Heritable differences in metabolic stability underpin thermal tolerance of threespine stickleback (*Gasterostues aculeatus)* ecotypes

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Abstract

Assessing intraspecific variation in thermal tolerance can provide insights on how rising global temperatures will differentially impact individual organisms and populations. The oxygen and capacity limited thermal tolerance hypothesis (OCLTTH) posits that aerobic scope (AS), the difference between standard and maximal metabolic rate, underpins other ecologically relevant metrics of performance. Here we assess short- and long-term thermal tolerance of benthic and limnetic ecotypes of threespine stickleback (*Gasterosteus aculeatus*) fish through common garden measurements of heritable variation in AS and parasite immunity. We predicted that benthic fish would display higher thermal tolerance resulting from adaptation to the relatively large thermal fluctuations in the shallow lakes that they inhabit. Based on the OCLTTH, we also expected that AS would decline with temperature and be a precursor for reduced condition. Although data connecting parasite immunity and temperature are limited, we predicted oxygen limitation would impair parasite resistance at higher temperature, particularly in the high-immunity limnetic fish. Despite exposing fish to temperatures below their thermal maxima, survival decreased with temperature and was most pronounced in limnetic fish at the highest temperature. The evolved thermal resilience of benthic stickleback was associated with their AS stability, suggesting that at least some populations of this keystone species are capable of withstanding hotter climates. However, contrary to the OCLTTH, temperature associated changes in AS were not associated with declines in fish condition or immunity.

Introduction

Global warming is altering species distributions (Chen et al., 2011) and driving population declines that can ultimately lead to extinctions (Hof et al., 2011). Phenotypic

plasticity is often proposed as a mechanism for resilience to global warming (Clement et al., 2023; Seebacher et al., 2015). However, theory regarding how plasticity may influence resilience to thermal challenge is conflicting. It is often argued that populations that evolve in variable environments are more likely to display adaptive plasticity, and therefore may be more robust to rapid environmental fluctuations (Botero et al., 2015). Alternatively, canalized phenotypes can sometimes be favored under fluctuating selection (Kawecki, 2000). If there is a physiological tradeoff between plasticity and thermal tolerance, then plastic populations may be more susceptible to heat stress (Bogan et al., 2024).

The oxygen- and capacity-limited thermal tolerance hypothesis (OCLTTH) posits that thermal tolerance results from the ability to increase transport of oxygen to tissues under elevated temperatures (Pörtner, 2001; Pörtner, 2002). The OCLTTH predicts that aerobic scope (AS), the difference between the minimum and maximum metabolic rate of an organism (Farrell, 2016), will increase until an optimal temperature (T_{opt}) is reached, after which AS will decline (Fig. 1A). Accordingly, the lower AS observed at extreme temperatures should result from less oxygen being supplied than is required to maintain homeostasis (i.e. higher standard metabolic rate (SMR)), as opposed to a change in maximum metabolic rate (MMR). Therefore, decreasing AS should be among the first indicators of decreased performance. Because of its emphasis on somatic maintenance, proponents of the OCLTTH highlight its ecological relevance (Pörtner et al., 2017). However, the OCLTTH remains contentious among ecophysiologists (Jutfelt et al., 2018). A meta-analysis of 73 studies found that almost half failed to find a thermal optima for AS, undercutting a major pillar of the hypothesis (Lefevre, 2016). Additionally, physiological reactions to thermal shifts are often duration dependent. Organisms display variation in their primary (immediate), secondary (long term maintenance of homeostasis), and tertiary

(dysregulation of biological systems) responses depending on how long they experience an altered environment (Franke et al., 2024). However, few studies have investigated the effects of chronic thermal stress on physiological plasticity (Clark et al., 2013). Given that organismal fitness depends on the duration of environments experienced throughout a lifetime, and that populations evolve in different environments, to understand how plasticity influences resilience to climate change requires testing physiological responses to stress in both temporal and population-specific contexts.

While hypotheses connecting respiration and temperature have received substantial experimental attention, less is known about the effect warming will have on fish immunity and parasite infection dynamics (Franke et al., 2024). It is hypothesized that global warming will exacerbate the impacts of parasitism by disrupting host immunity and facilitating parasite transmission, infectivity, and growth (Lõhmus & Björklund, 2015). The combination of heat and parasite infection may aggravate the detrimental effects of each stressor alone, amounting to a synergistically negative physiological response by the host (Earhart et al., 2022; Franke et al., 2024). Alternatively, heat may detrimentally impact fitness of parasites (Kirk et al., 2022), hindering their ability to complete their life cycle (Claar & Wood, 2020; Barber et al., 2016).

Threespine stickleback (*Gasterosteus aculeatus*) provide an opportunity to test the OCLTTH in the context of evolved differences in thermal tolerance and immune competency across populations. Multiple studies have found heritable differences in threespine stickleback thermal tolerance (Barrett et al., 2011; Dittmar et al., 2014). Much of this work has focused on contrasting different ecotypes of stickleback. For example, marine fish generally show less tolerance for low temperatures than freshwater populations (Barrett et al., 2011), but there is little evidence of evolved differences in thermal max (Barrett et al., 2011; Metzger et al., 2016;

Mottola et al., 2022). Benthic ecotypes are so named because they evolve in shallow lakes, habitats that can warm and cool rapidly. In contrast, limnetic ecotypes are adapted to large, thermally stratified lakes (Willacker et al., 2010). Given that many benthic and limnetic stickleback populations have lived in divergent temperature environments for thousands of years, they offer an opportunity to test for the evolution of divergence in thermal tolerance. To our knowledge, only one study has tested whether thermal tolerance differs among stickleback sampled from lakes of different size and mean summer temperature (Dammark et al., 2018). This work found only slight differences in short term thermal maximum and did not explicitly categorize fish by ecotype.

