared with animals at 5°C (ANOVA: $F = 11.05$; $df = 2, 12$; $P < 0.01$; Table 1).

Although *G. aculeatus* used for measuring AS and CS activity were maintained in the same tanks, the condition factor was 1.3- to 1.4-fold higher for animals used to measure CS at 5°C (unpaired t-test: $t = 5.50$; $df = 9$; $P < 0.001$), 12°C (Mann-Whitney: $U = 3$; $P < 0.05$), and 20°C (unpaired t-test: $t = 3.90$; $df = 9$; $P < 0.01$), and body mass was 1.5-fold higher in the 12°C animals used to measure CS compared with ones used to measure AS (unpaired t-test: $t = 4.94$; $df = 9$; $P < 0.05$) (Table 1).

3.2 Effect of temperature acclimation on aerobic scope

SMR differed among *G. aculeatus* in all temperature groups (ANOVA: $F = 79.64$; $df = 2, 14$; $P < 0.001$) and was highest in animals at 20°C and lowest in animals at 5°C (Figure 1a). Compared with the 5°C animals, SMR was 1.7- and 2.4-fold higher for animals at 12 and 20°C, respectively.

The MMR estimates for *G. aculeatus* were similar for both the traditional and truncated methods for the 5°C (paired t-test: $t = 0.01$; $df = 5$; $P = 0.99$), 12°C (paired t-test: $t = 2.07$; $df = 5$; $P = 0.09$), and 20°C animals (paired t-test: $t = 2.01$; $df = 5$; $P = 0.10$), but increased with acclimation temperature (Supplemental Figure 1). MMR estimates differed among all three temperature groups when calculated using the truncated method (ANOVA: $F = 1.00$; $df = 2, 15$; $P < 0.001$; Supplemental Figure 2a). When using the traditional method, MMR was 2.6-fold higher for animals at 20°C compared with animals at 5°C but similar between 5 and 12°C animals and between 12 and 20°C animals (Kruskal-Wallis: $H = 12.57$; $df = 3, 18$; $P < 0.001$; Figure 1b). Since only 8 of the 18 fish used in our study required the DO data to be truncated, we only describe results of AS obtained using the traditional method below, whereas estimates obtained with the truncated method are shown in Supplemental Figure 2b–c.

AAS was 2.0-fold higher in *G. aculeatus* at 12°C compared with fish at 5°C, 2.5-fold higher in animals at 20°C compared with fish at 5°C, and equivalent between animals at 12 and 20°C (Kruskal-Wallis: $H = 11.14$; $df = 3,17$; $P < 0.001$; Figure 2a). FAS was similar among fish in all temperature groups (ANOVA: $F = 0.55$; $df = 2,14$; $P = 0.59$; Figure 2b).

Over the duration of the experiments, there was no variation in SMR for the 5°C (linear regression: $F = 0.18$; $df = 1, 4$; $P = 0.69$; $r^2 = 0.04$), 12°C (linear regression: $F = 6.32$; $df = 1, 4$; $P = 0.06$; $r^2 = 0.61$), or 20°C animals (linear regression: $F = 4.18$; $df = 1, 3$; $P = 0.13$; $r^2 = 0.58$) (Supplemental Figure 3). There was also no variation in MMR for the 5°C (linear regression: $F = 0.10$; $df = 1, 4$; $P = 0.76$; $r^2 = 0.02$), 12°C (linear regression: $F = 3.75$; $df = 1, 4$; $P = 0.12$; $r^2 = 0.48$), or 20°C animals (linear regression: $F = 0.36$; $df = 1, 4$; $P = 0.58$; $r^2 = 0.08$) over the duration of the experiment (Supplemental Figure 3).

3.3. Aerobic metabolic capacity of heart ventricles

There was a significant effect of acclimation temperature on CS activity after controlling for the effect of the covariate, body mass (ANCOVA: $F = 6.45$; $df = 2, 11$; $P < 0.05$). CS activity was 1.4-fold higher in animals acclimated to 5°C compared with fish at 12°C, 2.0-fold higher in animals at 5°C compared with animals at 20°C, and equivalent between fish at 12 and 20°C when measured at a common temperature of 20°C (Figure 3a). When measured at the acclimation temperature, CS activity also differed among the acclimation groups after accounting for the effect of the covariate, body mass (ANCOVA: $F = 31.21$, $df = 2, 11$; $P < 0.001$). CS activity was lowest in hearts of animals acclimated to 5°C, highest in animals at 20°C, and intermediate in animals at 12°C (Figure 3b). The RVM of *G. aculeatus* was impacted by acclimation temperature and was significantly higher in animals acclimated to 12°C and 20°C compared with ones at 5°C (Table 1). When accounting for these differences in RVM, the maximal activity of CS activity per 100 g body was 5.1- or 1.6-fold higher for fish at 20°C compared with fish at 5 or 12°C, respectively (ANOVA: $F = 11.05$; $df = 2, 12$; $P < 0.01$; Figure 3c).

