DISSERTATION

THE EVOLUTIONARY ECOLOGY OF AQUATIC INSECT RANGE LIMITS:

A MECHANISTIC APPROACH USING THERMAL TOLERANCE

Submitted by

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ABSTRACT

THE EVOLUTIONARY ECOLOGY OF AQUATIC INSECT RANGE LIMITS:

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Understanding the effect of climate variability on species physiology and distribution is a longstanding and largely unresolved challenged in evolutionary ecology with important implications for vulnerability to climate change. My dissertation is focused on understanding the effects of temperature on physiological traits and genetic population structure of aquatic insects, to better understand the mechanisms that underlie their elevation range distributions.

For my first chapter, I tested the hypothesis proposed by Dan Janzen in 1967, that temperate mountain species should have broad thermal tolerances thus allowing them to disperse easily across elevation, unhindered by the novel temperatures they encounter. On the other hand, tropical species should exhibit narrower thermal tolerances in response to the stable climate they experience. They should be physiologically challenged to disperse and be restricted to a narrow elevation range distribution. I measured critical thermal limits (CT_{MAX} and CT_{MIN}) and thermal breadth (difference between CT_{MAX} and CT_{MIN}) in several phylogenetically related temperate (Colorado) and tropical (Ecuador) aquatic insect species. I found that, as predicted, species that encounter wider stream temperature ranges, such as temperate species and high elevation tropical species, have broader thermal breadths compared to their tropical and low elevation relatives.

Next, I tested how plastic the critical thermal maximum (CT_{MAX}) response was in a subset of aquatic insects. Greater acclimation ability is thought to allow species to withstand the large temperature fluctuations associated with different seasons. Implicit in Janzen's hypothesis,

is the assumption that temperate species have greater acclimation ability compared to tropical species. My experiments revealed that temperate and high elevation tropical mayfly species had greater acclimation ability compared to their relatives. However, we found no differences in acclimation capacity in stoneflies. Temperature may therefore not affect all species equally, and species acclimation ability may be a result of other factors such as body shape and evolutionary history.

I then measured a third trait, metabolic rate, to investigate how it varies with temperature in temperate and tropical mayflies. Metabolic rate is arguably one of the most important traits for species because it determines the amount of energy an animal has available for its activities. I found that metabolic rates vary between temperate and tropical mayflies, and that temperatures away from a certain optimum are stressful and sometimes lethal for tropical but not temperate mayflies.

Finally, I linked thermal tolerance to dispersal by correlating gene flow among populations with pairwise differences in the physiological trait CT_{MAX}. Analyses revealed that there was lower gene flow (higher F_{ST}) among populations in Ecuador than among populations in Colorado. Within Ecuador, differences in CT_{MAX} were highly correlated with maximum stream temperature, which was found to best explain tropical mayfly genetic structure. In Colorado, no environmental or physiological variable was found to explain population structure. Our results indicate, as Janzen predicted, that temperature can act as a significant barrier to dispersal among tropical populations but not in temperate ones. Thermal sensitivity measured as CT_{MAX} was also correlated with F_{ST} but was not significant.

As a whole, the results from my research lend support to Janzen's hypothesis and suggest that temperature plays an important role in determining range limits of aquatic insect species

through its effect of thermal tolerance traits. While this research addresses long standing questions in ecology and evolution, it also has conservation implications. Most importantly, as the effects of global climate change augment, the thermally sensitive tropical species from this study system are at particular risk for extreme population declines or even extinction.

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couldn't imagine a more beautiful "office" or a more fortunate choice for where to conduct this research. Every field foray into the rivers or jungles has felt like a spiritual experience. This work highlights my commitment to being a steward of these mountains and rivers and my wish to inspire future generations to strive tirelessly toward the conservation of these special places.

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1. INTRODUCTION

"But although life is everywhere diffused, and although the organic forces are incessantly at work in combining into new forms those elements which have been liberated by death; yet this fulness of life and its renovation differ according to differences of climate. Nature undergoes a periodic stagnation in the frigid zones; for fluidity is essential to life. Animals and plants, excepting indeed mosses and other Cryptogamia, here remain many months buried in a winter sleep. Over a great portion of the earth, therefore, only those organic forms are capable of full development, which have the property of resisting the considerable abstraction of heat, or those which, destitute of leaf-organs, can sustain a protracted interruption of their vital functions. Thus, the nearer we approach the tropics, the greater the increase in the variety of structure, grace of form, and mixture of colors, as also in perpetual youth and vigour of organic life." [1]

–Alexander von Humboldt 1850

The eighteenth century explorer and naturalist, Alexander von Humboldt, was one of the first to recognize the staggering difference in biodiversity between tropical and temperate mountains. Since then, generations of ecologists and evolutionary biologists have pondered the reasons for why patterns of biodiversity vary across the globe. One key component to resolving this question is understanding why *species ranges* vary, thus contributing to differences in biodiversity. Despite the fundamental nature of these ideas, and decades of research that address them, the causes and consequences of species range distributions remain poorly understood for most wild populations (Sexton et al., 2009).

The factors underlying limits to species geographic ranges have been proposed in a number of studies. For example, limitations on dispersal ability due to the presence of major geographic barriers can clearly be a constraint to a species' ability to colonize and occupy suitable habitat (Slatkin, 1973). However, most often one finds seemingly abrupt changes in species composition with no apparent physical barrier (Hoffmann & Blows, 1994; Irwin &

Otté, E.C. and Bohn, H.B. *translators* (1850). Views of Nature: or Contemplations on the Sublime Phenomena of Creation; with Scientific Illustrations, 3rd edn (Alexander von Humboldt), Henry G. Bohn.

Gibbs, 2002). Understanding these often invisble factors will fill a major gap in our knowledge of the ecology of species distributions and global patterns of biodiversity (Mayr 1963; Case & Taper, 2000; Hoffmann & Blows, 1994; Holt & Keitt, 2004; Janzen, 1967).

Abiotic factors such as temperature, salinity, and moisture are some of the well-studied determinants of species distributions (e.g. O'Brian 1994; Dippner 1997). Recently, studies have focused on temperature as an important determinant of range limits (reviewed in Bozinovic et al., 2011; Sunday et al., 2012; Sunday et al., 2015), and the link between temperature and poleward shifts associated with increasing global temperatures (Hickling et al., 2006) further emphasizes the impact of temperature on species distributions. Biologists have therefore argued that more studies are needed that quantify which *traits* are under strongest selection by temperature and how their interaction with the environment is manifested in patterns of distribution and gene flow (Hoffmann and Blows 1994; Blows and Hoffmann 2005).

Thermal sensitivity, for example, is a trait implicated in shaping an organism's ecological niche and thus its range limits (Huey & Stevenson 1979; Angilletta 2009). There are a number of techniques by which thermal sensitivity can be assessed, such as experimentally determining critical limits (CT_{MAX} and CT_{MIN}; Angilletta 2009), thermal acclimation (plasticity) (Ghalambor et al. 2007; Seebacher et al. 2015; Gunderson & Stillman 2015), various performance measures (e.g. Angilletta 2006), and metabolic rate (Brown et al. 2004; Pörtner 2002). Thermal sensitivity can therefore be a powerful tool in gaining a mechanistic understanding of range limit evolutionary ecology, especially along thermal gradients such as elevation and latitude. A combined ecological and thermal trait perspective of species distributions can not only provide a deeper understanding of a most fundamental biological phenomenon, but enable scientists to forecast adaptive potentials (if traits are found to be heritable), shifts in ranges, and extinctions

(Deutsch et al. 2008; Bozinovic et al. 2011) especially as a consequence of habitat fragmentation, degradation, and environmental change.

Aquatic insects as a study system

Freshwater aquatic insects (predominantly in larval form) present an excellent natural system in which to study range-limit evolutionary ecology. These ectotherms occur in freshwater streams from high elevation low-order tributaries to large lowland rivers, which can differ widely in temperature and dissolved oxygen. Aquatic insects are found to have high variability in spatial distribution and are reliant on water temperature for their survival and persistence. As study-organisms, aquatic insect species are reliably found throughout the year, can be collected unharmed with nets, and can be easily transported and housed in the laboratory for several days. Methods for rearing to adulthood are known, and various experimental tests have been conducted on them, making aquatic insects good candidates for lab and field manipulations. Freshwater ecosystems vary in the degree to which they are exposed to different temperature regimes. Thus, from a conservation perspective, studying the distribution of aquatic insects in streams with different thermal regimes can provide information about how freshwater communities and ecosystem functioning might be altered with the advent of rapid climate change.

Dissertation objectives: investigating the effect of temperature on physiology and distribution

My dissertation is focused on understanding the effects of thermal variability on physiological traits, or the mechanisms thought to determine the occupied elevation ranges of temperate aquatic insects from the Colorado Rocky Mountains and tropical aquatic insects from the Ecuadorian Andes. This body of work represents a first attempt at explaining freshwater

species distributions across broad geographic areas by incorporating physiological traits and population genetic structure.

My first study (Ch. 2) tested the seminal hypothesis proposed by Dan Janzen in 1967, in a paper entitled "Why Mountain Passes are Higher in the Tropics" (Janzen, 1967). The hypothesis states that wide fluctuations in temperature characteristic of temperate mountains should favor the evolution of species with broad thermal tolerances, enabling them to disperse freely to high and low elevations. By contrast, the stable climatic regime of tropical mountains should select for species with narrow thermal tolerances, restricting their dispersal (Janzen, 1967; Ghalambor et al, 2006). I found strong support for this hypothesis. At any given elevation, temperate aquatic insects had broader thermal breadths, i.e. higher critical maximum temperatures and lower critical minimum temperatures, compared to their tropical relatives. What is particularly interesting about these patterns is that even within a latitude, insects residing in streams with wide thermal variation also tended to have wider thermal breaths. This was most clearly evident at high elevation in the tropics, where stream temperatures can shift drastically even within a 24 – hour period. Insects from these streams typically displayed broader thermal tolerances. The pattern was reversed in the Rocky Mountains, where high elevations are more stable (consistently cold) compared to low elevation. Here, high elevation species had narrower thermal tolerances compared to their low elevation relatives. Thus, differences in thermal tolerance do not only occur at the scale of latitude, i.e., between temperate and tropical regions. They can also be applied at local scales, such as high and low elevation and perhaps even at still smaller scales.

One assumption implicit in Janzen's climate variability hypothesis is that organisms on temperate mountains should have the ability to acclimatize to changing temperature across seasons (Deutsch et al., 2008; Ghalambor et al., 2006a; Janzen, 1967). This ability is critical to

the persistence of populations in predictably or stochastically variable climates. I tested this hypothesis (Ch. 3) by subjecting temperate and tropical mayflies and stoneflies to warming treatments to assess changes in their critical thermal maxima. An increase in the critical thermal maximum in response to the warming treatment indicated the ability to acclimate. I found that temperate mayflies were able to acclimate to the warming treatment, but tropical mayflies could not cope with the temperature increase. These data suggest that tropical mayflies have a reduced ability to acclimate and may be far more vulnerable to warming than their temperate counterparts. However, tropical mayflies once again displayed higher acclimation ability than low elevation mayflies. The results for stoneflies in this study were less clear. Tropical stoneflies had an equivalent or greater acclimation ability than temperate stoneflies. This discrepancy could be the result of differences in morphology, respiratory physiology, life history, and evolutionary history, which may influence their physiological capabilities. Our results highlight the importance of studying a variety of thermal tolerance traits in organisms and caution against making generalizations based on studies of any single taxon.

In a final assessment of the effect of temperature on physiology, I turned to measures of metabolic rate (Ch. 4). I focused on mayflies from the same temperate and tropical streams and measured changes in metabolic rate at different temperatures. Because metabolic rate is often called "the rate of life" (Gillooly, Brown, West, Savage, & Charnov, 2001a) and is strongly influenced by temperature, it is a particularly important and relevant trait to assess (Johnston et al., 1991; Rangel & Johnson, 2018) . Theory suggests that metabolic rate should increase slowly over optimum temperatures, then rapidly over stressful temperatures (H. O Pörtner, 2002). If temperate mayflies truly have a broad thermal tolerance, the slow increase in metabolic rate should occur over a broader range of temperatures and they should experience stress only at very

high temperatures. In contrast, tropical mayflies should have a narrower range of "optimum" temperatures and experience stress at moderately high temperatures. I found support for these predictions, where not only did tropical mayflies display narrower thermal preferences, but they experienced high levels of stress and mortality at the higher test temperatures. Low elevation tropical mayflies appeared to be the most thermally sensitive as their Q_{10} values were high, and they suffered mortality at the lowest and highest temperatures.

By studying thermal breadth, acclimation ability, and metabolic rate in aquatic insects, we have evidence that temperature differentially affects tropical versus temperate insects. But even with a mechanistic understanding of how environmental factors such as temperature influence inter- and intraspecific distribution patterns, evolutionary ecologists are faced with the dilemma of explaining why species ranges are not continually expanding, given that natural selection acts to improve a population's ability to persist in an environment (Kirkpatrick & Barton, 1997). According to the Climate Variability Hypothesis, narrow thermal tolerance restricts the movement of tropical species and confines them to a narrow elevation range. Temperate species, however, should be able to occupy large geographic ranges, given their broad thermal tolerance (Janzen, 1967). To address this idea, I explored the effects of temperature on gene flow among temperate and tropical populations of mayflies (Ch. 5). I also assessed whether geographic distance, maximum stream temperature, or critical thermal maxima could best explain population structure at the two latitudes. As predicted, I found greater genetic population structure in the tropical mayfly populations compared to temperate populations. This suggests that there is restricted gene flow among populations in the tropics. Moreover, maximum stream temperature was the best explanatory variable for genetic structure in tropical populations. Temperate populations experience more gene flow, such that there is low genetic structure, and a

high level of admixture. None of the variables (distance, temperature, or CT_{MAX}) were found to explain overall genetic structure in temperate populations. These data suggest that temperate mayflies are likely to be able to expand their ranges as they are not limited by their physiological capacity or sensitivity to temperature. However, tropical mayflies must face a greater challenge if they are to move to elevations higher or lower than those they currently occupy.

Conclusions and significance

The primary goal of my dissertation was to empirically test the effects of temperature on the thermal physiology and distribution of species. To do this, I measured a number of physiological traits and linked environmental temperature to genetic structure in populations that were found to be thermally sensitive. The results from my physiological trait experiments showed overwhelming evidence of sensitivity to temperature in tropical aquatic insects. I found general consensus across all three traits which converge four main conclusions. First, tropical mayflies have narrower thermal breadths, decreased acclimation ability, and function over a narrower range of temperatures compared to related temperate mayflies. Second, of the tropical mayflies, high elevation populations have the broadest tolerances, whereas low elevation populations have the narrowest. Third, this pattern is reversed in temperate latitudes, where high elevation mayflies may be more thermally sensitive compared to low elevation populations. Fourth, there is a remarkable difference in genetic structure of populations of temperate and tropical mayflies, driven largely by the above mentioned differences in thermal tolerance traits. These results not only provide a mechanistic explanation for the differences in range sizes of aquatic insects (Gill et al., 2016), but also suggest that tropical aquatic insects are likely to be some of the most vulnerable species to global climate change.