In addition to inhabiting thermally divergent environments, benthic and limnetic stickleback differ in diet and, consequently, parasite exposure. Limnetic ecotypes tend to consume more plankton than benthic ecotypes, which drives higher exposure to the copepod-transmitted cestode *Schistocephalus solidus* (Bolnick et al., 2024; Stutz et al., 2014). To minimize the impacts of *S.solidus* infection, soon after parasite exposure many stickleback populations evolve an intense innate immune response that results in extensive peritoneal fibrosis (Hund et al. 2022; Weber et al., 2017b). However, severe fibrosis can be costly in terms of energy required to mount the response (Sasser et al., in review). These costs likely contribute to the evolved loss of fibrosis in some populations (Weber et al., 2022). Conversely, the protective effects of fibrosis may be particularly beneficial under warming conditions. Higher temperatures increase both the growth rate (Franke et al., 2017; Macnab & Barber, 2012) and fecundity (Scharsack et al., 2021) of *S. solidus*. One caveat is that some stickleback populations exhibit immune dysregulation at hotter temperatures (Scharsack et al., 2021), even in the absence of pathogens (Dittmar et al., 2014). Though complicated, these immune-temperature connections

should be most apparent in limnetic stickleback ecotypes, as they are expected to display both high cestode immunity (i.e., strong fibrosis) and limited thermal resilience.

We conducted an experiment to test three primary questions: 1) Do benthic and limnetic stickleback differ in their primary and secondary/tertiary responses to heat, 2) Are changes in AS across temperatures a reliable predictor of fish health (i.e. OCLTTH), and 3) Do increased temperatures impair fibrotic immune responses, thereby affecting parasite infections, aerobic capacity, and/or fish condition? We hypothesized that benthic fish would fare better at higher temperatures due to adaptation to fluctuating thermal environments in shallow lakes. Assuming that stickleback conform to predictions of the OCLTTH, we expected AS to decline as temperatures increased due to a higher SMR, leading to concomitant declines in condition metrics (Fig. 1A). Additionally, benthic thermal tolerance may be reflected by a shallower change in SMR with temperature. While we initially defined ecotypes based on morphological characters, in reality populations display a continuum of traits connected to environmental differences (Haines et al., 2022). Indeed, recent work suggests that fibrosis is best predicted by physical lake characteristics (Bolnick et al., 2024). Therefore, we also tested whether lake properties (i.e., depth and surface area) predicted variation in thermal tolerance and mortality. With respect to immunity, we expected that higher temperatures would lead to weakened fibrosis responses and higher incidence of cestode infections, and that these primary thermal responses would be most pronounced in cestode-immune limnetic fish. We also predicted that prolonged exposure to high temperatures would enhance parasite growth. Although we were unable to evaluate the energetic costs of fibrosis when it was induced early in the experiment, we predicted that its negative effects may lead to reduced AS and condition.

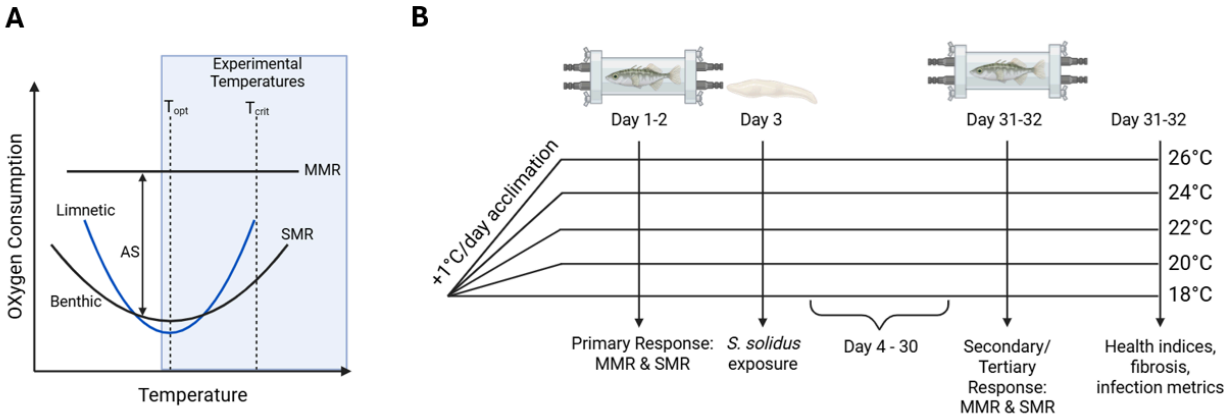

Figure 1: (A) According to the OCLTTH, SMR oxygen demand is lowest at the thermal optimum, causing AS to peak before declining at more extreme temperatures. We predict that limnetic fish (blue line SMR) will exhibit a more narrow thermal curve and have a lower critical temperature than benthic fish (black line SMR). **(B)** Summary of experimental setup. Graphics were created on BioRender.