4. DISCUSSION

The ability of temperate teleost fishes to compensate for seasonal changes in temperature varies among physiological processes, tissues, and species (Biederman *et al.*, 2021; Fangue *et al.*, 2008; Guderley, 1990; Rodnick & Sidell, 1997). Although previous studies in *G. aculeatus* have shown that the maximal activity of metabolic enzymes increases in oxidative and glycolytic muscles and liver in response to cold acclimation (Guderley *et al.*, 2001; Orczewska *et al.*, 2010; Vézina & Guderley, 1991), our results indicate that SMR and AAS are lower in *G. aculeatus* at 5°C compared with fish at 12 and 20°C. Studies have shown that AAS is linked to cardiac performance in fishes (Farrell, 2016; Farrell *et al.*, 2009), and consistent with this, we find that in general, the activity of CS in heart ventricle tissue per 100 g fish, indicative of cardiac aerobic metabolic capacity, increases with acclimation temperature along with MMR.

4.1 Metabolic rate is not maintained across habitat temperatures with acclimation

Standard metabolic rate represents the minimum energy required to support circulation, respiration, excretion, and muscle tone in an animal at rest in a post-absorptive state and is influenced by rates of protein turnover and ion leakage (Chabot *et al.*, 2016a; Clarke, 2016). SMR increased with acclimation temperature in *G. aculeatus*, although the thermal sensitivity was low (Q_{10} = 1.6 – 2.1; Table 2), suggesting an active acclimatory response, although we acknowledge that our study design precludes the ability to differentiate between passive and active acclimatory processes (Havird *et al.*, 2020). There is, however, ample evidence that active acclimation occurs in metabolism at the cellular level in *G. aculeatus*. A previous study using the same population of *G. aculeatus* found that mitochondrial density is 1.9-fold higher and CS activity is 1.7-fold higher in the oxidative muscle of *G. aculeatus* at 8°C compared with fish at 20°C, and in liver, CS activity is 2.0-fold higher in *G. aculeatus* at 8°C compared with fish at 20°C (Orczewska *et al.*, 2010). Similarly, CS activity increases in glycolytic muscle of anadromous *G. aculeatus* in response to cold acclimation (Guderley *et al.*, 2001). An increase in mitochondrial density, in addition to increasing ATP production, also enhances lipid metabolism and/or oxygen diffusion rates at cold temperature (Guderley, 1990). Standard metabolic rate, however, is likely set by rates of mitochondrial proton leak, ion leakage across membranes and protein turnover (Clarke, 2016) with mitochondrial density and aerobic metabolic capacity reflecting the energetic demands of these processes. Indeed, SMR is closely associated with proton leak in liver mitochondria in the brown trout *Salmo trutta* (Linnnaeus,1758) (Salin *et al.*, 2016). Thus, changes in the quality, in addition to the quantity, of mitochondria contribute to thermal adjustments of SMR. Additionally, temperature-induced changes in SMR are influenced by seasonal changes in reproduction, growth and food availability (Clarke, 2016). Nevertheless, it remains unclear what limits the extent of metabolic remodeling in SMR, especially in species such as *G. aculeatus* that regulate gene expression in response to temperature (Metzger & Schulte, 2018; Orczewska *et al.*, 2010) unlike some stenothermal fish species, which have diminished molecular responses to temperature as a result of genetic lesions arising during evolution in a stable environment (Bilyk *et al.*, 2018). Previous studies have shown that acclimation may be impaired by a low condition factor (Vézina & Guderley, 1991), but this was not the case in our study, as the 5°C acclimated *G. aculeatus* displayed the highest condition factor. Overall, the temperature specificity of metabolic regulation is intriguing and discovering the molecular mechanisms underlying the fine tuning of metabolism warrants further investigation.