This body of work represents a first attempt at explaining freshwater species distributions across broad geographic areas by incorporating physiological traits and population genetics. Broadly, I have found support for Janzen's hypothesis (1967), and conclude that mountain passes may indeed be "higher" in the tropics. Aquatic insects have largely been ignored in the literature of thermal physiology and evolutionary ecology, despite their immense importance to the healthy functioning of freshwater mountain streams (Covich et al, 1999). Thus, from a conservation standpoint, this work highlights the need for steps to mitigate rises in stream temperatures are necessary. These may include increasing canopy cover over streams, or preventing dam construction on rivers that harbor high levels of aquatic insect diversity. Though many important questions remain, my work serves to emphasize the importance of temperature in shaping the ecology of aquatic insect populations and the use of thermal tolerance traits in predicting the response of aquatic insect species to warming streams.

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2. CLIMATE VARIABILITY PREDICTS THERMAL LIMITS OF AQUATIC INSECTS ACROSS ELEVATION AND LATITUDE[2]

SUMMARY

Janzen's extension of the climate variability hypothesis posits that increased seasonal variation at high latitudes should result in greater temperature overlap across elevations, and favor wider thermal breadths in temperate organisms compared to their tropical counterparts. We tested these predictions by measuring stream temperatures and thermal breadths (i.e. the difference between the critical thermal maximum and minimum) of 62 aquatic insect species from temperate (Colorado, USA) and tropical (Papallacta, Ecuador) streams spanning an elevation gradient of *ca.* 2000m. Temperate streams exhibited greater seasonal temperature variation and overlap across elevations than tropical streams, and as predicted, temperate aquatic insects exhibited broader thermal breadths than tropical insects. However, elevation had contrasting effects on patterns of thermal breadth. In temperate species, thermal breadth decreased with increasing elevation because CT_{MAX} declined with elevation while CT_{MIN} was similar across elevations. In tropical insects, by contrast, CT_{MAX} declined less sharply than CT_{MIN} with elevation, causing thermal breadth to increase with elevation. These macrophysiological patterns are consistent with the narrower elevation ranges found in other tropical organisms, and they extend Janzen's climate variability hypothesis to freshwater streams. Furthermore, because lowland tropical aquatic insects have the

narrowest thermal breadths of any region, they may be particularly vulnerable to short-term extreme changes in stream temperature.

INTRODUCTION

A fundamental goal of integrative biology is to understand how variation in climate shapes the thermal physiology, behavior, and geographic distribution of organisms (Spicer and Gaston 2009; Angiletta 2009; Bozinovic, Calosi & Spicer 2011). The "climate variability hypothesis" (CVH) predicts that more variable climates select for organisms with broader thermal tolerances, whereas less variable (stable) climates select for narrower thermal tolerances (Dobzhansky 1950; Janzen 1967; Stevens 1989; Gaston & Chown 1999; Angiletta 2009). Thus, compared to the climatically stable tropics, mid-to-high latitude temperate environments should select for organisms with broader thermal breadths because of the seasonal variation between warm summer and cold winter temperatures (Angiletta 2009). In an extension of the CVH, Janzen (1967) incorporated the influence of elevation on thermal breadth. In temperate mountains, seasonal temperature changes cause low and high elevation localities to have similar annual ranges, which should allow species to physiologically tolerate a wide range of temperatures. However, in tropical mountains at any given elevation, a narrow annual range of temperatures should lead to a narrow thermal tolerance. The narrower thermal breadth of tropical species should lead to reduced dispersal and a reduced elevation distribution. Thus, Janzen (1967) proposed that mountain passes should be "higher" for lowland tropical species because colder temperatures at higher elevations would be a greater physiological barrier to dispersal compared to temperate lowland species (Huey 1978; Ghalambor et al. 2006; McCain 2009, Chan et al. 2016).

Macrophysiological studies of terrestrial species have shown general support for the predicted relationship between thermal tolerance and latitude (Brattstrom 1968; Feder 1978; van Berkum 1988; Addo-Bediako, Chown & Gaston 2000; Calosi et al. 2008; Deutsch et al. 2008; Sunday, Bates & Dulvy 2011) and elevation (Addo-Bediako et al. 2000; Ghalambor et al. 2006; Bozinovik et al. 2011; Sheldon & Tewksbury 2014). For most organisms, this relationship is driven by maximum (CT_{MAX}) and minimum (CT_{MIN}) critical thermal limits that track maximum and minimum environmental temperatures, respectively (Angiletta 2009). In the warmer lowland tropics, organisms should exhibit higher CT_{MAX} and CT_{MIN} values compared to organisms from colder, temperate regions. High elevation tropical species may have low CT_{MIN} values similar to those found in temperate species. Consequently, we predict that temperature variation across elevation and latitude should shape macrophysiological patterns in thermal limits. The generality of such patterns remains undescribed for most taxa (Gaston et al. 2009), and studies of temperate and tropical freshwater ectotherms are particularly lacking (Chown, Duffy & Sørensen 2015; Gutíerrez-Pesquera et al. 2016).

Freshwater streams naturally occur along elevation gradients and exhibit global scale variation in thermal regimes that can be similar to those observed for air temperatures (Ward 1985). As with air temperature, increasing elevation is accompanied by a decrease in stream temperatures as a function of the adiabatic lapse rate (Dillon, Frazier & Dudley 2006). However, due in part to the high heat capacity of water, stream temperatures are more stable and do not vary linearly with air temperatures (Mohseni & Stefan 1999). Across latitude, lowland tropical streams may be generally warmer and temporally more stable than temperate streams (Boulton et al. 2008). This variation in stream thermal regime likely drives several aspects of aquatic insect life-history, such as dormancy, growth rate, and timing of emergence (Ward & Stanford 1982).

Other factors also interact with stream temperature to impose unique challenges on the thermal tolerance of aquatic ectotherms, most notably oxygen availability, which might vary with elevation and stream flow (Clark, Webb & Ladle 1999; Pörtner & Knust 2007; Jacobsen, Rostgaard & Vásconez 2003; Verberk et al. 2011). The degree to which global scale climatic variability is mitigated by such local scale effects on thermal breadth in temperate and tropical stream organisms is not known for aquatic insects, but has important implications for our understanding of how aquatic and terrestrial organisms differentially cope with temperature variation, and for their vulnerability to climate change (Verberk et al. 2016).

Here, we test two important components of Janzen's (1967) climate variability hypothesis in temperate and tropical stream insects. We first test the assumption that tropical stream temperatures are less variable and exhibit reduced overlap across elevation compared to temperate zone streams. Second, we test the prediction that tropical aquatic insects have narrower thermal breadths relative to their temperate counterparts, by measuring the difference between the maximum (CT_{MAX}) and minimum (CT_{MIN}) critical thermal limits (Huey & Stevenson 1979). We focus on species of aquatic insects that occur across a range of elevations in temperate Colorado, U.S.A and tropical Ecuador.

METHODS

Study Sites

Our study streams were distributed at a mid-latitude temperate location, the Rocky Mountains of northern Colorado, USA (40° 35' N, 105° 5' W), and a low latitude tropical location, the Andes Mountains of Ecuador (0.3° 10' S, 78° 28' W). We selected five streams from 1,992 to 3,166m above sea level (a.s.l.) in the Cache la Poudre drainage in Colorado and seven

streams from 1,845 to 3,898m a.s.l. in the Papallacta-Quijos drainage (headwaters of the Amazon

Basin) in Ecuador. To control for any confounding effects of stream flow, water depth, or width,

we only sampled low-order (wadeable) streams that were similar in size.

Quantifying Stream Temperature Variability

At each stream, (1,992m, 2,212m, 2,950m, 2,798m, and 3,166m in Colorado and 1,845m,

2,694m, and 3,683m in Ecuador) we submerged temperature loggers (3001, Solinst Canada Ltd.

Georgetown, Ontario) to record hourly fluctuations in water temperature over a period of several

months (Oct-Apr). Loggers could not be submerged in streams at 2,957m and 3,387m in

Ecuador. Landslides and flooding of the Andean streams prevented loggers from collecting data

for an entire year. We also measured stream temperature with a field meter (Pro ODO, YSI

Incorporated, Yellow Springs, Ohio) at every site during each insect collection.

Aquatic Insect Collection, Identification, Transport, and Housing

We focused on species of aquatic insects from three ecologically important orders:

Ephemeroptera (mayflies), Plecoptera (stoneflies), and Trichoptera (caddisflies). Because the

majority of freshwater insect life cycles are spent in the water as juveniles, we tested this stage in

our experiments and not the short-lived flying adult stage. While the climatic variation in water

experienced by the juvenile stage is likely reduced compared to what the adults experience in air

(Kingsolver et al. 2011), successful emergence for the adult stage is dependent on the growth and

survival challenges of the juvenile larval stage, which for some species, can last several years.

Thus, we focused our efforts on testing the effects of climate variability on thermal tolerance

during the juvenile stage. We selected species from families that occur at multiple elevations at

both latitudes, were members of various functional feeding groups (Appendix Table S1.1), and

were numerically abundant to ensure adequate sample sizes. Insects were collected, acclimated, and tested from one elevation at a time. We collected aquatic insects between 8:00AM and 11:00AM using a D-frame kick net (mesh size: 500 μm). Insects were identified based on morphology to the lowest taxonomic level possible in the field without a microscope (i.e. morphological taxonomic units, MTUs), usually to genus and in some cases to species.

After collection from the stream, insects were transported to a laboratory (Fort Collins in the Colorado Rockies or the town of Baeza in the Ecuadorian Andes). In the lab, we maintained insects at the average temperature of stream from which they were collected. For a given elevation, these temperatures were the same between the two latitudes. A moderate water current was generated within the holding cooler using an aquarium pump. Insects remained in mesh-enclosed containers for 48 h on a 12:12 h light-dark cycle. This period allowed insects to acclimate to laboratory conditions and ensured that all individuals experienced a similar environment prior to experiments.

Determining Critical Thermal Limits

Our goal was to test if there is a relationship in the field between the amount of temperature variability at a given site and the observed patterns of thermal breadth (i.e. difference between CT_{MIN} and CT_{MAX}). We measured critical thermal limits using thermal ramping (Lutterschmidt & Hutchinson 1997; Duarte et al. 2012). While estimates of critical thermal limits can be sensitive to the measurement protocol (Terblanche et al. 2007; Rezende et al. 2011), the same methodology was used for all species to facilitate comparisons. One aspect of the experimental design that requires particular attention is the choice of acclimation and starting temperatures. Because comparisons were made across species from very different thermal environments, all species were acclimated to average stream temperature at the time of collection

and that same temperature was used at the start of each experiment. Thus, what we report are the natural "un-manipulated" patterns of CT_{MIN} and CT_{MAX} across elevation and latitude that likely reflect a combination of genetic and plastic influences. Using the stream temperature at the time of collection also allowed us to avoid the confounding effects of deteriorating body condition associated with acclimating all species to a single temperature outside their preferred temperature range (see Rezende et al. 2011). Indeed, during separate experiments designed to test the degree of plasticity in critical thermal maximum rates, we found the use of acclimation temperatures outside the range normally experienced resulted in obvious stress and mortality, particularly in tropical species, making comparisons less meaningful (Shah et al. *unpublished*). See Appendix for more information regarding methods.

CT_{MAX} experiments were conducted during the months of January–March 2013 and 2014 in Baeza, Ecuador (1,795m) and from June–August 2014 and 2015 in Fort Collins, Colorado (1,524m) (Appendix Methods). We ramped temperature at the rate of 0.3°C per min until a pre-determined, sub-lethal behavior was observed (Appendix Table S1.2). In each experiment, we tested up to 12 individuals (mean = 8.34, min = 4) per MTU per stream site (n=847 individuals, 23 MTUs). When individuals approached CT_{MAX}—determined by a loss of righting response— we transferred individuals to aerated water at normal stream temperature for recovery.

We conducted CT_{MIN} experiments on 299 individual insects (11 MTUs), during October–December 2014, in Ecuador, and June–August 2015 in Colorado (Appendix Methods). In Ecuador, all insects could not be collected at two elevations (1,845 and 2,798m a.s.l.), where CT_{MAX} data were collected, due to an oil pipeline construction project and a severe landslide. To measure $CT_{MIN,}$ we ramped temperature down at the rate of ~0.3 °C min^{-1}. Each CT_{MIN} trial

consisted of testing a maximum of 9 individuals per MTU (mean = 8.5, min = 5). We used data from only those insects that recovered from the experiments, i.e. resumed normal activity.

Taxonomic Identification and Phylogeny Reconstruction

To establish the taxonomic identity of every individual in our study, we used a hierarchical approach to species assignment. First, we DNA barcoded (Hoffmann & Blows, 1994; Irwin & Gibbs, 2002) a subset of specimens (n = 323 out of 956) following standard protocols from the Canadian Center for DNA barcoding (Hajibabaei et al., 2005; Natalia V Ivanova, DeWaard, Hajibabaei, & Hebert, 2006; NV. Ivanova, DeWaard, & Hebert, 2006). Second, in cases where no specimens were DNA barcoded for a MTU and site, we used a larger data set of DNA barcoded specimens to determine the known species composition at that site. These data were used to assign a species to every non-barcoded MTU. In some cases (n = 21), MTUs that were not barcoded could not be assigned to a single species because more than one cryptic species was present at a site. In these cases, we assumed proportional representation and distributed the MTUs evenly between the possible species present. This combination of morphological, genetic, and geographic information was then used to develop a phylogenetic hypothesis for all specimens in the study (see Appendix I for details).

Statistical Analyses

All statistical analyses were conducted in R version 3.1.3 (R Core Team 2013). To validate the assumption that temperature regimes are more variable in the temperate streams than in the tropical streams in our study, we calculated the coefficient of variation (CV) in temperature for each stream. Next, we used a general linear model (GLM) to test the effect of

elevation, latitude, and their interaction on stream temperature range. Lastly, we conducted ANOVAs for each latitude, to explore how stream temperature varies with elevation.