Methods

Animal husbandry and thermal manipulations

We assayed lab-reared fish descended from 4 lakes in southcentral Alaska: Finger (lat: 61.6085, long: -149.2675), Spirit (60.5951, -150.9995), Watson (60.5365, -150.4628), and Wik (60.7160, -151.2479). Morphological analyses assigned Watson and Finger as benthic ecotypes, Spirit as moderately limnetic, and Wik as highly limnetic (Haines et al., 2022; Hendry et al., 2024). Lake depth corresponds with the classical benthic-limnetic paradigm, with Spirit and Wik being considerably deeper (21m and 24.4m, respectively) than Finger and Watson (13.4m and 4.3m, respectively), but variation in lake surface area is not perfectly associated with depth and could also affect thermal dynamics. The surface areas (ha) of Spirit, Wik, Finger and Watson Lakes are, respectively, 123.2, 74.1, 135.7, and 22.8.

During the experiment, fish (n = 32 Wik, n = 72 Spirit, n = 34 Finger, n = 73 Watson) were placed into mixed-family, population-specific tanks. Each population was represented by

3-4 families that were spread evenly across temperature treatments. We acclimated the fish to their experimental treatments (18-26°C) by increasing water temperature 1°C per day (Fig. 1B) to prevent mortality associated with a rapid change in temperature (Earhart et al., 2022). The temperature range was selected because it is below the reported thermal maxima for this species (Barrett et al., 2011; Dammarck et al, 2018; Dittmar et al., 2014) and spans the projected mean and maximum air temperatures in the Kenai, AK area during the hottest month of the year in 2060 under a moderate emissions scenario (RCP 6.0; https://snap.uaf.edu/tools/community-charts). Following thermal acclimations, we maintained fish at experimental temperatures for 31-32 days to measure primary and secondary physiological responses, as well as cestode resistance (Fig. 1B; details below). Fish were monitored daily, and any animals showing signs of distress (i.e., erratic swimming, immobility, loss of equilibrium, or gasping) were immediately euthanized using an overdose of pH buffered MS-222. If a fish died or was euthanized prior to the end of the experiment, it was dissected to assess *S. solidus* presence. We similarly euthanized all fish at the end of the experiment and performed dissections to measure infection outcome and tertiary physiological responses. All procedures were approved by the University of Wisconsin-Madison IACUC (protocol #L006460).

Respirometry

We assessed primary physiological response to heat 1-2 days after thermal acclimation and secondary response 31-32 day post acclimation by measuring mass specific SMR and MMR via intermittent flow respirometry (Supplementary methods). R:FishResp (Svendsen et al., 2019) was used to calculate AS as the difference between SMR and MMR (Clark et al. 2013).

Respirometry traits were measured in one benthic and one limnetic population at the secondary timepoint (Supplemental Table 1).

Experimental cestode exposures

Following Weber et al. (2022), batches of cyclopoid copepods (*Acanthocyclops robustus*) were exposed to lab-reared *S. solidus* coracidea descended from individuals caught in Walby Lake, Alaska (61.6178, -149.2138). We measured infection rates by screening a subset of each batch two weeks post exposure. After initial respirometry assays, we transferred individual fish into temperature-controlled containers and exposed them to infected copepods containing a total of ~7 cestodes. At the time of exposure, the fish had been fasted for 48 hours. To guarantee the opportunity to observe uninfected fish, 86% of the fish were exposed to parasites. We could not distinguish exposed from unexposed individuals in the final analysis.

Health indices, infection outcome, and fibrosis

Spleenosomatic (SSI), hepatosomatic (HSI), and gonadosomatic (GSI - for males only) index were calculated by dividing the log transformed organ mass by the log transformed wet mass of the fish. SSI is a metric of immune condition, HSI is a proxy for energy storage, and GSI is informative for reproductive investment (Zapata et al., 2006). Fulton's body condition was calculated using $K = (Mass/SL^3)x100$, where K = body condition. During dissections we screened for cestode infection and scored fibrosis (Hund et al., 2022). Ordinal scores were 0 for no visible fibrosis, 1 if organs were attached to the swim bladder, 2 if organs were fused together, 3 if organs were fused to the side of the body cavity, and 4 if the body cavity was entirely filled with fibrotic tissue.

Statistical Analysis

We used generalized linear models to test whether primary and secondary (i.e., start and end of experiment) respirometry traits (MMR, SMR, and AS) responded to temperature, ecotype, and temperature*ecotype interactions. Although models for secondary traits were limited to data from two fish populations, we tested for additive effects of fibrosis presence and score on respirometry. Stickleback can only be definitively sexed by observing the gonads during dissection, so sex effects were only included for secondary metabolic and fibrosis data.

We fit linear mixed effect models (R package nlme v. 31-164; Bates et al., 2015) to test whether condition metrics (body condition, SSI, GSI, HSI) were predicted by AS, ecotype, and their interaction. Sex was included as a fixed effect and tank density was a random effect to account for differential mortality throughout the experiment. In the absence of evidence to support the OCLTTH (i.e., no effect of AS), temperature could still influence condition metrics through other biological pathways. Therefore, we again used linear mixed effect models to directly test the effects of temperature, ecotype, temperature*ecotype, and sex on each condition phenotype, with tank density as a random effect. Cox regression was used to assess whether mortality varied between ecotypes, across temperature treatments, or between ecotypes within a temperature treatment. Additional details regarding animal husbandry, respirometry, and analysis can be found in the supplementary information.