MMR was significantly lower in animals at 5°C compared with ones at 20°C and when analyzed using the truncated method, MMR was significantly different among all acclimation groups and increased with acclimation temperature (Supplementary Figure 2a). MMR is influenced by multiple components of the oxygen cascade including gill ventilation, oxygen transport by hemoglobin, and cardiac performance (Farrell *et al.*, 2009), any one (or more) of which may limit thermal compensation in MMR. Our data suggest that cardiac aerobic metabolic capacity may contribute the limits of thermal plasticity in MMR.

The heart is considered a central mediator of MMR and AS because of its essential role in oxygen delivery (Farrell *et al.*, 2009; Gamperl & Farrell, 2004). Previous studies have shown that temperature acclimation results in remodeling of cardiac mitochondria, as well as changes in the activity of aerobic metabolic enzymes, including CS, which enhance cardiac performance in some fish species (Pichaud *et al.*, 2017). For example, the activity of cardiac CS is positively correlated with heart rate scope in *O. mykiss* and the European perch *Perca fluviatilis* (Linnaeus 1758) in response to an increase in temperature, although the time course for remodeling differs, with changes in metabolism occurring more quickly than cardiac function (Ekström *et al.*, 2016; Pichaud *et al.*, 2017; Sandblom *et al.*, 2016). In our study, aerobic metabolic capacity, as assessed by measurements of maximal activities of CS per g tissue, increases in response to warm acclimation along with RVM so that CS activity per 100 g fish is significantly higher in *G. aculeatus* at 20°C compared with fish at 12 and 5°C, and higher in fish at 12 than at 5°C. The increase in cardiac aerobic metabolic capacity with thermal acclimation parallels that of MMR, suggesting that cardiac CS activity may be a good indicator of MMR, although our results would be strengthened by measuring CS activity in the same individuals as those used to measure MMR. We also acknowledge that cardiac CS activity parallels changes in SMR in response to acclimation, but aerobic metabolic activity of the liver, and especially mitochondrial leak, seem to be more important in defining SMR in fishes than aerobic metabolic activity of the heart (Norin & Malte, 2012; Salin *et al.*, 2016).

AAS estimates for *G. aculeatus* increased with acclimation temperature and were equivalent for animals acclimated to 12 and 20°C, but for animals acclimated to 5°C, AAS was 2.0- and 2.5-

fold lower than at 12 and 20°C, respectively. Similar to our study, Bruneaux *et al*. (2014) found that SMR and MMR declined with a decrease in acclimation temperature in *P. pungitius* acclimated to 6 or 11°C with the exception of a cold pond population that experienced little seasonal temperature variation, with temperatures ranging between 2.4 to 13.7°C. We anticipated that the cold freshwater population of *G. aculeatus* we used would also maintain AS at low temperature. However, maintaining AAS at 5°C may be unnecessary for the *G. aculeatus* used in this study, which overwinter under the ice and in near complete darkness. Although several fish species become less active and suppress their metabolic rate during winter (Shuter *et al.*, 2012), AAS was reduced in the 5°C acclimated *G. aculeatus* in this study without any of the cues that might elicit metabolic suppression in the wild, such as short daylength, winter hypoxia, or reduced food availability (Shuter *et al.*, 2012).