Effects of Latitude and Elevation on Critical Thermal Limits

To test the effects of latitude and elevation on CT_{MAX}, CT_{MIN}, and thermal breadth we used phylogenetic generalized least squares regression (Grafen, 1989) fit with an Ornstein–Uhlenbeck model of trait evolution (OU; (Butler & King, 2004; Hansen, 1997). We chose the OU model based on its low AIC and higher log likelihood values in a model selection analysis (Appendix Table S1.4). We ran PGLS for CT_{MAX}, CT_{MIN}, and thermal breadth separately, using a model that included latitude, elevation, dry weight (Gaston & Spicer 1988), and an interaction between latitude and elevation. We included elevation in the model because it was highly correlated with maximum and average stream temperature (Appendix Fig. S1.1) and dry weight as a covariate to account for differences in thermal limits due to body size. Because thermal breadth was calculated from different individuals used in the CT_{MAX} and CT_{MIN} experiments, we used a mean dry weight of individuals. Finally, we used separate PGLS analyses for each latitude to test if there was a relationship between stream temperature range and thermal breadth while controlling for dry weight.

RESULTS

Stream Temperature Variability

Tropical streams were less thermally variable than temperate streams regardless of elevation (Figs 2.1A, B; Table 2.1). The major qualitative difference between the temperate and tropical regions is the clear influence of seasons in the temperate streams. Cold winters result in all streams converging on 0°C, at all elevations (Fig. 2.1A). At lower elevations temperate

streams warm more quickly and reach higher values in the transition from winter to spring and summer. But there is also a signal of spring snowmelt runoff that returns seasonally rising temperatures to near freezing for approximately three weeks between February and March. In contrast, tropical streams exhibit no seasonal patterns in temperature, and their thermal coefficients of variation (CV) are largely driven by diel variation in stream temperature (Fig. 2.1B). Annual thermal CV were consistently lower for tropical streams compared to temperate streams (Welch's Two-Sample T-Test corrected for unequal variance: $t_{4.9} = 11.435, p<0.0001$). For any given elevation, temperate streams had higher maximum and lower minimum annual temperatures (Table 2.1). Yet, on average, temperate streams were colder (mean annual temperature $4.2 \pm 0.99°C$) than tropical streams (mean annual temperature $10.5 \pm 0.83°C$). We found a significant elevation by latitude effect on stream temperature range (GLM: $F_{1,16} = 73.14, p<0.001$). Specifically, tropical stream temperature range remains stable across elevation (although it exhibits an increasing trend, ANOVA: $F_{1,5} = 1.00, p=0.362$), but temperate stream temperature range decreases with elevation (ANOVA: $F_{1,3} = 19.57, p=0.021$; Fig. 2.1C).

Effects of Latitude and Elevation on Critical Thermal Limits and Breadth

The PGLS analysis revealed that latitude was a significant predictor of CT_{MAX} estimates (PGLS: $t_{82}= -2.745, p=0.007$). Specifically, CT_{MAX} was lower in tropical compared to temperate stream insects (Fig. 2.2; Appendix Fig. S1.2). CT_{MAX} also decreased with increasing elevation in temperate and tropical aquatic insects (Fig. 2.2). Dry weight was not a significant covariate. For CT_{MIN}, we found a significant latitude by elevation interaction (PGLS: $t_{46}= -6.473, p<0.001$) reflecting the fact that CT_{MIN} remains relatively constant and low across elevation in temperate aquatic insects, but decreases with elevation in tropical aquatic insects (Fig. 2.3; Appendix Fig. S1.2). Dry weight was not a significant predictor of CT_{MIN}.

The results of the PGLS analysis for thermal breadth revealed a significant interaction between latitude and elevation on thermal breadth (PGLS: $t_{46}= 3.937, p<0.001$; Appendix Table S1.3). This interaction reflects the large difference in thermal breadth between temperate and tropical species at low elevations and the convergence of thermal breadth at high elevations (Fig. 2.4A; Appendix Table S1.3). Lastly, we found that stream temperature range predicted aquatic insect thermal breadth (PGLS: $t_{48}=7.550, p<0.001$) (Fig. 2.4B; Appendix Table S1.3). This relationship was also significant *within* Colorado (PGLS: $t_{19}=3.525, p=0.003$) and Ecuador (PGLS: $t_{32}=2.326, p=0.027$), when controlling for phylogeny and dry weight. Thus, insects that experienced the widest stream temperature ranges had the broadest thermal breadths (Fig.2.4B; Appendix Table S1.3).

DISCUSSION

Janzen (1967) predicted that reduced climatic variability across an elevation gradient would result in tropical organisms with narrower thermal breadths compared to their temperate counterparts. Narrower thermal tolerance in the tropics has implications for patterns of dispersal, elevation range sizes, and rates of speciation (Janzen 1967; Ghalambor et al. 2006). We tested the relationship between temperature variability on patterns of thermal breadth ($CT_{MAX} - CT_{MIN}$) and found support for Janzen's predictions; tropical aquatic insects on average have narrower thermal breadths compared to their temperate relatives. Indeed, these results complement recent findings that tropical aquatic insects have reduced dispersal and smaller elevation ranges (Gill et al. 2016). However, variation in thermal breadth is not simply a response to different latitudes, but instead reflects the magnitude of temperature variation experienced at a given site. For example, the difference in thermal breadths are greatest when comparing temperate and tropical

species at lower elevations, and as elevation increases the relative differences in thermal breadth across latitude decline (Fig. 2.4A). The close relationship between temperature variation and thermal breadth is thus influenced not only by large-scale latitudinal differences, but also by local scale effects such as elevation. Such relationships appear to be a general pattern in freshwater systems, as Gutierrez-Pesquera et al. (2016) report a similar pattern in a comparison of temperate and tropical tadpoles. We explore these findings in greater detail below.

Patterns of Stream Temperature Variation Across Elevation and Latitude

Temperate and tropical streams can exhibit substantial variation in characteristics such as width, depth, groundwater input, and insolation (Ward 1985). We controlled for much of this variation by focusing on low-order mountain streams. While individual streams exhibit local variation, our comparative results indicate that tropical stream temperatures are 1) less seasonally variable than temperate streams, and 2) exhibit less overlap across elevation (Fig. 2.1). Aquatic organisms in these tropical streams experience considerably less annual variation in mean daily temperature (mean tropical range = 5.4°C; mean temperate range = 17.3°C, Table 2.1). Subsequently, there is reduced overlap in tropical stream temperature range across elevation, where the primary source of thermal variation appears to occur at the diel scale, particularly at higher elevations (Fig. 2.1C; Table 2.1). Temperate streams, however, exhibit reduced annual temperature variability with increasing elevation because maximum stream temperatures decrease with elevation, while minimum temperatures are similar across elevations (converging on 0 °C). Thus, organisms living in lower elevation temperate streams experience the widest variation in stream temperature, because these streams are warmer in summer and approach freezing temperatures in winter. In contrast, lower elevation tropical streams have the narrowest

temperature ranges, as the variation of water temperature is low year-round. This variation in stream temperature explains patterns of thermal breadth across latitude and elevation (Fig. 2.4).

Effects of Latitude and Elevation on Thermal Breadth

We found strong support for the prediction that thermal breadth is narrower in tropical compared to temperate streams (Fig. 1.4). However, across the elevation gradient, temperate and tropical aquatic insects exhibit contrasting patterns in their range of thermal tolerance. In tropical streams, insect thermal breadth increases with elevation as the difference between CT_{MAX} and CT_{MIN} increases, mostly driven by a sharper decrease in CT_{MIN} with elevation (Figs. 2.2 & 2.3). In temperate streams, the unchanging CT_{MIN} and the decreasing CT_{MAX} with elevation result in a narrowing of thermal breadth as elevation increases (Figs. 2.2 & 2.3). Thus, the low elevation temperate insects in this study system have the broadest thermal breadths, while low elevation tropical insects have the narrowest thermal breadths (Fig. 2.4). Had we been able to include even lower elevation tropical streams, we suspect thermal breadths could become narrower still. For high elevation species at both latitudes, in contrast, thermal breadths converge (Fig. 2.4A).

These patterns in thermal breadth reflect how stream temperatures change with elevation in each region. In the tropics, the average annual minimum and maximum temperatures declines with increasing elevation, but the difference between them remains constant (Table 2.1). The exception to this pattern occurs at the highest elevation above tree-line, where the grass-dominated tropical páramo receives high insolation, and stream temperatures show greater thermal variation than lower elevation forest-canopied streams (Table 2.1; Finn, Encalada, & Hampel 2016; see also Huey et al. 2009). In temperate streams, thermal breadth decreases with increasing elevation as the difference between annual minimum and maximum temperatures also decreases with elevation (Fig. 2.4A). In Colorado our highest sampling point was at 3100m a.s.l.

and additional work is required to determine whether thermal breadth continues to decrease at even higher elevations. Nevertheless, such results contrast with studies of terrestrial insects that find thermal breadth decreases with elevation because cold tolerance increases while heat tolerance remains unchanged (Gaston & Chown 1999).

Effects of Latitude and Elevation on Critical Thermal Limits

We found that temperate species exhibited greater tolerance for high temperatures compared to their tropical counterparts (Fig. 2.2). This result may appear counterintuitive at first because temperate streams are colder on average than tropical streams. However, at any given elevation, annual maximum temperatures are higher during the temperate summer (Table 2.1, Fig. 2.1), and the greater CT_{MAX} values in the temperate species reflect these maximum temperatures. Indeed, previous studies have shown high maximum environmental temperatures to be correlated with higher CT_{MAX} values for ectotherms (eg. Wu & Kam 2005; Kaspari et al 2015; Turriago, Parra, & Bernal 2015). Within latitude, CT_{MAX} is also sensitive to water temperatures, as it declines with elevation (Fig. 2.2). These results are intriguing because studies of terrestrial ectotherms have generally found CT_{MAX} to be largely invariant across elevation and latitude (e.g. Gaston & Chown 1999; Addo-Bediako et al. 2000; Ghalambor et al. 2006; Hoffmann, Chown, & Clusella-Trullas 2013). In marine ectotherms, however, a meta-analysis by Sunday et al. (2012) showed CT_{MAX} to closely track changes in ocean temperature. An increase in CT_{MAX} was also detected across latitude in freshwater diving beetles (Calosi et al. 2010). Thus, aquatic ectotherms may differ from terrestrial ectotherms in maintaining more variation in their physiological sensitivity to maximum temperatures. While the causes of this difference have yet to be explored, they may be linked to differences in oxygen availability. Water has 33 times less oxygen than air, which severely reduces the amount available for respiration (Jones

1972). Unlike air, warm water holds less oxygen than cold water and this property has been implicated in limiting thermal tolerance in aquatic organisms due to decreased oxygen supply (Pörtner 2001; Verberk et al. 2011).

Ectotherm CT_{MIN} values typically track minimum temperatures in both terrestrial and aquatic environments (Addo-Bediako et al. 2000; Sunday et al. 2012), and we found similar patterns in aquatic insects. In tropical stream insects, CT_{MIN} values decreased with increasing elevation (Fig. 2.3). Thus, high elevation tropical species are considerably more cold-tolerant than low elevation species. For instance, tropical Ephemeroptera at the highest elevation could withstand short periods of freezing during these experiments (A. Shah *pers. obs.*). This high cold tolerance reflects the persistent cold temperatures in the tropical highlands (Fig. 2.1B). In temperate streams, by contrast, near freezing temperatures routinely occur at all elevations in the winter, and not surprisingly, CT_{MIN} values for the temperate taxa were near freezing and invariant across elevation (Fig. 2.3). Indeed, our experiments with temperate insects often had to be terminated due to ice formation in the experimental water bath rather than the cessation of locomotor function. Such high levels of cold tolerance in temperate species call into question the ecological relevance of CT_{MIN}, and suggest that many temperate species could be freeze-tolerant. Although the streams we sampled never freeze solid and water temperatures remain at or above 0°C, many aquatic and semi-aquatic insect species have been documented to be freeze tolerant (e.g. Sinclair et al. 2003: Danks 2007).

The measurement of critical thermal limits can be affected by methodological factors like the acclimation temperature and ramping rate (Terblanche et al. 2007; Rezende et al. 2011). We avoided this problem by using the same methodological approach for all species. The only source of environmental variation across species was acclimating species at the average stream

temperature at the time of capture. While such an approach minimizes the effects of thermal stress on estimates of critical thermal limits, it does not allow us to partition variation across species due to genetically based evolved differences, versus plastic responses to the acclimation temperatures. However, certain comparisons suggest plasticity alone cannot explain the patterns. For example, our temperate and tropical low elevation sites had similar stream temperatures at the time of the experiments, and all species were acclimated at ~15°C. Despite the same acclimation temperature, there are very large differences in CT_{MAX} among species between latitudes (Fig. 2.2). The estimates for CT_{MIN} are also informative in this regard, because CT_{MIN} is thought to be very sensitive to acclimation temperatures (Chown 2001; Chown & Terblanche 2007; Terblanche et al. 2007). Despite different acclimation temperatures along the elevation gradient, almost all temperate species have approximately the same CT_{MIN} (Fig. 2.3). Thus, even when we restrict ourselves to comparisons of species reared under the same temperatures, or compare similar responses of species held under different acclimation temperatures, we still find the influence of latitude and elevation on species differences.

Implications for dispersal and vulnerability

Determining critical maximum and minimum limits lends insights into the physiological mechanisms driving species distributions and vulnerability of organisms to climate warming (Chown & Gaston 2015). Janzen (1967) predicted that the narrow thermal tolerances of tropical organisms result in reduced dispersal and narrower elevation ranges. Linking physiological tolerance to elevation range size is challenging (Bozinovic et al. 2011), but our results suggest that the wider thermal breadths of temperate Ephemeroptera may permit increased dispersal ability and broader elevation distributions compared to their tropical counterparts (Gill et al. 2016). Other studies have documented similar differences in the elevation range sizes of

temperate and tropical organisms (e.g. McCain 2009; Chan et al. 2016), but the degree to which physiology alone determines geographic distributions remains a topic of debate (Bozinovic et al. 2011; Jankowski et al. 2013).

Narrow thermal breadths are predicted to increase species vulnerability to rapid global climate change (e.g. Calosi et al. 2008; Deutsch et al. 2008; Chan et al. 2016; Garcia-Robledo et al. 2016). A key result from our study is that lower elevation tropical aquatic insect populations appear to be especially vulnerable to short-term extreme warming (or cooling) events. Consequently, even though the magnitude of projected warming is predicted to be relatively small in the tropics, such reduced thermal tolerance may increase vulnerability (see Deutsch et al. 2008). The degree to which dispersal to higher elevations could ameliorate such impacts in aquatic insects remains open to debate, because most long-distance dispersal is confined to the short-lived adult stage (Kovats, Ciborowski, & Corkum 1996). Perhaps surprisingly, the least vulnerable group of aquatic insects may be low elevation temperate species that naturally experience wide seasonal fluctuations in stream temperature, possess relatively broad thermal breadths, and appear to have the potential to disperse to higher elevations (Finn et al. 2016).