Results

Primary Response: Both ecotype and lake ecology interact with temperature to explain variation in SMR and AS

Immediately following acclimation, MMR was best explained by a linear increase with temperature (Fig. 3A, Supplemental Table 2 & 3). Although there was no effect of fish ecotype, MMR did vary depending on lake surface area (Supplemental Table 2 & 4, Supplemental Fig. 1A). Specifically, MMR increased more with temperature in populations that originated from lakes with a smaller surface area (temperature * surface area: $t = -2.393$, $p = 0.0177$).

In contrast to MMR, SMR was best explained as an effect of quadratic temperature and increased significantly with temperature (Fig. 3B, Supplemental Table 2 & 3). Limnetic fish tended to have a lower SMR than benthic fish between 18-22°C (ecotype: $t = -2.213$, $p = 0.0281$), but experienced a dramatic increase at 24°C (temperature * ecotype: $t = 2.266$, $p = 0.0247$). Another difference between measures of metabolic rate was that mean lake depth, not surface area, best predicted temperature-related shifts in SMR (Supplemental Table 2 & 4, Supplemental Fig. 1B). Fish from shallower lakes tended to have a higher SMR, but their SMR did not increase with temperature as much as fish from lakes with a higher depth (temperature*mean depth: $t = -2.803$, $p = 0.00561$).

Like SMR, the best model for AS included temperature as a quadratic term (Fig. 3C, Supplemental Table 2 & 3). AS followed a concave association with temperature, peaking at 22°C. Limnetic individuals displayed ~8% higher AS than benthic fish on average, but limnetic AS decreased precipitously at 26°C (temperature * ecotype: $t = -2.426$, $p = 0.01626$). Surface area was the best lake attribute to predict AS (Supplemental Table 2 & 4, Supplemental Fig. 1C). Populations from lakes with a larger surface area tended to have a higher AS, but a more stable AS across temperature (temperature * surface area: $t = -3.482$, $p = 0.000623$).

Secondary & Tertiary Response: Limnetic fish display substantial mortality at high temperature related to decreases in AS

Although this experiment was not designed to test survival under heat stress, mortality rates significantly increased with chronic exposure to higher temperature ($z = 2.091$, $p = 0.0365$). Temperature had a greater effect on the survival of limnetic fish (ecotype: $z = 1.949$, $p = 0.0512$, Fig. 2). Much of this ecotype difference was driven by the 89% mortality of limnetic fish at 26°C, compared to just 11% mortality among benthic fish.

Although we only examined secondary (end of experiment) metabolic rates in two populations at three temperatures, we found significant linear and quadratic effects of temperature on MMR, SMR, and AS, with all three metrics reaching a minimum at 22°C (Fig. 3D-F, Supplemental Table 2 & 5). While there was not a significant effect of ecotype or sex (Supplemental Table 5, Supplemental Fig. 2), when averaged across temperatures limnetic individuals displayed ~11% higher MMR and ~21% higher AS than benthic fish.