4.2 A modified method for measuring MMR

Methods to elicit fatigue and measure MMR in fishes vary greatly among studies (Killen *et al.*, 2017; Norin & Clark, 2016; Zhang *et al.*, 2020). One common approach is to manually chase the fish in a bucket or circular "track" until it is unresponsive to touch (Norin & Malte, 2012; Rummer *et al.*, 2016); however, this "chase" protocol failed to consistently induce swimming in the *G. aculeatus* used in this study, and the fish often did not respond to touch at all (personal observations). Another approach for eliciting MMR is to calculate the critical swimming speed (U_{crit}), begin swimming the fish at a fraction of U_{crit}, and then continue ramping the water velocity until the fish is exhausted (Rummer *et al.*, 2016; Zhang *et al.*, 2020), but not all fish swim well in a swim tunnel (Peake & Farrell, 2006). In our experience, some animals actively swam against the water current as the velocity was incrementally increased, while others did not attempt to swim, even at low velocities when the animal was capable of swimming against the current. To induce *O. mykiss* to swim, Zhang *et al*. (2020) modified a static respirometer to include a chasing device (i.e., a soft brush that the researcher could move to startle the fish). To achieve a similar effect, we used a rapid change in velocity to provoke swimming, which, to our knowledge, is a technique that has not previously been used. Although it is difficult to assess whether MMR is truly being measured using any method (Norin & Clark, 2016), our estimates for MMR are similar to those generated for stickleback in other studies (Bruneaux *et al.*, 2014; Dalziel *et al.*, 2012). For example, Dalziel *et al.* (2012) used the U_{crit} method to measure MMR in two resident stream populations of *G. aculeatus* at 15°C and obtained values of 15 and 19 µmol O_2 g^{-1} h^{-1}, which were similar to our values for 12°C acclimated *G. aculeatus* where MMR was 19 µmol O_2 g^{-1} h^{-1} when calculated using the traditional method or 21 µmol O_2 g^{-1} h^{-1} when calculated using the truncated method. Also, our values for MMR in 5 and 20°C acclimated *G. aculeatus* (10 and 27 µmol O_2 g^{-1} h^{-1}, respectively using the traditional method and 12 and 28 µmol O_2 g^{-1} h^{-1}, respectively using the truncated method) were similar to MMR measured in *P. pungitius* using the chase protocol, which ranged from 15 to ~ 30 µmol O_2 g^{-1} h^{-1} in animals acclimated to 6 and 19°C, respectively (Bruneaux *et al.*, 2014). Although these are not perfect comparisons given the differences among studies in species and locale, the data suggest that using quick changes in velocity may elicit MMR and be a useful approach for encouraging otherwise unwilling fishes to swim in a respirometer.

In summary, our study shows that despite long-term acclimation, AS is not maintained over a range of habitat temperatures in *G. aculeatus*. Factors limiting AS at the molecular level, especially in a eurythermal species such as *G. aculeatus*, remain to be elucidated. We find, however, similar to other studies in temperate fishes, that cardiac aerobic capacity is associated with AS. Measuring the maximal activity of CS in cardiac muscle may be a useful proxy for cardiac performance that can be used in addition to, or in place of, measuring heart rate to assess thermal performance in fishes. Further, the alternative approach we suggest for inducing swimming in a swim tunnel respirometer using rapid changes in water velocity may be a useful tool for researchers studying other species of reluctant swimmers.

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CONTRIBUTIONS

K.R. collected and analyzed the respirometry data. J.S. collected and analyzed data from CS assays. K.R., L.C. and K.O. prepared and edited the manuscript. K.O. conceived of the study and obtained funding for it. All authors approved of the final manuscript.

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FIGURE LEGENDS

FIGURE 1. Metabolic rate of threespine stickleback (*Gasterosteus aculeatus*) acclimated to three temperatures. Standard metabolic rate (SMR; n = 6, 6, and 5 for 5, 12, and 20°C respectively) (a) and maximum metabolic rate (MMR; n = 6 for each temperature group) (b). Boxes designate quartiles, line designates median, whiskers designate minimum and maximum values, and dots represent individual measurements. Letters denote significant differences among temperature groups as determined by an ANOVA followed by a Tukey's Honest Significant Difference test or a Kruskal-Wallis test followed by a Dunn's multiple comparison test ($P < 0.001$).

FIGURE 2: Aerobic scope of threespine stickleback (*Gasterosteus aculeatus*) acclimated to three temperatures. Absolute aerobic scope (AAS) (a) and factorial aerobic scope (FAS) (b). Boxes designate quartiles, line designates median, whiskers designate minimum and maximum values, and dots represent individual measurements (n = 6, 6, and 5 for 5, 12, and 20°C respectively). Letters denote significant differences among temperature groups as determined by an ANOVA followed by a Tukey's Honest Significant Difference test or a Kruskal-Wallis test followed by a Dunn's multiple comparison test ($P < 0.05$).

FIGURE 3. Maximal activity of citrate synthase in hearts of threespine stickleback (*Gasterosteus aculeatus*) acclimated to three temperatures. Maximal activity of citrate synthase (CS) at a common temperature of 20°C (a) and at the acclimation temperature (b). Relative ventricular mass differed in response to acclimation, so CS activity was also calculated per 100 g body mass at the acclimation temperature (c). Dots represent individual measurements

(n=5 for each temperature group). Error bars indicate s.e.m. Letters denote significant differences among temperature groups as determined by an ANCOVA followed by a Tukey's Honest Significant Difference test ($P < 0.05$).