In summary, the interaction between elevation and latitude provides a cautionary lesson when generalizing across temperate and tropical organisms, particularly with respect to vulnerability and sensitivity to changes in temperature. Physiologically, high elevation temperate and tropical aquatic insects may exhibit similarly narrow thermal breadths, whereas low elevation temperate and tropical species represent the ends of the thermal tolerance continuum with the broadest and narrowest thermal breadths, respectively. Acknowledging these differences and incorporating them into future climate scenarios will likely provide more accurate predictions of how different populations will be impacted by warming.

Table 2.1. Elevations, annual maximum, minimum, range, and average temperatures of our study streams. Coefficients of variation (CV) were calculated for each stream.

	Stream Elevation (m)	Maximum (°C)	Minimum (°C)	Range (°C)	Average (°C)	CV (%)
Temperate (Colorado, U.S.A.)	1992	22.0	-0.4	22.4	6.77	89.69
	2212	21.4	-0.3	21.7	4.84	95.16
	2590	18.1	-0.4	18.5	4.64	108.64
	2798	11.5	-0.2	11.8	2.83	99.38
	3166	11.9	-0.1	12.1	2.04	134.46
Tropical (Papallacta, Ecuador)	1845	16.9	10.8	6.1	13.53	6.27
	2003	16.4	12.9	3.5	13.95	4.4
	2694	13.3	7.2	6.1	10.02	8.86
	2957	10.3	8.9	1.4	9.62	n/a
	3387	12.7	9.1	3.6	10.9	n/a
	3683	13.1	4.6	8.5	8.57	11.0
	3898	11.5	3.0	8.5	6.59	20.02
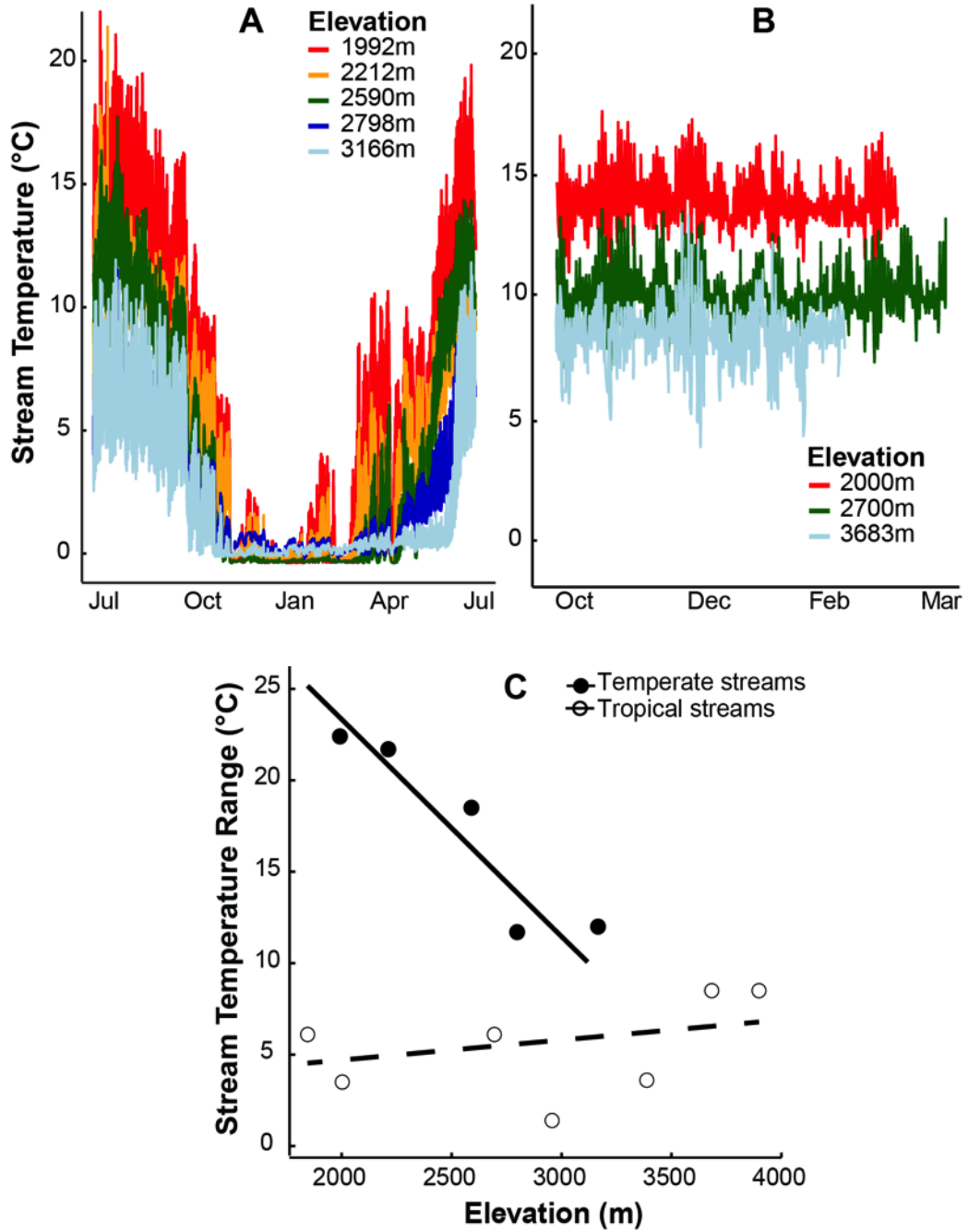
Figure 2.1. Stream temperature profiles for low-to-high elevation temperate streams, showing increased overlap in temperature, especially in the winter months (A), tropical streams with reduced overlap (B). C shows the relationship between stream temperature range (annual maximum - minimum) and elevation, where it decreases with elevation in temperate streams, but has an increasing trend with elevation in tropical streams.
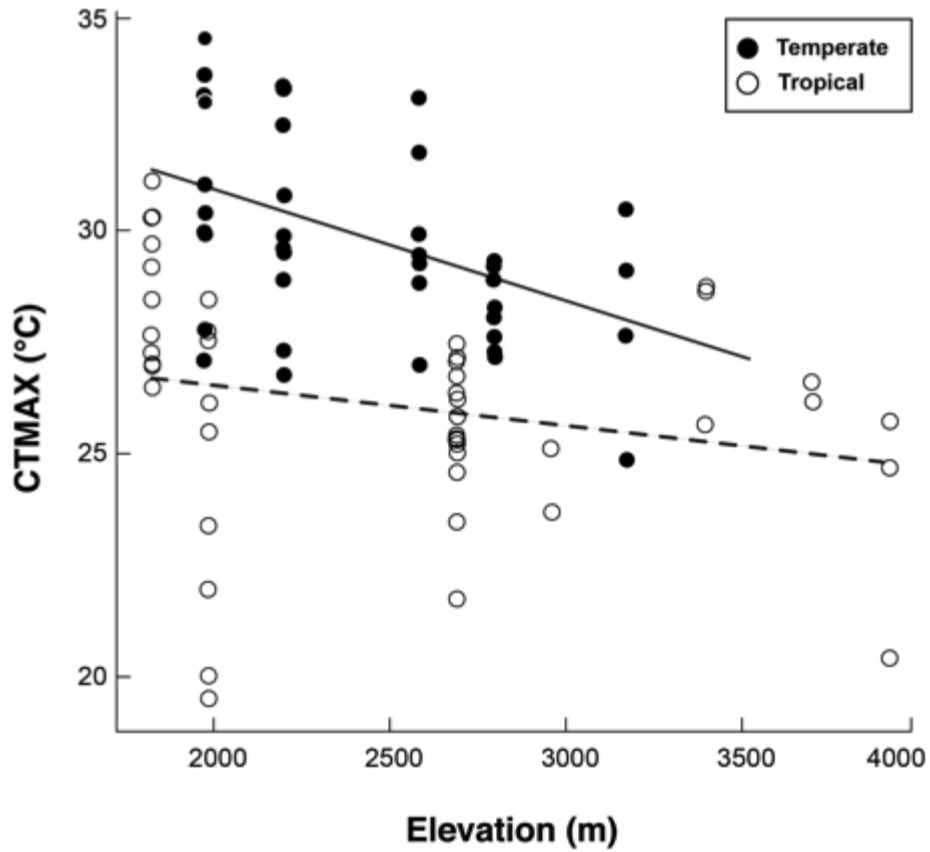
Figure 2.2. CT$_{MAX}$ declines with increasing elevation in Ecuador and Colorado. The regression lines, generated from the PGLS analysis do not show a significant interaction. In general, CT$_{MAX}$ was higher in insects from Colorado than in those from Ecuador.
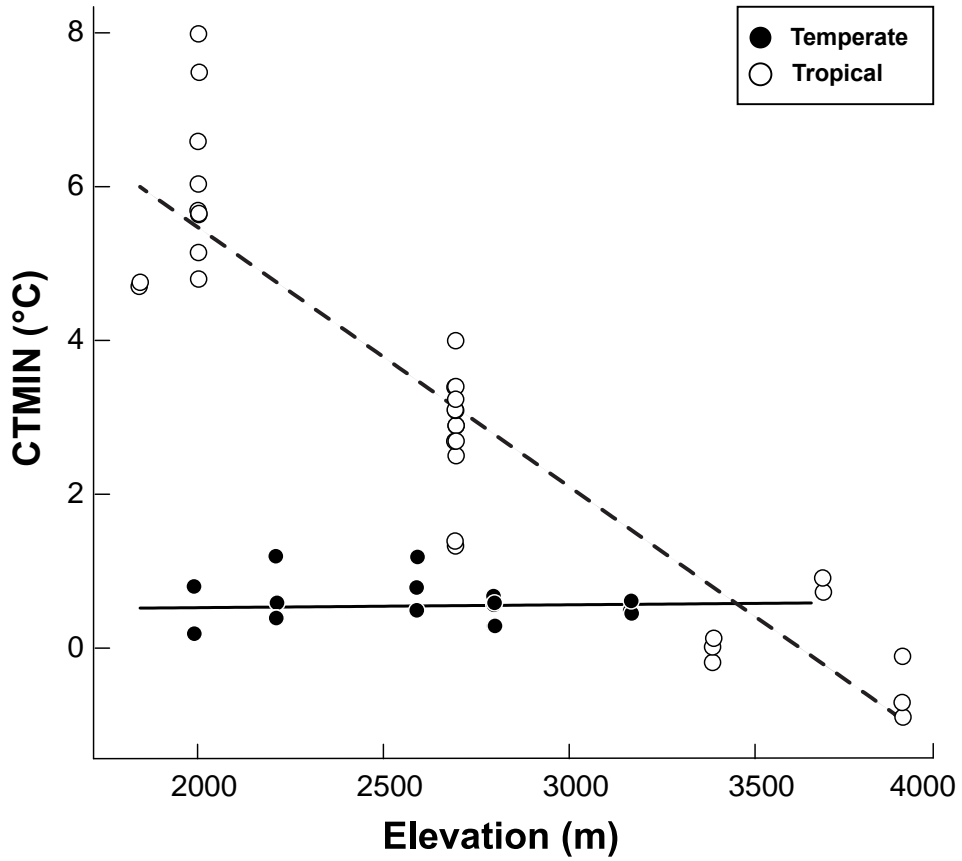
Figure 2.3. CT_MIN declines with increasing elevation only in Ecuador. In Colorado, there is no change in CT_MIN across elevation, resulting in a significant interaction between latitude and elevation. All regression lines were generated from the PGLS analysis.
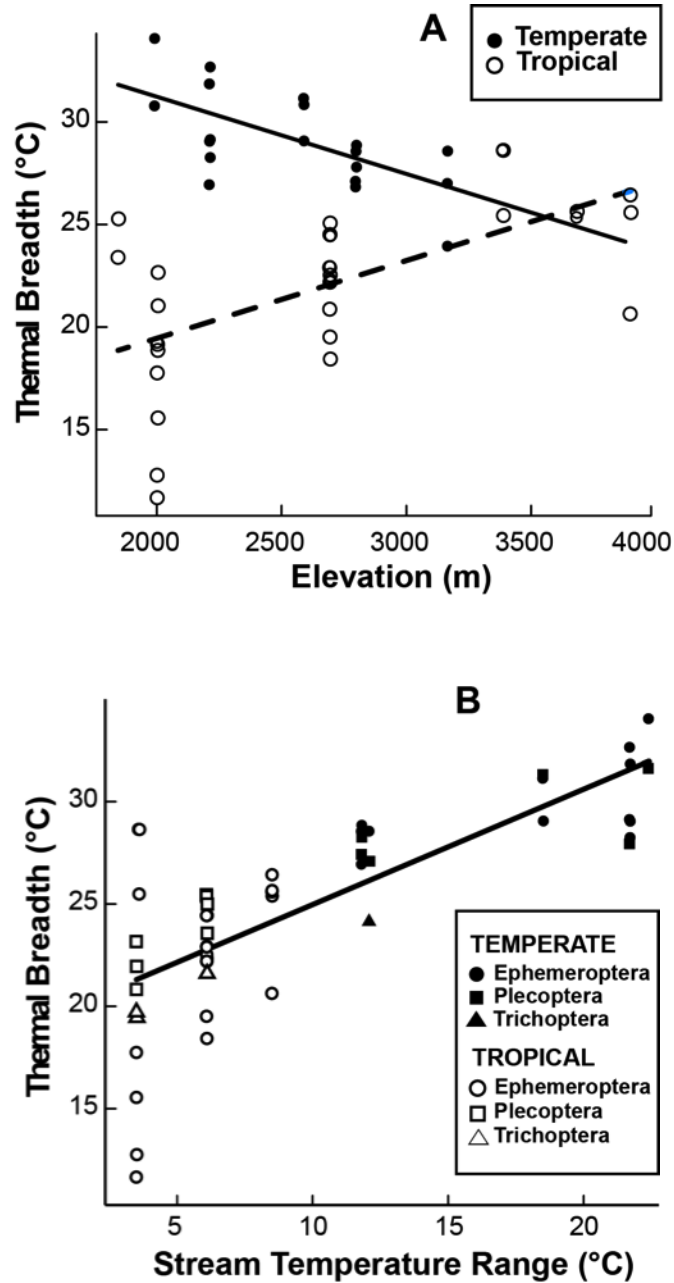
Figure 2.4. Thermal breadth shows a decreasing trend in Colorado, but an increasing trend in Ecuador (A). This matches the patterns of stream temperature range variation suggesting that within latitude stream temperature variation plays a role in shaping thermal breadth. B shows that between latitudes, stream temperature range predicts thermal breadth in aquatic insects. Colorado insects that experience on average wider temperature ranges also have wider thermal breadths compared to their relatives in Ecuador where temperature range is narrow.