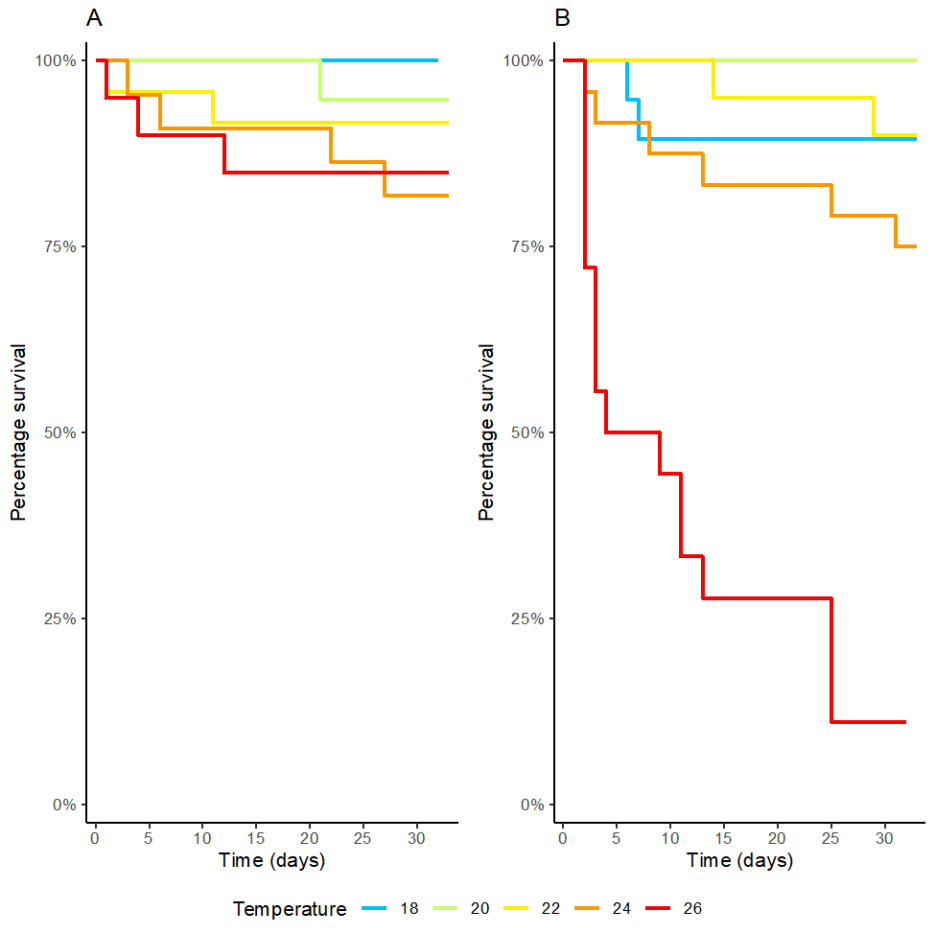

Figure 2: Probability of survival as function of temperature and time for **(A)** benthic and **(B)** limnetic stickleback. Survival probability significantly decreased at higher temperatures ($z = 2.091$, $p = 0.0365$) and there was a marginally significant effect of ecotype ($z = 1.949$, $p = 0.0512$).

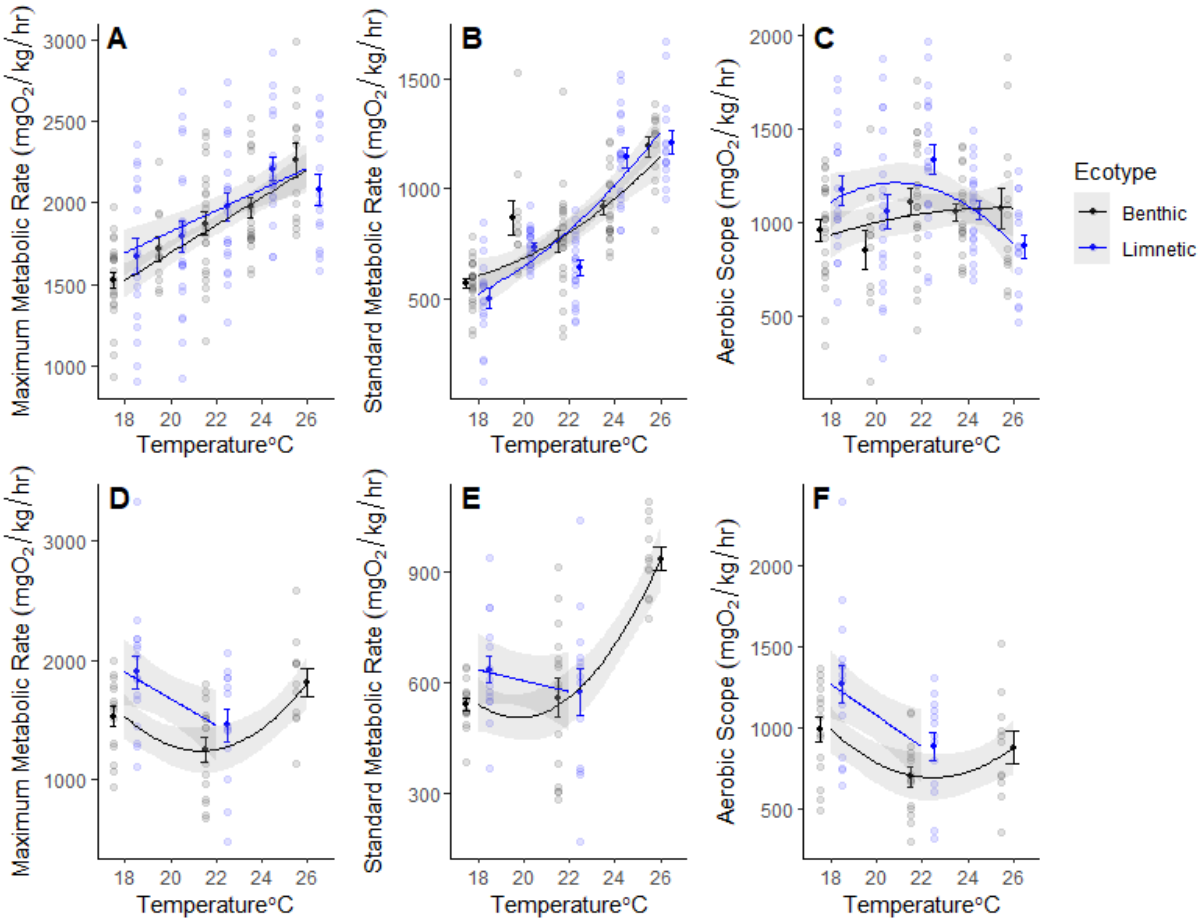

Figure 3: Metabolic data from the start **(A-C)** and end **(D-F)** of the experiment. **(A)** MMR and **(B)** SMR initially increased with temperature. **(C)** Limnetic fish displayed an overall higher AS, which increased to a peak at 22°C followed by a sharp decline. The initial AS of benthic fish increased with temperature. **(D)** After 31-32 days MMR decreased from 18C to 22C in both ecotypes, but then increased at 26°C for benthic fish. **(E)** SMR responded similarly to MMR, marked by a strong increase at 26°C in benthic fish. **(F)** Like MMR and SMR, AS decreased at 22°C but increased slightly at 26°C.