Figure 1

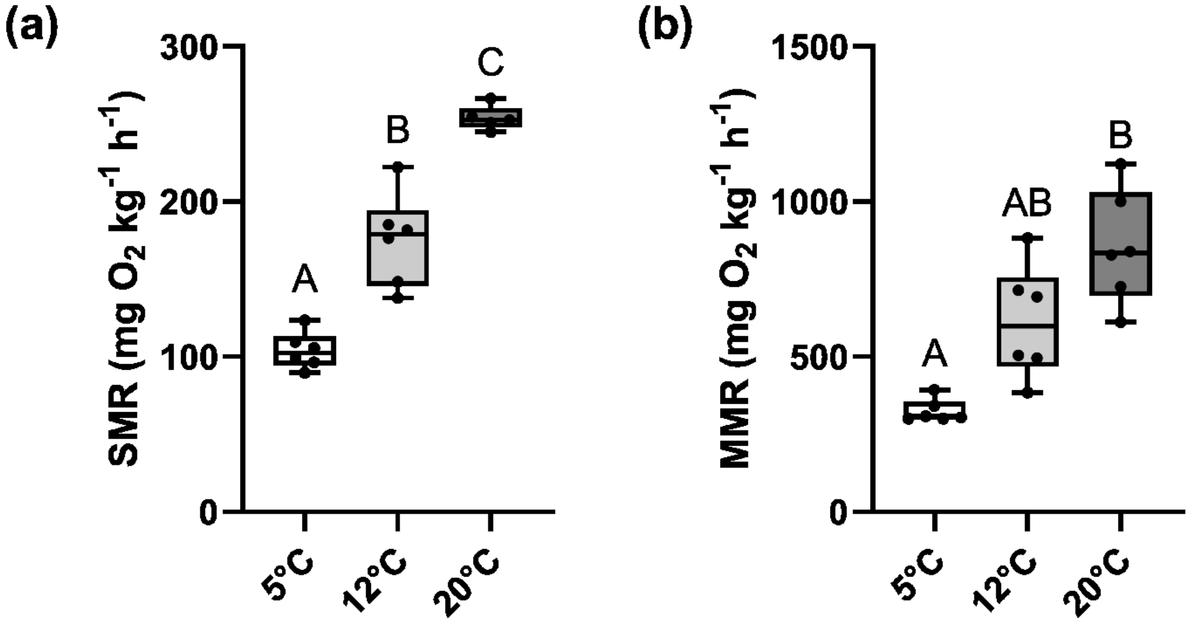


Figure 2

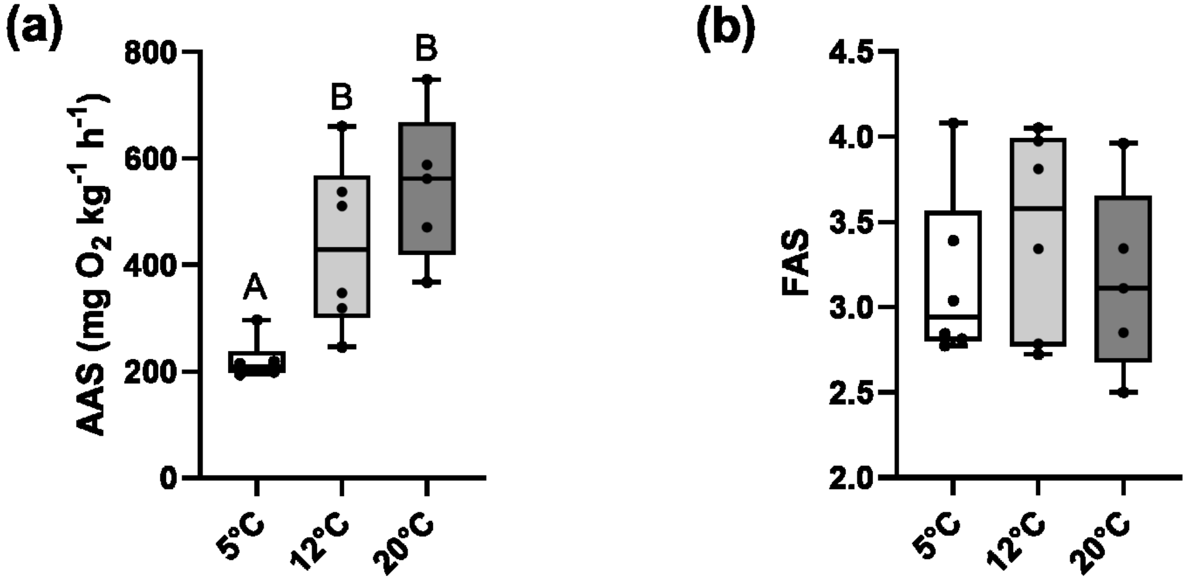


Figure 3

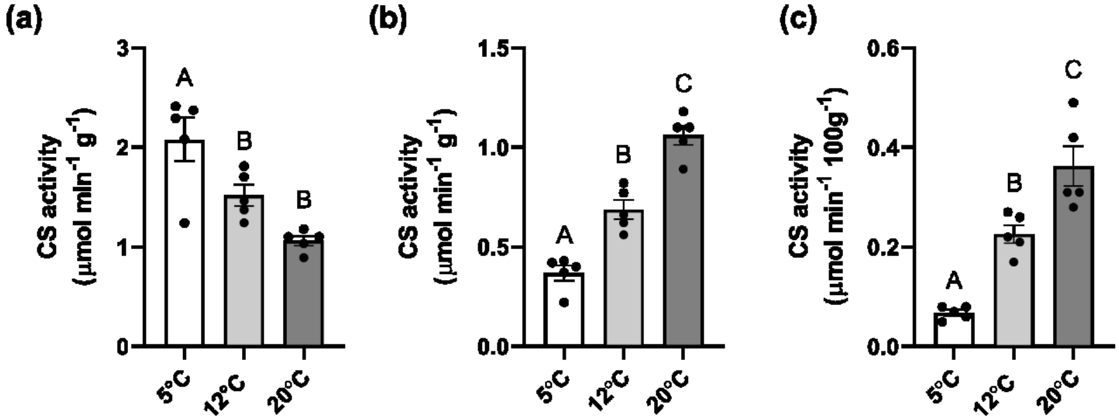


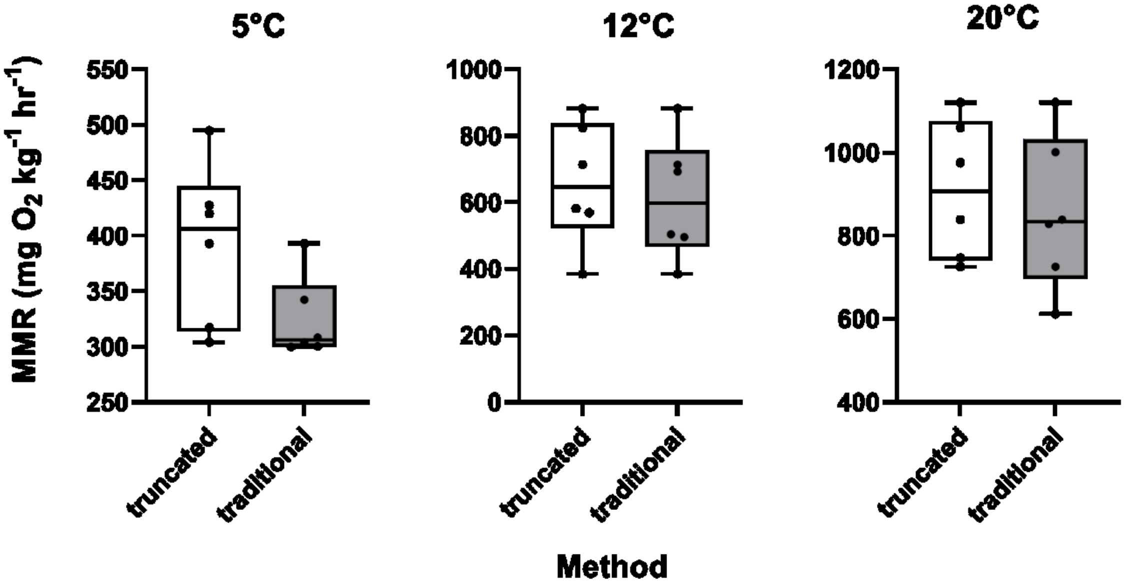
TABLE 1: Physical characteristics of threespine stickleback (*Gasterosteus aculeatus*) acclimated to three temperatures. Data are mean ± sd. Different groups of animals were used for respirometry and citrate synthase (CS) assays. The superscript letters A and B indicate significant differences among acclimation groups for animals used in respirometry experiments, whereas superscript letters X and Y indicate significant differences among acclimation groups

for animals used to measure cardiac CS activity as identified by a one-way ANOVA or Kruskal-Wallis test followed by a Tukey's Honest Significant Difference test or a Dunn's multiple comparison test, respectively ($P < 0.05$). Asterisks indicates significant differences between animals used for the respirometry versus CS assays within each temperature group as determined by an unpaired t-test or Mann-Whitney test ($P < 0.05$). n = sample size, L_S = standard length, RVM = relative ventricular mass.