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3. THERMAL ACCLIMATION ABILITY VARIES IN TEMPERATE AND TROPICAL AQUATIC INSECTS FROM DIFFERENT ELEVATIONS[3]

SUMMARY

It has long been recognized that populations and species occupying different environments vary in their thermal tolerance traits. However, far less attention has been given to the impact of different environments on the capacity for plastic adjustments in thermal sensitivity, i.e., acclimation ability. One hypothesis is that environments characterized by greater thermal variability and seasonality should favor the evolution of increased acclimation ability compared to environments that are aseasonal or thermally stable. Additionally, organisms under selection for high heat tolerance may experience a trade-off and lose acclimation ability. Few studies have tested these non-mutually exclusive hypotheses at both broad latitudinal and local elevation scales in phylogenetically paired taxa. Here, we measure short-term acclimation ability of the critical thermal maximum (CT_{MAX}) in closely related temperate and tropical mayflies (Ephemeroptera) and stoneflies (Plecoptera) from mountain streams at different elevations. We found that stream temperature was a good predictor of acclimation ability in mayflies, but not in stoneflies. Specifically, tropical mayflies showed reduced acclimation ability compared to their temperate counterparts. High elevation tropical mayflies had greater acclimation ability than low elevation mayflies, which reflected the wider temperature variation experienced in high elevation streams. In contrast, temperate and tropical stoneflies exhibited similar acclimation responses. We found no evidence for a trade-off between heat tolerance and acclimation ability in either

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[3] Shah, A. A., Funk, W. C., & Ghalambor, C. K. (2017). Thermal acclimation ability varies in temperate and tropical aquatic insects from different elevations. *Integrative and Comparative Biology*, 57(5), 977-987.

taxonomic order. The acclimation response in stoneflies may reflect their temperate origin or foraging mode. In combination with previous studies showing tropical taxa have narrower thermal breadths, these results demonstrate that many lower elevation tropical aquatic insects are more vulnerable to climate warming than their temperate relatives.

INTRODUCTION

Thermal acclimation is a form of plasticity that enables organisms to adjust their physiology following chronic or brief exposure to thermal stimuli (Michael J. Angiletta, 2009). Yet, the extent to which organisms exhibit thermal acclimation varies among species (e.g. Brattstrom 1968; Addo-Bediako et al. 2000; Deutsch et al. 2008; Beaman et al. 2016), types of traits (e.g. CT_{MAX}, CT_{MIN}, metabolic rate, performance; Johnson and Bennett 1995; Stillman 2003; Terblanche et al. 2005; Terblanche and Chown 2006; Calosi et al. 2008), and habitats (e.g. temperate, tropical, elevation; Feder 1978, 1982; Tsjui 1988). One fundamental challenge is therefore to understand what selection pressures act on and shape acclimation ability in organisms (Beaman et al. 2016).

Theoretical models suggest that populations from more thermally variable environments should be under greater selection for the capacity to be plastic, because the presumed benefits of maintaining thermal plasticity exceed any potential costs (e.g. Levins 1968; Gavrilets & Scheiner 1993; Hoffmann 1995; Angilletta 2009). At macrophysiological scales, this theory has led to the general prediction that organisms living at low, tropical latitudes should have a reduced acclimation capacity compared to those from temperate environments, because temperate latitudes undergo greater seasonal changes in temperature (Janzen 1967; Ghalambor et al 2006; Tewksbury et al 2008; Angilletta 2009; Gunderson and Stillman 2015). Indeed, the role of

temperature or climate variability in shaping species acclimation ability between high and low latitudes is one of the primary predictions of Daniel Janzen's "climate variability hypothesis" (1967). Janzen proposed that organisms from temperate mountains should evolve broad thermal breadths and greater acclimation abilities in response to the large seasonal fluctuations and overlap in temperature across elevations. Selection for broader thermal breadths or increased acclimation responses should subsequently enable temperate organisms to disperse across elevations, unhindered by the changes in temperature they encounter. In contrast, the lack of seasonal variation and reduced overlap in temperature across elevations in the tropics should favor the evolution of narrower thermal breadths and reduced acclimation ability (reviewed in Ghalambor et al. 2006). However, the relationship between thermal breadths and acclimation capacity can be shaped by other factors. It has been proposed that the evolution of high thermal tolerance should come at the expense of acclimation ability (Cavicchi et al. 1995; Stillman 2003; Overgaard et al. 2011; Gunderson & Stillman 2015). The "trade-off hypothesis" (sensu Gunderson & Stillman 2015) therefore predicts a negative relationship between increased thermal breadths (particularly for tolerance to warmer temperatures) and acclimation ability, whereas the climate variability hypothesis predicts a positive relationship.

Macrophysiological comparisons of temperate and tropical organisms provide ideal conditions for testing how thermal limits and acclimation ability vary independently or jointly. To date, there is growing evidence that tropical organisms have narrower thermal breadths compared to temperate species (Ghalambor et al. 2006; Deutsch et al. 2008; Tewksbury et al. 2008; Gaston et al. 2009; Huey et al. 2009; Sunday et al. 2011). However, evidence for differences in acclimation ability due to climate variability (i.e. in support of the climate variability hypothesis) is mixed (Angilletta 2009). For example, while some studies have found

reduced acclimation ability in tropical organisms (Feder 1978, 1982; Tsuji 1988; García-Robledo et al. 2016), other studies have found little or no difference in acclimation responses of temperate and tropical species (Hoffmann & Watson 1993; Gunderson & Stillman 2015; van Heerwaarden et al. 2016; Seebacher et al. 2015). Similarly, some studies have found evidence for the trade-off between thermal tolerance and acclimation ability (e.g. Cavicchi et al. 1995; Stillman 2003), but other studies do not find such evidence (e.g. Calosi et al. 2008; Gunderson and Stillman 2015). These mixed results could, in part, reflect how differences in local temperature variation shape thermal acclimation. For example, tropical high elevation sites can exhibit temperature fluctuations on a daily basis that are similar to those experienced between summer and winter in temperate regions (Mani 1967; Ghalambor et al. 2006; Shah et al. 2017). Thus, latitudinal comparisons of critical thermal limits and acclimation ability could be complicated by the degree of local variation in temperature due to elevation.

Understanding what factors ultimately shape patterns of thermal plasticity requires comparisons across diverse regions and taxa (Seebacher et al. 2015). Here, we focus on temperate and tropical aquatic insects; a group that plays an important ecological role in freshwater habitats, but have been largely ignored compared to terrestrial insects (Chown et al. 2015). We test predictions from two non-mutually exclusive hypotheses: 1) the climate variability hypothesis, which predicts that increased local or regional temperature variability is positively correlated with increased acclimation ability (e.g. Brattstrom 1968; Patterson 1984; Rogowitz 1996), and 2) the trade-off hypothesis, which predicts that increased thermal tolerance is negatively correlated with acclimation ability (Stillman 2003). In addition to addressing the long-standing question of what factors shape patterns of thermal physiology in organisms,

understanding geographic variation in acclimation ability is paramount to assessing vulnerability and species response to climate change (Sgrò et al. 2016).

We measured the short-term acclimation response (48 h; defined in Bowler 2005) of critical thermal maximum temperatures (CT_{MAX}) in two orders of freshwater aquatic insects, mayflies (Ephemeroptera) and stoneflies (Plecoptera). Previous work has shown that the thermal breadths (the difference between CT_{MAX} and CT_{MIN}) of these insects closely track environmental temperature variation (Shah et al. 2017). Here, we focused on short-term acclimation because both temperate and tropical aquatic insects experience short-term changes in temperature, whereas only temperate species experience long-term, seasonal changes in temperature. For instance, insects in high elevation temperate and tropical streams can experience rapid temperature changes because of the combined influences of reduced vegetation cover, cloud cover, and weather events that persist for several days (see Methods). The same taxonomic families can also be found in temperate and tropical regions facilitating phylogenetically paired comparisons. Such conditions make freshwater streams an excellent model system to test how large-scale climate variability and local variation along an elevation gradient shapes patterns of thermal acclimation.

METHODS

Study sites and Species

We collected aquatic insects from shallow paired streams at ~2000m ("low elevation", hereafter) and ~2800m ("mid elevation", hereafter) in the Colorado Rocky Mountains and the Ecuadorian Andes between the months of June and December 2014 and 2015. We also collected tropical mayflies from a "high elevation" stream in Ecuador (~3683m) but were unable to collect

from an equivalent high elevation in the Colorado study site because of a lack of accessibility. To assess how stream temperature ranges differed across latitude and elevation, we recorded temperature in each stream using HOBO loggers (Onset Corporation). We calculated temperature range from the logger data collected over an 8-12-month period. We collected mayflies (Ephemeroptera) within the family Baetidae (*Baetis spp*. in Colorado and *Andesiops spp*. in Ecuador) and stoneflies (Plecoptera) within the families Perlidae (*Hesperoperla sp*. in Colorado and *Anacroneuria spp*. in Ecuador) and Perlodidae (*Megarcys sp*. and *Kogotus sp*. in Colorado). Most of these species occur at both latitudes, multiple elevations, and are numerically abundant in streams. Insects from each species were size-matched to reduce any effects of developmental stage on thermal physiology.

CT$_{MAX}$ Acclimation Experiments

In the lab, we acclimated the field-caught insects for 48 h at one of two temperature treatments, "home" or "warming", which were the same at both latitudes for a given elevation. We were specifically interested in determining if CT$_{MAX}$ increased in the "warming" treatment, suggesting an acclimation response. The "home" temperature treatments, calculated as the average stream temperature from the HOBO logger data, were 13°C and 10°C for low and mid elevation streams, respectively. The "warming" temperature treatments were 5°C higher than the "home" temperatures. These were 18°C and 15°C for low and mid elevations, respectively. Thus, temperatures by elevation were paired *across* latitudes, because at these elevations the temperate and tropical sites had the same average temperatures. The use of different temperature treatments between elevations *within* latitudes ensured insects, particularly the sensitive tropical populations, were unstressed in their "home" temperature treatments (Rezende et al. 2011). We also collected mayflies from an un-paired high elevation tropical site (3683m, home temperature

treatment = 7°C; warming treatment = 12°C). Because we did not have insects from a similar high elevation in Colorado, this population was analyzed separately (see Statistical Analyses).

We chose a 5°C increase from the "home" stream temperature as the "warming" treatment because it is within the range of natural variation experienced by most aquatic insects (Alan et al. 1991; Shah et al. 2017). The acclimation period of 48 h was used because it was the duration for which insects could be kept in the laboratory without exhibiting any visible signs of stress due to food deprivation (see Rezende at al. 2011; Shah et al. 2017). For example, when we attempted longer acclimation periods (> 4 days), we found that insects experienced high mortality. All insects were starved during the "home" and "warming" acclimation period to ensure individuals were tested in a similar nutritional and post-absorptive state.

We measured CT_{MAX} following the 48-h acclimation period by placing up to 12 insects in separate mesh containers and immersing them in a water bath fitted with water and air pumps to provide flow and maximum oxygenation. The mesh allowed water to flow through the containers, but prevented insects from escaping during the experiment. We ramped temperature in the water bath at the rate of 0.3°C min^{-1} (Dallas and Rivers-Moore 2012) using a temperature controller (16C-2, Dwyer Instruments Inc.) connected to a 500watt titanium heating rod. As temperature increased, we recorded changes in behavior (see Shah et al. 2017 for detailed documentation of behavioral changes) until insects displayed a loss of righting response (LRR, Hutchinson & Lutterschmidt 1995; Angilletta 2009). After exhibiting LRR, each insect was returned to cooler water for recovery. Only data from insects that recovered from the experiments were used in the analyses. After recovery, insects were euthanized in 95% EtOH, dried for 24 h at 56°C, and weighed to obtain individual dried body mass measurements.

Statistical Analyses

We used R version 3.1.3 (R Core Team 2013) for all statistical analyses. Between latitudes, the acclimation temperatures were the same at each elevation, facilitating direct statistical comparisons between temperate and tropical insects under the same temperature treatments. For all analyses, an insect was considered to have acclimated if CT_{MAX} increased in the "warming" treatment compared to the "home" treatment. All model parameters were treated as fixed effects and effect sizes (partial eta^2) were also calculated.

To test the influence of climate variability on acclimation ability, we first ran an ANOVA with CT_{MAX} as our response variable. Parameters for this analysis included the fixed factors latitude, treatment, elevation, taxonomic order, all interactions, and dry weight as a covariate to account for body size. Next, we assessed the effect of temperature variability on acclimation ability by directly comparing the reaction norms of the different insect populations. We did this by conducting separate analyses for each taxonomic order, to control for phylogeny, and each elevation sampled across both latitudes. The models included latitude and treatment as fixed effects, an interaction between latitude and treatment, and dry weight as a covariate. We were particularly interested in finding if there was a significant interaction indicating that acclimation ability (i.e. the slope of the reaction norm) differed between temperate and tropical populations of insects from a given elevation. Third, for the un-paired high elevation stream in Ecuador, we ran an analysis in which we only tested the treatment effect, because there was no Colorado counterpart. Fourth, we analyzed within-latitude differences in acclimation ability separately for each taxonomic order to assess the effect of elevation on acclimation ability. If thermal variability at a given elevation influences acclimation ability, we predicted a stronger acclimation response with increasing temperature variation. We included elevation and treatment

as fixed effects with an interaction between elevation and treatment. As with the previous analysis with latitude, a significant elevation by treatment interaction would indicate that there are differences in acclimation ability among insects from different elevations. However, because different acclimation temperatures were used at different elevations, such comparisons must be interpreted cautiously. Finally, we tested the directional hypothesis that increased stream temperature variability is correlated with the percent change in CT_{MAX} (i.e. acclimation ability) for the two taxonomic orders.

To assess the if there is a trade-off between thermal breadth and acclimation ability, we tested the directional hypothesis that the percent change in CT_{MAX} decreases with increasing CT_{MAX}. For these analyses, we considered a decrease in CT_{MAX} after acclimation to be a non-acclimatory response.

RESULTS

Temperature loggers placed in each stream at both latitudes revealed that stream temperature range was greater in temperate (Colorado) streams than in tropical (Ecuador) streams (Fig. 3.1A). Within the temperate latitude site, temperature ranges in low elevation streams were greater than mid elevation streams. However, the pattern was reversed in tropical streams where low and mid elevation streams had a narrower temperature range compared to the high elevation stream (Fig. 3.1B; also see Shah et al. 2017).