Limited cestode infection across temperatures and ecotypes

Despite exposing 178 fish to *S. solidus*, we only recovered a single infected fish. Notably, this fish died before the end of the experiment. We did however find that 72 fish exhibited fibrosis, which includes individuals from all four populations. We also observed a few *S. solidus* encysted in fibrotic tissue. Although some stickleback populations, including Finger Lake,

display fibrosis without parasite exposure (Sriramulu et al., 2025), observing fibrosis in 34% of cases suggests that exposures were successful but the parasites failed to establish infection after burrowing into the body cavity of the fish. Contrary to our prediction, temperature had no effect on fibrosis presence or severity (Supplemental Fig. 3E-F). However, fibrosis presence was associated with lower AS ($t = -2.392$, $df = 63$, $p = 0.0197$), regardless of fibrosis score or fish ecotype (Fig. 4E-F).

Health metrics decline with AS in limnetic fish, but temperature is a poor predictor of condition

Based on the OCLTTH, we predicted that decreased AS would be a precursor to decline in a number of metrics of somatic condition. Body condition did not vary based on AS. However, there was a significant difference between ecotypes in the relationship between AS and condition (AS * ecotype: $t = -2.254$, $df = 61$, $p = 0.0278$, Fig. 4A); condition tended to decrease with higher AS in limnetic fish, but this relationship was not significant ($t = -1.828$, $df = 61$, $p = 0.0724$). Males overall also had a higher body condition than females regardless of temperature ($t = 5.972$, $df = 61$, $p < 0.0001$, Supplemental Fig. 4). We found the same ecotype difference in the slope of AS associations across HSI (AS * ecotype: $t = -2.094$, $df = 60$, $p = 0.0405$, Fig. 4B), GSI (AS * ecotype: $t = -2.016$, $df = 28$, $p = 0.0535$, Fig. 4C), and SSI (AS * ecotype: $t = -1.947473$, $df = 60$, $p = 0.0562$, Fig. 4D).