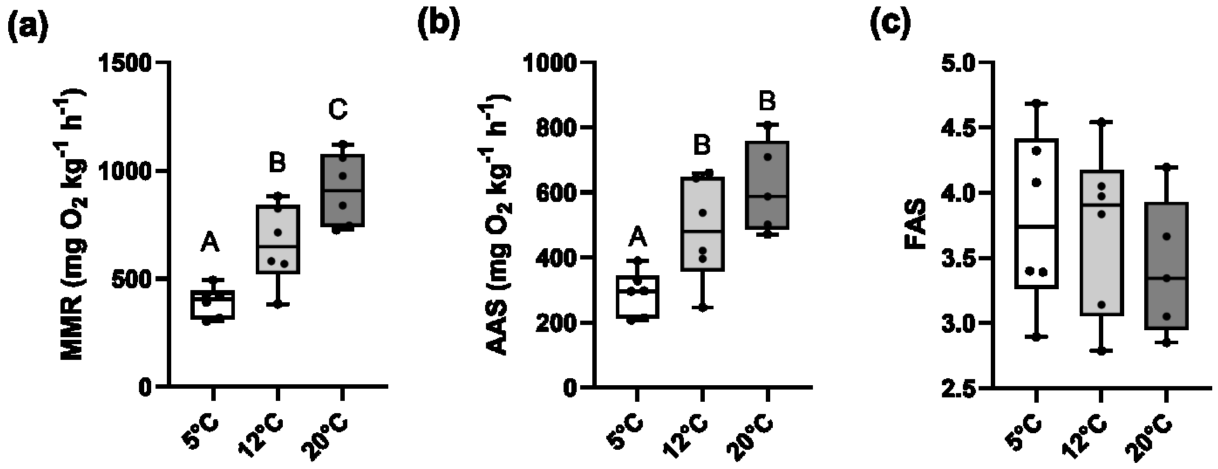
Group	Method	n	L_S (cm)	Body mass (g)	Heart mass (mg)	RVM	Condition Factor
5°C	respirometry	6	5.55 ± 0.40	2.17 ± 0.58	NA	NA	1.24 ± 0.08^{*A}
	CS	5	5.38 ± 0.29	2.76 ± 0.29^{X}	5.34 ± 1.11^{X}	0.19 ± 0.04^{X}	1.78 ± 0.23^{X}
12°C	respirometry	6	5.48 ± 0.25	1.90 ± 0.28*	NA	NA	1.15 ± 0.13^{*AB}
	CS	5	5.64 ± 0.26	2.80 ± 0.33^{X}	9.06 ± 1.53^{Y}	0.32 ± 0.05^{Y}	1.59 ± 0.34^{XY}
20°C	respirometry	6	5.42 ± 0.34	1.63 ± 0.32	NA	NA	1.02 ± 0.08^{*B}
	CS	5	5.38 ± 0.47	2.01 ± 0.44^{Y}	6.90 ± 2.03^{XY}	0.34 ± 0.07^{Y}	1.28 ± 0.15^{Y}

TABLE 2: Temperature coefficients (Q_{10s}) of metabolic rate and aerobic scope estimates for threespine stickleback (*Gasterosteus aculeatus*). T1 = temperature 1, T2 = temperature 2, SMR = standard metabolic rate, MMR = maximum metabolic rate, AAS = absolute aerobic scope, FAS = factorial aerobic scope. N = 6, 6, and 5 for 5, 12, and 20°C, respectively.