The full ANOVA model results showed that latitude, treatment, elevation, and taxonomic order had a significant effect on CT_{MAX}. Dry weight and all interactions were non-significant (Table 3.1). When we tested if acclimation varied among our paired temperate and tropical taxa at the same elevation, we found the latitude x treatment interaction to be significant for all but

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the low elevation stoneflies (Table 3.2). Specifically, when examining the thermal reaction norms, we see that temperate, but not tropical mayflies increased CT_{MAX} in response to the warming treatment at the low and mid elevations (Figs. 3.2A, B; Table 3.3). Three of the stonefly populations exhibited a significant acclimation response across latitude (Fig. 3.3A, B), with no differences in the slope between latitudes at the low elevation site (Fig. 3.3A; Table 3.4). Interestingly, a significant latitude x treatment interaction at the mid elevation revealed that the tropical species had a greater acclimation response compared to its temperate counterpart (Fig. 3.3B; Table 3.2). When we compared the acclimation response of the low and mid-elevation mayflies to the unpaired high elevation population, we found only the high elevation population exhibited a significant acclimation response (Table 3.2; Fig. 3.4).

Finally, we found a positive correlation between stream temperature variation and percent change in CT_{MAX} (r = 0.85; one-tailed p-value = 0.036) for mayflies, consistent with predictions from the climate variability hypothesis, but no correlation in stoneflies (r= -0.31; one-tailed p-value = 0.347; Fig. 3.5A). We found no evidence of the trade-off hypothesis in mayflies (r = -0.01; one-tailed p-value = 0.492) or in stoneflies (r= -0.79; one-tailed p-value = 0.105; Fig. 3.5B).

DISCUSSION

The capacity for thermal acclimation is thought to be an adaptive response that allows organisms to adjust their physiology and track variable environmental temperatures (Kingsolver and Huey 1998; Gunderson and Stillman 2015; Seebacher et al. 2015). Nevertheless, the relationships between environmental thermal variability, thermal limits, and acclimation ability continue to be debated. Here, we tested two mutually non-exclusive hypotheses that have been

proposed to explain variation in whole organism acclimation ability. The "climate variability hypothesis" posits that organisms from more variable climates should be under selection for greater thermal acclimation ability than those from stable climates (Janzen 1967; Ghalambor et al. 2006; Angilletta 2009). The "trade-off hypothesis" proposes that organisms that evolve high levels of thermal tolerance do so at the expense of their acclimation ability (Stillman 2003). We found support for the prediction that more variable thermal environments select for greater acclimation ability in mayflies. Temperate mayflies and high elevation tropical mayflies that experience more variable temperatures exhibit a significant acclimation response (~2°C increase in CT_{MAX} over a 5°C warming treatment), whereas tropical mayflies from less variable lower and middle elevations do not exhibit an acclimation response (~0-0.5°C increase in CT_{MAX} over a 5°C warming treatment; Figs. 3.2A, B; 3.4). In contrast, the stoneflies generally exhibit a similar acclimation response at all latitudes and elevations despite having relatively high upper critical temperatures (Figs. 3.3A, B). Thus, climate variability predicted acclimation ability in mayflies, but not in stoneflies (Fig. 3.5A). We did not find support for the trade-off hypothesis, as there was no general relationship between increasing thermal tolerance and reduced acclimation ability (Fig. 3.5B). The contrasting results between mayflies and stoneflies may reflect differences in evolutionary history, functional roles, challenges associated with aquatic life, or some combination of these factors. Below we discuss these results within the context of how temperature varies across latitude and elevation.

Across latitude, tropical and temperate streams exhibit significant differences in temperature range (Figs. 3.1A, B). The warm summers and cold winters that characterize Rocky Mountain seasons result in wide ranges of annual stream temperatures. This range (i.e., from freezing to the warmest summer temperatures) is typically experienced from May, just before the

spring-melt, through late August. Small ectotherms, such as insects, are highly responsive to their thermal environment because their body temperature closely matches that of their surroundings. Unlike terrestrial insects, aquatic insect larvae can remain active year-round and experience the full range of temperature variation at a given site. For example, stonefly larvae often live for 2-3 years, thus encountering repeated seasonal shifts in temperature. Similarly, while mayflies overwinter as eggs, they hatch in early June when snowmelt causes stream temperatures to be very low. These larvae rapidly develop and emerge as flying adults a few months later, when temperatures peak in the summer, thus experiencing the full range of seasonal variation during their development. In the aseasonal tropics, streams typically exhibit far less temperature variation (Figs. 3.1A, B; see also Shah et al. 2017) except in the highest elevation tropical streams (Fig. 3.1B), where diel changes in temperature are much greater than those observed at lower tropical elevations. Thus, if temperature variation selects for acclimation ability in the tropics, it would be predicted to be more common only at high elevations, as we observed (Fig. 3.4).

We found greater acclimation ability in temperate mayflies compared to their tropical counterparts at similar elevations, whereas tropical stoneflies exhibited mostly similar acclimation ability compared to temperate stoneflies (Figs. 3.2A, B; 3.3A, B; 3.4). In tropical streams, neither low nor mid elevation mayflies acclimated after the 48-h period (Fig. 3.2). In fact, not only was there a lack of an acclimation response in the low elevation tropical mayflies (genus *Andesiops*), but they also experienced ~55% mortality during the 48-h acclimation period prior to experimentation. We did not document such high rates of mortality in any of the other populations of insects in this study. These results suggest that even a seemingly moderate 5°C increase in temperature can be stressful for low elevation populations. Thus, despite experiencing

only slightly warmer year-round temperatures than their mid-elevation counterparts, low

elevation tropical mayflies have the most reduced capacity for short-term thermal acclimation.

Our results also suggest that differences in CT_{MAX} across elevations are unlikely to reflect plastic

responses to different stream temperatures (Shah et al. 2017). For example, short-term

acclimation to warmer temperatures does not result in mid-elevation mayflies increasing their

CT_{MAX} temperatures to match that of the low elevation mayflies (Fig. 3.2), suggesting these

populations have evolved different upper thermal limits.

Further support for the role of temperature variability in selecting for greater acclimation

ability is supported by the observation that high elevation tropical mayflies show a significant

acclimation response (Fig. 3.4). High elevation tropical stream temperatures are significantly

colder than lower elevation streams, but they can also vary by ~7°C on a daily, weekly, or

seasonal basis (A.A Shah, *pers. obs.*) exposing insects to a remarkably large and rapid

temperature change in an otherwise thermally stable environment. Shah et al. (2017*)* found that

high elevation tropical aquatic insects had thermal breadths that were more like high elevation

temperate species, rather than other tropical species. Thus, the observed plasticity in high

elevation tropical mayflies is consistent with the view that it is the degree of local temperature

variability that selects for thermal breadth (Shah et al. 2017) and plasticity, rather than simply

latitudinal position. Indeed, there is a positive relationship between the amount of temperature

variation at a given site and the magnitude of the acclimation response in mayflies (Fig. 3.5A).

Collectively, such results suggest that mayfly acclimation responses are shaped by both large-

scale seasonal changes in temperature and local temperature variation.

Tropical stoneflies acclimate as well as (Fig. 3.3A) or better than (Fig. 3.3B) their

temperate relatives. These results stand in sharp contrast to the mayfly results. While there was

no general support for the trade-off hypothesis (Fig. 3.5B), as a group, temperate stoneflies are good candidates for tests of the trade-off hypothesis, as they have some of the highest CT_{MAX} values among all aquatic insects (Shah et al. 2017*)*. A broader comparison of stoneflies throughout their geographic range and across a more diverse set of thermal environments is ultimately needed to test if thermal limits trade off against acclimation ability. In other aquatic organisms, studies have found evidence for acclimation capacity-thermal breadth trade-offs (e.g. Stillman 2003), but others have not (e.g. Calosi et al. 2008; Simon et al. 2015). Thus, the generality of this hypothesis remains to be explored.

The evolutionary history of stoneflies may also provide some insight into the similar acclimation capacities between temperate and tropical species. The order Plecoptera has a northern Pangean origin, and is thought to have split into two lineages, Arctoperlaria and Antarctoperlaria relatively recently compared to mayflies (McCulloch 2010). The Antarctoperlaria subsequently dispersed to South America (Zwick 2000). If tropical stoneflies retained their ancestral traits, then high CT_{MAX} values and acclimation abilities of tropical stoneflies may reflect the temperate origin of this lineage.

Differences in thermal physiology among orders could also result from alternative evolutionary responses and constraints in how respiratory physiology, life-history, foraging, and habitat use impact heat tolerance via oxygen limitation (Pörtner 2001) and oxygen availability (Verberk et al. 2011). For example, stoneflies are active predators that presumably have high oxygen demands when hunting, and maintaining acclimation ability could be necessary for their foraging success (Grigaltchik et al. 2012). In comparison, the herbivorous mayflies that 'drift' along with the water flow may not require such precise thermoregulation. Stoneflies and mayflies also emerge at different times of the year, so some of the variation we observed could

also be linked to differences associated with various stages of development, particularly those close to molting (Camp et al. 2014). Additional studies that include several species from multiple temperate and tropical drainages could be used to further explore the variation in acclimation of thermal limits between different taxonomic groups.

Summary and Implications

We find evidence that climate variability plays an important role in determining acclimation ability in phylogenetically related temperate and tropical mayflies but not stoneflies. More work comparing different groups of temperate and tropical insects are needed before broader generalizations can be made. Nevertheless, in combination with measures of thermal breadth (i.e. the difference between CT_{MAX} and CT_{MIN}) across a large number of temperate and tropical aquatic insect species (Shah et al. 2017*)*, the mayfly results provide support for the role of climate variability in shaping thermal physiology across latitude and elevation gradients, as predicted by Janzen (1967).

We note that whole organism acclimation ability is only a first step in identifying how animals respond to changes in their thermal environment. Although beyond the scope of this study, a next step would include quantifying the responses to temperature at the molecular and biochemical level to better understand the physiological mechanisms driving shifts in whole organism thermal sensitivity (Hochachka and Somero 2002; Somero 2005, 2010).

These results also have important implications for how aquatic insect taxa, which are vital to healthy freshwater stream communities (Allan & Castillo 2007), will respond to warming global temperatures. Studies designed to estimate organismal responses to such changes often fail to incorporate acclimation capacity (see Gunderson et al. 2016). Freshwater ecosystems are predicted to respond the warming of atmospheric temperatures (Daufresne et al. 2007) and

increase by a range of +1 °C to +3.7 °C (IPCC 2007, 2013). Forecasts of climate change also suggest that thermal and natural disturbance regimes in small streams are likely to change in the near future, with warming being the most proximate effect (IPCC 2007, 2013). Yet, thermal tolerance for freshwater aquatic invertebrates is not well understood (Chown et al. 2015)

Human exploits of stream environments – riparian clearing, dams, and urbanization to name a few – have also been shown to increase stream temperatures well above their natural range (e.g. Brown & Krygier 1970) over relatively short periods of time. In our study system, landslides due to heavy rains and clear-cutting at the mid-elevation tropical stream resulted in a temperature increase of ~ 10°C in just 24 h (*pers. obs.*). Our results indicate that while some acclimation ability exists in tropical aquatic insects, they are likely to be more vulnerable to warming than their temperate counterparts. This is especially true of low and mid elevation tropical mayflies. In fact, even the moderate increase in CT_{MAX} after acclimation in high elevation tropical mayflies may not be enough to buffer those insects from warming streams. Although tropical stoneflies display some thermal plasticity, they too have lower CT_{MAX} temperatures compared to temperate species, which suggest greater sensitivity to warming. Future work should therefore address how temperature sensitivity determines species vulnerability, and alters species interactions, to better predict potential shifts in community composition of freshwater ecosystems.

Table 3.1. Output of the full model ANOVA in which latitude, treatment, and order are fixed effects, and dry weight is a covariate. The fixed effects are all significant but the interactions are not. Dry weight is not a significant covariate.

Factor	F(df)	p-value	Partial eta^2 (effect size)
Latitude	68.49 (1, 366)	**<0.001**	0.016
Treatment	56.74 (1, 366)	**<0.001**	0.133
Elevation	62.87 (2, 366)	**<0.001**	0.240
Order	78.54 (2, 366)	**<0.001**	0.305
Dry weight	0.26 (1, 366)	0.61	0.61
Latitude x Treatment	1.24 (1, 366)	0.27	0.27
Latitude x Elevation	0.002 (1, 366)	0.96	0.96
Treatment x Elevation	0.20 (2, 366)	0.81	0.81
Latitude x Treatment x Elevation	0.36 (1, 366)	0.55	0.55

Table 3.2. Output of models assessing the latitude x treatment interaction for mayflies and stoneflies from different elevations.

Factor	F(df)	P-value	Partial eta^2 (effect size)
Low Elevation Mayflies			
Latitude	14.41 (1, 26)	**<0.001**	0.35
Treatment	0.10 (1, 26)	0.75	0.00
Dry weight	1.00 (1,26)	0.32	0.00
Latitude x Treatment	4.62 (1, 26)	**0.04**	0.17
Mid Elevation Mayflies			
Latitude	2.52 (1, 119)	0.12	0.02
Treatment	17.34 (1, 119)	**<0.001**	0.13
Dry weight	0.05 (1,119)	0.82	0.00
Latitude x Treatment	5.97 (1, 119)	**0.02**	0.05
High Elevation Mayflies			
Treatment	8.23 (1, 21)	**0.01**	n/a
Dry weight	6.42 (1, 21)	**0.02**	n/a
Low Elevation Stoneflies			
Latitude	50.05 (1, 20)	**<0.001**	0.71
Treatment	12.57 (1, 20)	**0.002**	0.39
Dry weight	1.79 (1,20)	1.20	0.08
Latitude x Treatment	0.70 (1, 20)	0.41	0.03
Mid Elevation Stoneflies			
Latitude	200.36 (1, 93)	**<0.001**	0.68
Treatment	8.65 (1, 93)	**0.004**	0.09
Dry weight	3.55 (1, 93)	0.06	0.04
Latitude x Treatment	5.17 (1, 93)	**0.03**	0.05

Table 3.3. Output of models assessing the elevation x treatment interaction for mayflies and stoneflies from temperate and tropical latitudes.