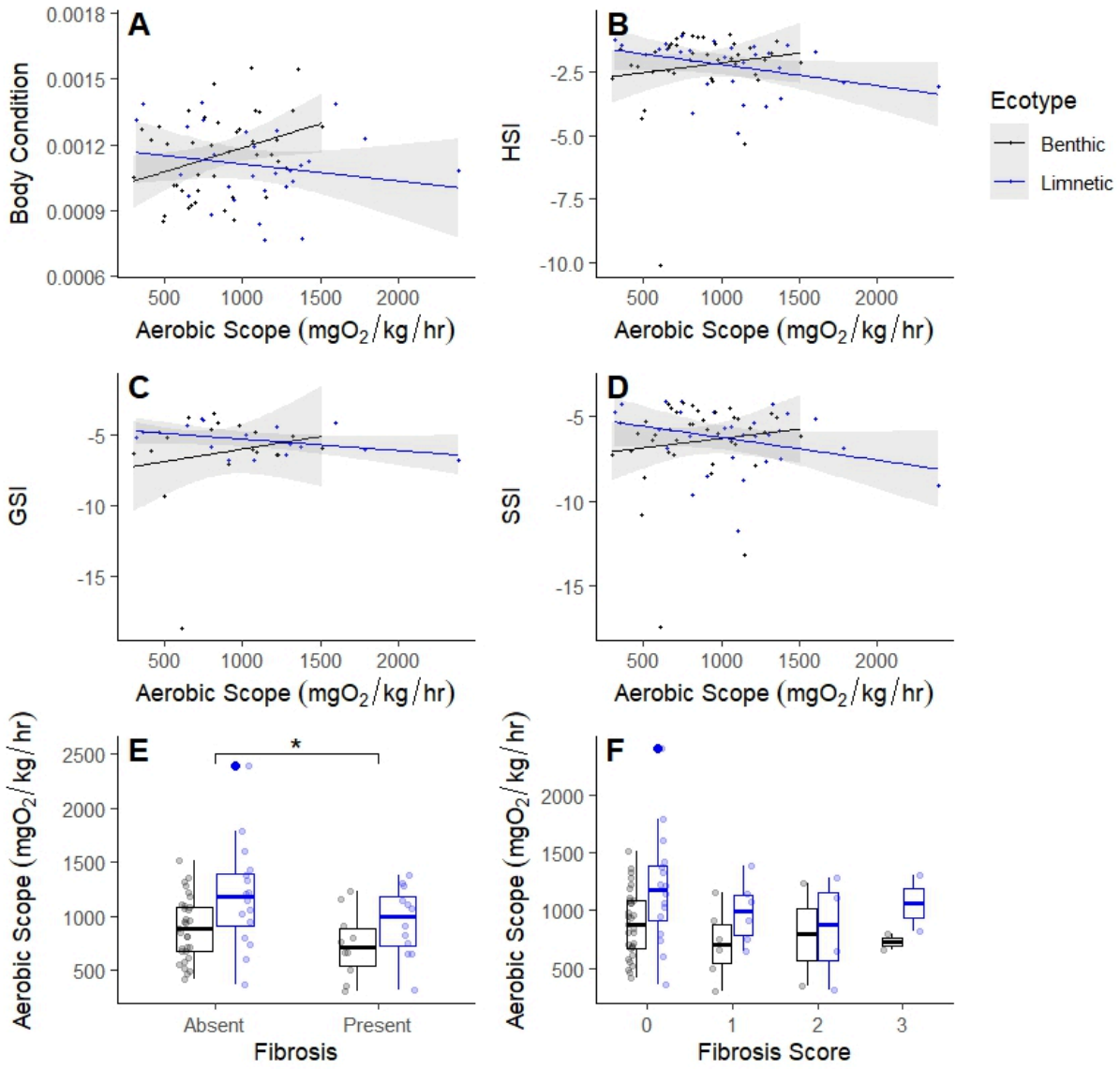

Figure 4: Performance metrics as a function of aerobic scope for each ecotype. **(A)** Body condition, **(B)** HSI, **(C)** GSI, and **(D)** SSI decreased with AS in limnetic populations. **(E)** Higher AS was associated with lower incidence of fibrosis. **(F)** AS was not associated with fibrosis severity, but limnetic fish fibrosed more severely than benthic fish. Significance levels: *P < 0.05; P > 0.05 (not significant)

Discussion

Thermal tolerance is used to project changes in geographic range (Sunday et al., 2012; Smith et al., 2022), population declines (Roeder et al., 2021), and identify species at risk of either

local or global extinction (Turko et al., 2021). However, studies often assess tolerance in relation to critical thermal maxima (CT_{max}), where temperature is steadily increased until the fish is unable to maintain equilibrium. While this approach identifies an upper lethal limit, it can obscure more ecologically relevant differences caused by prolonged exposure to less extreme temperatures. Additionally, thermal tolerance may be affected by additional stressors that wild individuals are likely to experience simultaneously, such as pathogen exposure. We therefore assayed thermal tolerance across four benthic and limnetic populations of threespine stickleback after exposing them to the common cestode *S. solidus*. Consistent with our prediction, we found that benthic populations are more resilient to hotter temperatures. However, we found limited support for the OCLTTH. Aerobic scope (AS) decreased at the highest temperature treatment, but only for limnetic fish and was a poor predictor of condition. Although we observed no successful infections, nor any impact of temperature on fibrosis, AS significantly decreased in fibrotic fish. When combined with temperature-associated declines in AS, this suggests that variation in immune responses warrant additional attention when estimating thermal tolerance.

Stable AS may enable benthic populations to survive at high temperatures

As noted previously, we designed our temperature challenge to fall well below the reported CT_{max} for stickleback. The only other comparison of freshwater stickleback from variable thermal environments suggested that there would be no differences in severe stress experienced at 26°C (Dammark et al., 2018). Yet, we observed significantly lower survival at higher temperatures, with the impact being most severe in the 26°C limnetic ecotype treatment (Fig. 2). It is unlikely that wild Alaskan stickleback will be continuously exposed to this high of temperature, and all populations showed high survival immediately after acute stress (i.e.

immediately after acclimation). Regardless, our results highlight that stickleback populations respond differently to prolonged temperature increases, with limnetic fish suffering potentially large and negative consequences from even relatively small (i.e., compared to CT_{max}) but chronic temperature shifts. These longer term consequences were also related to ecotype-specific metabolism differences observed immediately after thermal acclimation. At the start of the experiment, AS peaked in limnetic fish at 22°C and declined by 36% at the highest temperature (Fig 3C). Another study on long-term thermal responses used a different limnetic population from Alaska and, though not spanning the same temperatures, also found substantial plasticity in AS (Ressel et al., 2021). Meanwhile, initial AS remained relatively stable across all experimental temperatures for benthic fish. It is important to note that the trajectory of change in AS with temperature was more telling than absolute AS values. Both ecotypes had equivalent measures of AS at 24°C, but the downward trajectory of limnetic AS likely reflects the early stages of physiological dysregulation (i.e. tertiary response).

It is also instructive to consider whether our metabolic results might explain why benthic populations exhibited low mortality rates even after 30+ days at high temperatures. Although our end of experiment data are limited to two populations and three temperatures, they still contain several interesting trends. As with the primary physiological responses, at the end of the experiment benthic populations maintained a more stable AS than limnetic populations across temperatures. The secondary benthic AS-temperature curve does shift to a convex shape, but likely the more important relationship is the limited change between initial and end AS values. When AS is averaged across temperature in the Watson Lake benthic fish, there is a 19% decrease by the end of the experiment. Although this suggests that benthic fish suffered

consequences from chronic thermal stress, they generally maintained homeostasis while limnetic fish experienced physiological dysregulation.

But how did the two ecotypes come to differ in their chronic responses to heat? Previous work has identified morphological and physiological changes connected to stickleback migration, with nonmigratory stream fish evolving lower MMR than migratory marine ecotypes (Dalziel et al, 2012a; Dalziel et al., 2012b). Migration-associated ecotype differences in SMR are also connected to heritable changes in thyroid hormone signaling (Kitano et al., 2010). Although all lake fish in the present study are non-migratory, similar mechanisms may underlie both thermal tolerance and migratory ability. To differentiate between canalization in benthic populations or gain of plasticity in limnetic populations, future research will need to examine long-term thermal responses of marine stickleback as a proxy for the ancestral state. We favor the hypothesis that benthic populations lost maladaptive plasticity, enabling them to better regulate AS across temperatures and adapt to the small, shallow ponds they inhabit. This prediction fits with other examples of thermal canalization, including adaptation to urban heat islands in anoles (Campbell-Staton et al., 2021). It will also be important to extend our results by studying a more geographically diverse set of benthic and limnetic populations. The only other study testing for local adaptation to high temperature found little evidence for the evolution of short-term thermal tolerance in stickleback from Denmark (Dammark et al., 2018). But, interestingly, fish from the naturally hottest lake in that study did display the highest CTmax. Our results predict that chronic heat challenge may yield more distinctive adaptive patterns than short term assays.