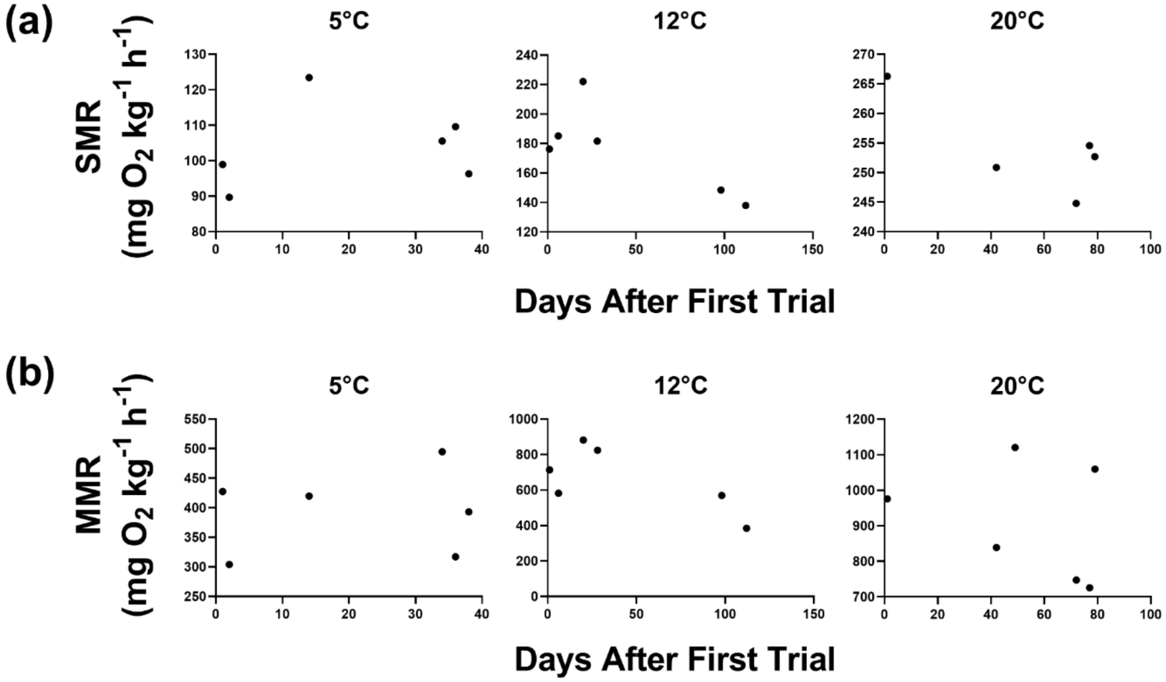
T 1	T 2	SMR	MMR	AAS	FAS
5°C	12°C	2.1	2.5	2.7	1.1
12°C	20°C	1.6	1.5	1.3	0.9



SUPPLEMENTAL FIGURE 1. Comparison of maximum metabolic rate (MMR) estimates for threespine stickleback (*Gasterosteus aculeatus*) acclimated to three temperatures. The "truncated" method measured the highest rate of $\dot{M}O_2$ within each measurement period (minimum length = 100 s). The "traditional" method included all DO measurements within each measurement period. Boxes designate quartiles, line designates median, and whiskers designate minimum and maximum values. MMR estimates did not differ by method for any temperature group (paired t-test: $P > 0.05$). N=6 for each temperature group.



SUPPLEMENTAL FIGURE 2: Maximum metabolic rate (MMR) and aerobic scope of threespine stickleback (*Gasterosteus aculeatus*) acclimated to three temperatures calculated using the truncated MMR method. MMR (a), absolute aerobic scope (AAS) (b), and factorial aerobic scope (FAS) (c). Boxes designate quartiles, line designates median, and whiskers designate minimum and maximum values (MMR: n = 6 for all temperatures, AAS and FAS: n= 6, 6 and 5 for 5, 12, and 20°C, respectively). Letters denote significant differences among temperature groups as determined by an ANOVA followed by a Tukey's Honest Significant Difference test ($P < 0.001$, $P < 0.01$ and $P < 0.05$ for MMR, AAS and FAS respectively).



SUPPLEMENTAL FIGURE 3. Metabolic rate of threespine stickleback (*Gasterosteus aculeatus*) based on when the trial was conducted relative to the date of the first trial for each temperature. Standard metabolic rate (SMR) (a) and maximum metabolic rate (MMR) (b). Each point represents the metabolic rate estimate for one animal. Day 0 = 20 and 21 weeks of acclimation for 5 and 20°C animals, respectively. Animals were held at 12°C prior to acclimation, so Day 0 = 44 weeks after collection.