Factor	F(df)	P-value	Partial eta^2 (effect size)
Tropical Mayflies			
Elevation	5.47 (2, 58)	**0.01**	0.16
Treatment	0.06 (1, 58)	0.81	0.00
Dry weight	3.11 (1, 58)	0.08	0.05
Elevation x Treatment	3.00 (2, 58)	**0.05**	0.09
Tropical Stoneflies			
Elevation	230.77 (1, 44)	**<0.001**	0.84
Treatment	47.39 (1, 44)	**<0.001**	0.52
Dry weight	2.08 (1,44)	0.12	0.05
Elevation x Treatment	1.69 (1, 44)	0.20	0.04
Temperate Mayflies			
Elevation	31.41 (1, 109)	**<0.001**	0.22
Treatment	24.32 (1, 109)	**<0.001**	0.18
Dry weight	0.02 (1, 109)	0.89	0.00
Elevation x Treatment	0.02 (1, 109)	0.90	0.00
Temperate Stoneflies			
Elevation	8.72 (1, 69)	**0.004**	0.11
Treatment	1.96 (1, 69)	0.17	0.03
Dry weight	12.43 (1, 69)	**<0.001**	0.15
Elevation x Treatment	1.56 (1, 69)	0.22	0.02
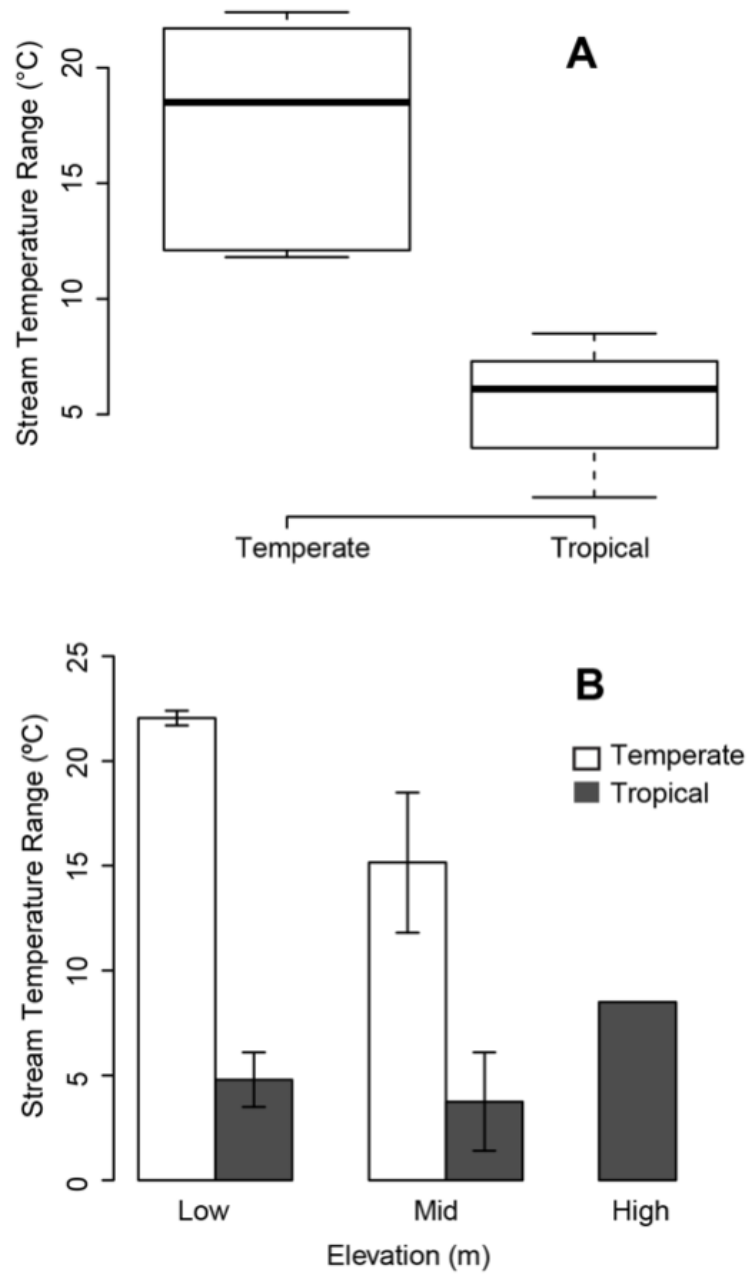
Figure 3.1. Average stream temperature range (annual maximum – minimum) at temperate and tropical latitudes (A) and across low, mid, and high elevations (B).
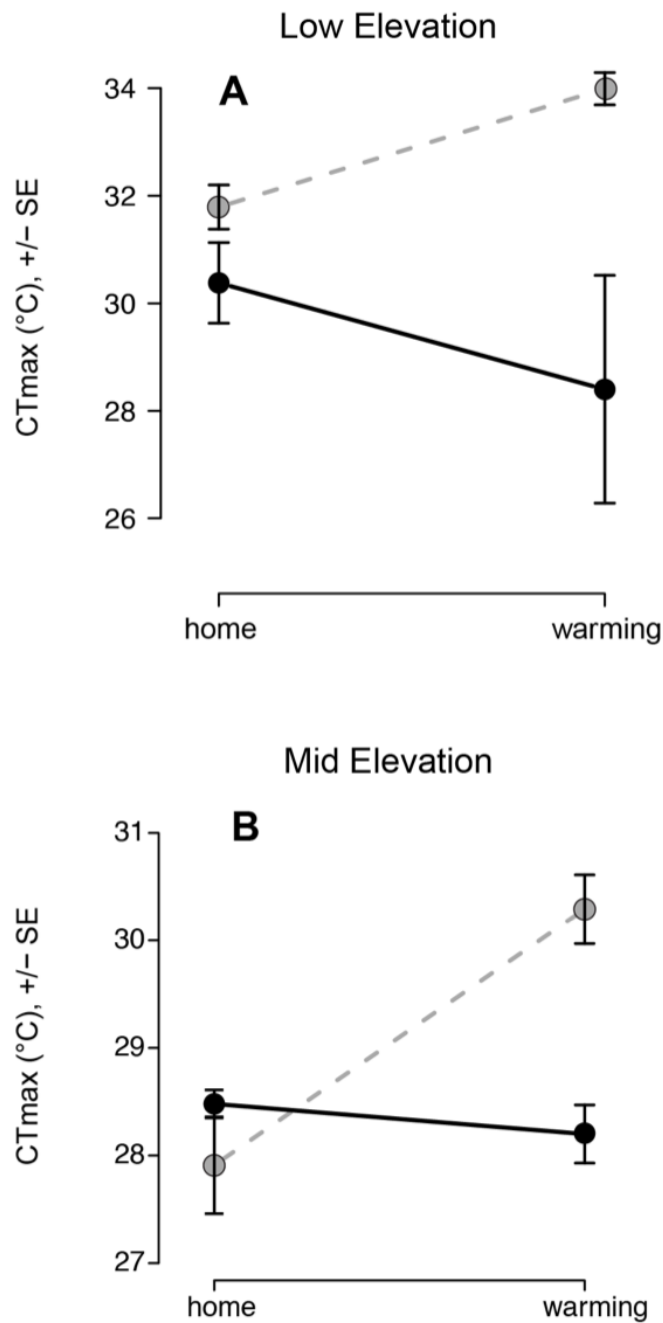
Figure 3.2. Reaction norms showing acclimation response in mayflies from low elevation (A; home = 13°C, warming = 18°C) and mid elevation (B; home = 10°C, warming = 15°C). Temperate mayflies (gray circles, dashed line) exhibited a greater acclimation response than tropical mayflies (black circles, solid line).
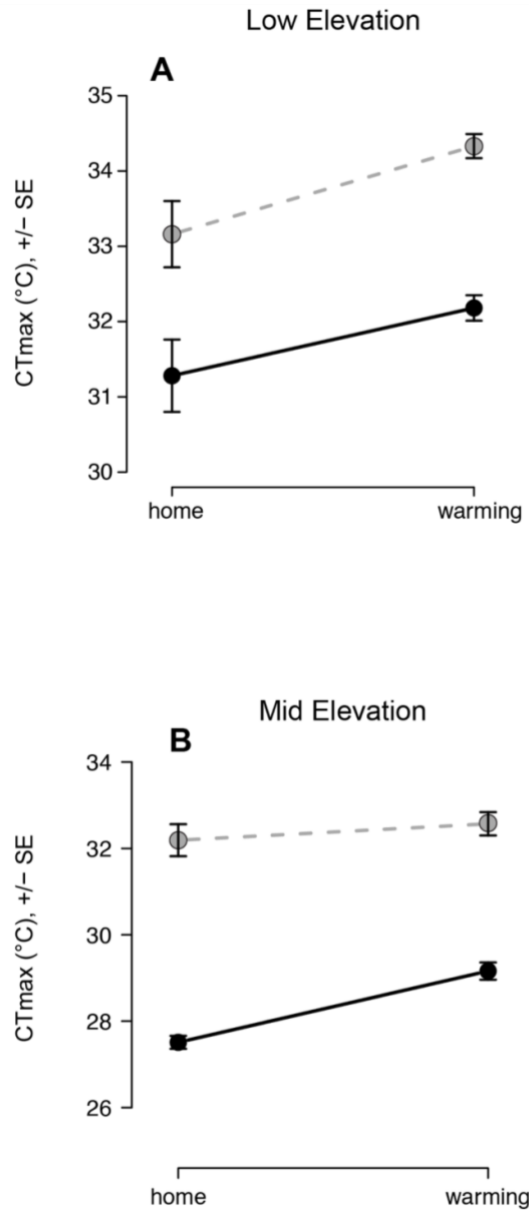
Figure 3.3. Reaction norms showing acclimation response in stoneflies from low elevation (A; home = 13°C, warming = 18°C) and mid elevation (B; home = 10°C, warming = 15°C). There were no differences in acclimation responses between low elevation temperate (gray circles, dashed line) and tropical (black circles, solid line) stoneflies, but a borderline significant difference between the two groups at mid elevation, where tropical stoneflies acclimated better than their temperate relatives.
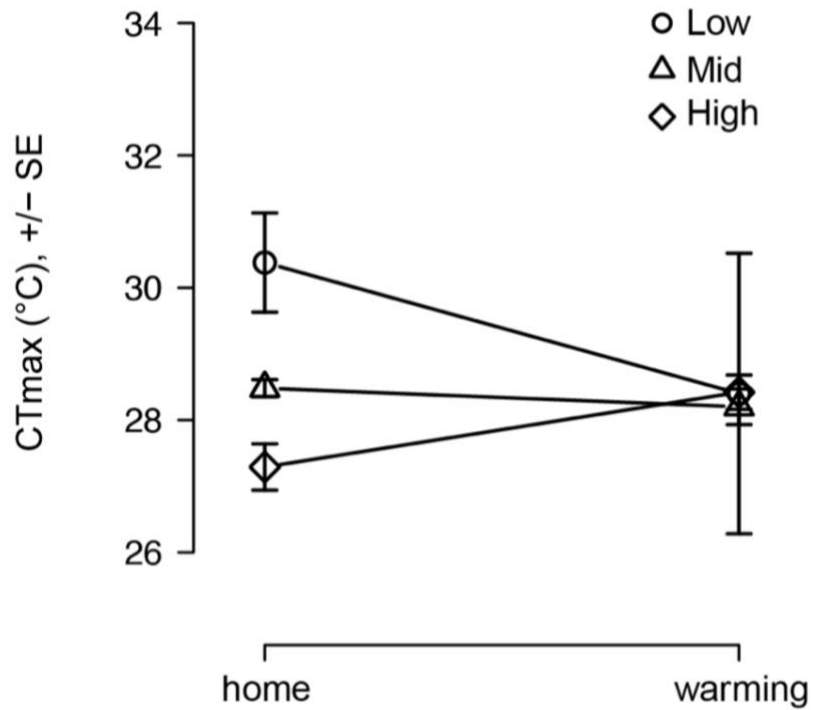
Figure 3.4. Reaction norm showing differences in acclimation responses for mayflies from low (home = 13°C, warming = 18°C), mid (home = 10°C, warming = 15°C) and high (home = 7°C, warming = 12°C) elevations. Although no acclimation capacity was seen in mid and low elevation populations, high elevation mayflies exhibited an acclimation response.
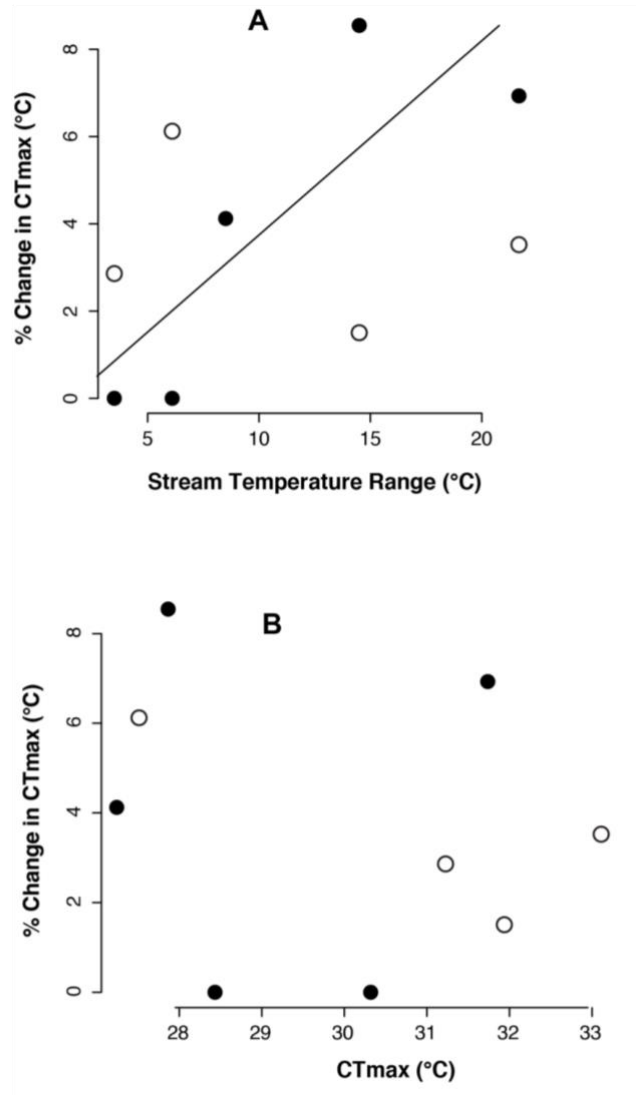
Figure 3.5. Percent change in CT_{MAX} (showing acclimation ability) as a function of stream temperature variation to test the climate variability hypothesis (A). While the trend is positive for mayflies (filled circles), there is no relationship for stoneflies (open circles). B shows acclimation capacity as a function of CT_{MAX} to test the trade-off hypothesis. We find no evidence for this hypothesis in mayflies (filled circles) or stoneflies (open circles).

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4. THE RELATIONSHIP BETWEEN METABOLIC RATE AND TEMPERTURE IN TROPICAL AND TEMPERATE MAYFLIES: IMPLICATIONS FOR VULNERABILITY AT DIFFERENT ELEVATIONS

SUMMARY

Rapid global warming is one of the greatest threats to biodiversity. Effects of warming are already being seen in a number of ectotherms in temperate latitudes especially. However, very little information about tropical ectotherms exists and studies on the vulnerability of freshwater tropical species are few and far between. Here, we compare sensitivity to temperature in temperate and tropical aquatic insects (mayflies) by measuring routine metabolic rate (RMR), a trait known to vary with temperature and with important implications for survival. We compared three phases of insect metabolic rate profiles, i.e. the ascending phase, peak, and descending phase to better understand i) how thermal regime shapes metabolic rate profiles at various temperatures and ii) what conclusions can be drawn about the vulnerability of mayflies to warming stream temperatures. We found that tropical mayflies, that typically experience stable climatic regimes, showed greater sensitivity to temperature. Regardless of the elevation of origin, these mayflies had overall steeper increases and decreases in metabolic rate and higher peaks compared to their temperate counterparts. Further, Q_{10} effects were generally higher in tropical mayflies compared to temperate relatives. Within latitude, low elevation tropical and high elevation temperate mayflies showed the greatest thermal sensitivity. Our data indicate that mayflies experiencing thermally stable environments (tropical or high elevation temperate streams) may be the most vulnerable to warming.