Temperature did not affect infection rates or fibrosis

Temperature impacts immune responses across a wide range of teleosts (Scharsack & Franke, 2022), including stickleback (Dittmar et al., 2014; Franke et al., 2019; Scharsack et al., 2021). Thus, we were surprised to find no connection between temperature and infection or fibrosis. However, fibrosis was associated with lower AS at the end of the experiment. While fibrosis is initiated within days of cestode exposure (Hund et al., 2022), it can have long-term effects on mobility (Matthews et al., 2023) and gene expression (Weber et al., 2022). Therefore, lower AS may result from increased energy demands in immobile fish or metabolic costs of fibrosis maintenance.

Although we did not want to bias our infection results by using cestodes from one of the four host populations, using a foreign cestode (i.e., from Walby Lake) may explain the total lack of infection in our study. Stickleback tend to be better at resisting infection from foreign populations of *S. solidus* (Bolnick et al., 2024; Weber et al., 2017a). Future work that incorporates *S.solidus* population genetic and host specificity data across benthic and limnetic lakes will help address the lack of infection in this study.

Limited support for OCLTTH

In line with the OCLTTH, AS declined in limnetic populations as temperature increased toward their maximum. However, AS peaked at 22°C in limnetic fish at the start of the experiment, which would indicate that 22°C is the T_{opt} for limnetic populations under the OCLTTH. This agrees with another study showing that AS increases up to at least 20°C (Ressel et al., 2021). However, both the historical lake temperature and developmental temperature of these populations is well below 22°C, and previous work in other stickleback ecotypes suggests their T_{opt} is between 13-17°C (Barrett et al., 2011; Dittmar et al., 2014; Franke et al., 2017,

2019). Metrics of fish health also declined with AS, in line with the OCLTTH, but this relationship was only present in limnetic fish and was marginally significant. The lack of change in benthic fish is clearly connected to their high AS stability, indicating the OCLTTH may have limited relevance to populations adapted to fluctuating temperatures. Another explanation involves differences in energetic demand and uptake between lab and wild environments. Costs may be masked when resources are plentiful but apparent when they are scarce, as is likely the case in natural settings (Boots, 2011). Our laboratory fish were fed to satiation and likely had much greater energy stores than wild stickleback, perhaps leading to higher MMR than would be seen in resource limited animals. Further tests of the OCLTTH will benefit from greater attention to evolved differences in AS, and from testing the impact of resource limitation on performance across temperatures .

Limitations

Several features of our experimental design limited our ability to test connections between temperature and metabolism. First, we were unable to individually mark the fish used in this experiment, and therefore could not directly connect AS at the start of the experiment to survival or growth rate. Second, our choice to acclimate fish to constant temperatures was practical but not ecologically realistic. The chronic impact of high temperatures in this experiment likely exceeds what stickleback experience in the wild. Conversely, acclimating fish to high temperatures also appears to buffer the negative impacts of thermal stress compared to rapid environmental change (Earhart et al., 2022) Additionally, rapid temperature fluctuations are expected to become more frequent, eliminating the opportunity to acclimate (Diffenbaugh & Field, 2013). Although technically challenging, future studies that mimic daily oscillations in

temperature over longer periods without using an acclimation period may provide more accurate predictions regarding impacts of natural heat waves. Lastly, when assaying MMR we were limited to exercising the fish for one minute in compliance with animal care and ethics guidelines. Ideally, fish will be unresponsive to agitation of the caudal fin before MMR is measured (Clark et al., 2013). Some, but not all, fish in this experiment reached this state of exhaustion. Although MMR is expected to remain stable across temperatures, our observations of increases in primary MMR could have resulted from fish not being reliably exercised to exhaustion. Despite this limitation, all fish were exercised for an equal amount of time. Although we did not specifically record the presence of exhaustion, there was no obvious difference between fish challenged at the start and end of the experiment. It is also noteworthy that our 20°C MMR values were higher than those reported from wild stickleback assayed at the same temperature and collected from the same region of south central Alaska (Ressel et al., 2021). Furthermore, MMR is notoriously difficult to measure using standardized methods (Ressel et al., 2021). Therefore, while our MMR estimates may not be perfectly comparable with other studies, they were still comparable between treatment groups.

Conclusions

In contrast to previous work on short term thermal tolerance, this work demonstrates that threespine stickleback have evolved large differences in long term thermal tolerance. These differences are closely connected to ecotype variation in thermal habitats, suggesting that they may result from local adaptation. This work also provides a valuable test of the OCLTTH and suggests that AS stability, potentially a result of evolved SMR canalization, underpins resilience to chronic heat in benthic stickleback. Future work should aim to identify the mechanistic basis

of AS stability and whether benthic and limnetic ecotypes commonly evolve differences in chronic thermal tolerance.

Data Accessibility

All data and analysis scripts can be found at https://github.com/EmilyKerns/ThermTol.

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Author Contributions:

All authors contributed to the design of the study and writing of the manuscript. KTS and EVK led data collection and analysis. JNW assisted in analysis and supervised and funded the work.

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