INTRODUCTION

Rapid global climate warming is already having adverse effects on many organisms (Colwell et al. 2008; Parmesan & Yohe, 2003; Root et al., 2003; Walther et al., 2002), but most importantly ectotherms (Clusella-Trullas et al. 2011; Deutsch et al., 2008; Dillon et al. 2010; Paaijmans et al. 2013). Recent studies predict that high latitude organisms may be the most vulnerable (Parmesan 2007; Rosenzweig et al. 2008) to warming because temperatures are forecast to rise higher and more rapidly there (Meehl et al. 2007). However, there is little empirical information assessing the impacts of warming on tropical species (Deutsch et al. 2008; Pounds et al. 1999) even though many studies predict high vulnerability to warming in tropical ectotherms due to their narrow thermal tolerances (Deutsch et al., 2008; Ghalambor, Huey, Martin, Tewksbury, & Wang, 2006b; Janzen, 1967). Further, an understanding of the vulnerability of tropical aquatic ectotherms is particularly lacking and urgently needed because freshwater ecosystems are some of the most biodiverse on earth (Dudgeon et al. 2006) and harbor some of the highest numbers of endemic species (Collen et al. 2013). In fact, aquatic insects that reside in mountain streams are vital to the healthy functioning of stream ecosystems, yet surprisingly few studies have systematically assessed their vulnerability to warming (Chown et al. 2015). Thus, studies that assess and compare vulnerability in closely-related temperate and tropical aquatic insects are imperative to better predict how these taxa will respond to global warming.

Assessing vulnerability in temperate and tropical ectotherms requires an understanding of the sensitivity of various important physiological traits that are shaped by temperature (Huey et al. 2009). One vital physiological trait is metabolic rate. Often referred to as the "rate of life" (Gillooly et al. 2001) , metabolic rate is a key physiological mechanism that determines the

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amount of energy an organism has to devote to a variety of activities such as movement, growth, tissue maintenance, and development. Thus, metabolic processes can have far-reaching effects for organisms by ultimately determining geographic distributions and abundance (Andrewartha & Birch 1954).

Though it is itself determined by a complex set of variables, metabolic rate depends heavily on body mass and temperature and these factors have received considerable attention (Brown 1990; Clarke & Johnston 2002; Gillooly 2001; Hemmingsen 1950; Kleiber 1947; Robinson et al. 1983). Emerging from this previous work is a general consensus on the theoretical predictions for how metabolic rate varies with temperature (Fig. 4.1). Metabolic rate profiles typically have three regions (Portner 2002; Schulte 2015): an ascending phase, a peak that likely indicates stress, and a descending phase in which an organism is thought to experience metabolic depression (Guppy and Withers 1999). When measured at the whole-organism level, metabolic rate begins to rise as temperatures increase from a given critical thermal minimum (CT_{MIN}), marking the beginning of the ascending phase (Fig. 4.1). While rising metabolism may even be optimal over a certain range of temperatures, it is also accompanied by a demand for higher food intake, and an organism must continually meet its body's food requirements to sustain high levels of metabolic activity. Ultimately, increased food intake cannot be maintained indefinitely (Gutow et al. 2016) and as temperature continues to rise, the organism begins to experience the onset of stress which can be visualized as a peak or maximum on the curve (Fig. 4.1). At these high temperatures the organism may begin to experience a mismatch between oxygen supply and demand (Pörtner 2002) or a failure of biochemical pathways and other functions, such as ventilation, to operate efficiently. A limited capacity of the circulatory and ventilatory systems to keep pace with increasing oxygen demands are thought to result in a

reduction of aerobic scope (i.e. the difference between maximum active and minimum resting or routine metabolic rates), and allow less energy to be devoted to other activities, such as feeding and growth (Fry 1971; Pörtner & Knust 2007). Beyond these increasingly stressful temperatures, a decrease in metabolic rate occurs (descending phase; Fig. 4.1) and an ectotherm experiences metabolic depression, which can help conserve energy (Guderley & St-Pierre 2002). Although the cause of this metabolic depression is not always clear, it is perhaps due to a combination of reduced energy and the ceasing of biochemical reactions or other neuromuscular functions (e.g., in a heat coma; (Clarke 2000). Finally, an ectotherm may encounter its critical thermal maximum (CT_{MAX}) and will now have little energy left for anything else but short-term survival (Fig. 4.1).

Despite the similarity in the overall response of metabolism to temperature across ectotherms (aquatic and terrestrial), there is still a large amount of variation in the shape of the metabolic rate curve among species (Dell, Pawar, & Savage, 2011). Variation may arise from an array of sources, such as methodology used by researchers to measure routine metabolic rate, developmental stage (e.g. Pough & Kamel, 1984), acclimation (e.g. Paganini, Miller, & Stillman, 2014), as a well as adaptive differences (e.g. Dahlhoff & Somero, 1991). For example, different climatic regimes in temperate and tropical latitudes should favor the evolution of ectotherms with different thermal sensitivities (Deutsch et al., 2008; Dillon et al., 2010). Thus, we might expect metabolic rate profiles and therefore sensitivity to temperature to differ greatly between temperate and tropical species. Here, we compare metabolic rate profiles between temperate and tropical aquatic insects – mayflies – to assess their sensitivity to temperature and vulnerability to global warming. We deconstruct metabolic rate curves into their three phases, i.e. ascending, descending, and peak phases and compare each component between temperate Rocky Mountain and tropical Andes mountain mayflies from high elevation, mid elevation, and low elevation.

Several predictions arise in making these comparisons between related mayflies. First, we predict that there will be differences between the ascending and descending phases of temperate and tropical mayfly metabolic profiles. The steepness of the slopes indicate how thermally sensitive temperate versus tropical species are by showing how rapidly metabolic rate changes with temperature. Temperate species are thought to be less sensitive to temperature due to the broad seasonal fluctuations they experience in temperate regions (e.g. Ghalambor et al 2006; Janzen 1967; Dobzhansky 1950). Thus, the ascending and descending phase of their metabolic rate profiles may be shallower for temperate species but steeper for tropical species.

Second, we predict that peak routine metabolic rates will be higher for tropical species because their narrower thermal breadths (Deutsch et al. 2008; Shah et al. 2017a) suggest that these species may operate closer to their maximum active metabolic rates even during low levels of activity. This has implications for the overall energetic economy of mayflies and that tropical species may have lower reserves of energy or a reduced aerobic scope when stream temperatures rise (Rowe & Crandall 2017).

Finally we predict that the Q_{10} values (a widely used measure of sensitivity to temperature), should be greater in tropical insects. Q_{10} is the factor by which metabolic rate (or any physiological trait value) increases over 10°C. It serves as a way to standardize changes in trait values across a variety of taxa to facilitate comparisons of thermal sensitivity (e.g. Rangel & Johnson 2018). A large Q_{10} value would suggest a greater change in metabolic rate and thus greater sensitivity to temperature. tropical streams and included some high, possibly stressful temperatures.

METHODS

Site Information

We collected two genera of mayflies (Family: *Baetidae*) from low-order, wadeable streams in the Cache La Poudre river drainage (Genus: *Baetis*; Rocky Mountains, Colorado, USA) and the Papallacta river drainage (Genus: *Andesiops*; Andes, Napo Province, Ecuador). Stream sites were matched by elevation, enabling comparison at high elevations (>3000m), mid elevations (>2000m), and low elevations (>1000m) from both latitudes (Table 4.1). At any given elevation, streams at both latitudes were approximately the same temperature at the time of collection. Experiments were conducted between January and April of 2013 in the Andes and June to August of 2013 in the Rockies.

Lab Acclimation

We brought field-caught insects back to the lab (~1600m at both latitudes) and placed them in containers full of filtered stream water from their native streams without food for 48h. We maintained natural levels of oxygen and flow using air and water pumps. Acclimation temperatures were chosen based on average tropical stream temperatures (Table 4.1). We acclimated high elevation insects to ~6°C, mid-elevation insects to ~10°C, and low elevation insects to ~14°C. Because acclimation temperatures were different between elevations, we only compared pairs of elevations across latitudes. We could not acclimate all insects from both latitudes to a single temperature because high and low elevation tropical insects were remarkably sensitive to temperatures outside average stream temperature. We often detected some stress (jerking movements, unnatural swimming patterns) especially in high elevation tropical insects

acclimated to 12°C after 48 h, and similar types of responses in low elevation tropical insects

acclimated to 10°C (both possible choices for a common acclimation temperature for this study).

Metabolic Rate Experiments

We used a closed-respirometery system (Unisense A/S, Aarhus, Denmark) to measure

rate of oxygen consumption, traditionally used as a proxy for metabolic rate (Appendix Fig.

S2.1). After the 48 h acclimation period, mayflies were slowly (1-2 h) introduced to one of nine

test temperatures, i.e. 5°C, 7.5°C, 10°C, 12.5°C, 15°C, 17.5°C, 20°C, 22.5°C, or 25°C. An

average of 10 mayflies were tested at each of these temperatures (Table S2.1, S2.2). Once at the

test temperature, we placed each mayfly individually into 4mL chambers filled with UV-filtered

stream water (to reduce microbial respiration) held at the test temperature. Chambers were fitted

with a magnetic stir bar to evenly distribute dissolved oxygen throughout the chamber and a

plastic mesh for the insect to cling to (Appendix Fig. S2.1). We allowed insects to acclimate to

the small chamber space for another 1 h before sealing each chamber to prevent any flux of

oxygen with the surrounding water. Once sealed, the chambers were lowered into a water bath

set at the desired test temperature (Appendix Fig. S2.1). A single control chamber (without an

insect) was included in each experiment to measure background respiration rates (these

background rates would be subtracted from each insect's respiration rate prior to statistical

analyses). We then used a single Clark's oxygen probe attached to a signal amplifier and a

computer to measure changes in oxygen concentration in each chamber. The probe was first

lowered into the control chamber at the start of the experiment. After reading oxygen

concentration continuously for 2 minutes, we retracted the probe and moved it to the next

chamber containing an insect (Appendix Fig. S2.1). In this way, the probe was moved to each

subsequent chamber every 2 min. Each chamber was typically measured 3-6 times although

more measurements were taken if possible. We terminated experiments after chambers reached 80% dissolved oxygen so as not to confound the effect of temperature on metabolic rate with hypoxic conditions. While recording oxygen concentration, we also made general behavioral observations of insects inside the chambers, noting any stress responses or mortality, which proved critical to our interpretation of the results (see Results). Following all experiments, insects were removed from chambers, dried, weighed, and stored in 95% EtOH for other analyses. Oxygen concentration data were output as *csv* files from the Unisense software program and downstream statistical analyses were performed using these raw data files.

Statistical Analyses

We calculated an average rate of consumption for each individual insect by measuring rate of oxygen consumption across all 2-min measurements taken in a given chamber (see Appendix 2 for methods and Fig. S2.2) for an explanation of this calculation). Using this same method, we then averaged the control values for each temperature such that we had a single control rate for each of the nine test temperatures from each latitude. We subtracted this control rate from insect consumption rates to correct for any background microbial respiration and to arrive a single best estimate of respiration rate (i.e. metabolic rate) for each insect. We conducted all statistical analyses on these "control-corrected oxygen consumption rates" (oxygen consumption, hereafter).

We first graphed the least-square means of mass-corrected oxygen consumption values to visualize the metabolic rate profiles measured for insects at all elevations (Figs. 4.2 A, B, C). Note that we overlaid critical thermal limit values measured in Shah et al. (2017a) to facilitate a comparison between the two physiological trait responses. Next, to compare metabolic rates during the ascending phase between temperate and tropical mayflies, we ran a general linear

model in R with oxygen consumption as the response variable for each elevation type (i.e. high, mid, and low) separately. For this analysis, we used only the temperatures 5°C, 7.5°C, 10°C, 12.5°C, 15°C and 17.5°C because these temperatures appeared to be within the ascending phase of the metabolic rate curve for all populations. The model included the fixed effects of test temperature and latitude, and their interaction, with body mass as a covariate. We did not divide metabolic rate estimates by body mass because this a common statistical error that results in artificially inflated degrees of freedom and increased Type I error rates (Packard & Boardman 1999). We graphed the least square means from each model to visualize the effect of temperature on metabolic rate, accounting for body mass (Fig. 4.2).

We conducted a similar analysis for the descending phase of the profiles, and used temperatures 17.5°C and 20°C as these were the only two temperatures that encompassed the descending phase of the curve in our populations. During experiments, behavioral observations suggested that some of the highest temperatures (i.e. 22.5°C and 25°C) were especially stressful for tropical mayflies. We therefore did not include these temperatures in this analysis and instead inspected them separately using Q_{10} calculations (see below).

To compare the peak routine metabolic rates at each elevation between temperate and tropical mayflies, we first identified temperatures at which routine metabolic rates were highest. We then compared these values using an ANOVA for each elevation pair separately. We used oxygen consumption rate as our response variable, latitude as the fixed effect and body mass as a covariate.

We finally tested differences in sensitivity to non-stressful versus stressful temperatures at high, mid, and low elevation for temperate and tropical mayflies. We calculated Q_{10} using the for mayflies from each elevation and latitude at 7.5°C, 15°C, and 25°C. Q_{10} values for 7.5-15°C

represented the change in metabolic rate across "non-stressful" temperatures and those at 15-

25°C represented change at "stressful" temperatures. We used the following formula to calculate

Q_{10} :

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

where R_1 and R_2 are reaction (oxygen consumption) rates measured at two different

temperatures, T_1 and T_2, respectively.

RESULTS

Behavioral observations

Our observations of mayfly behavior during experiments indicated that temperate

mayflies experienced little-to-no stress at any of the temperatures, but tropical mayflies showed

obvious signs of stress at several test temperatures (Tables S2.1, S2.2). Stress was often followed

by death at the end of an experiment in tropical mayflies. Importantly, we documented 100%

mortality at high elevation after the 22.5 and 25°C experiments and at low elevation after the 5°C

experiment.

Metabolic Rates

We found that metabolic rate profiles differed between temperate and tropical mayflies at

all elevations (Fig. 4.2). In general, temperate mayfly metabolic rates did not change

significantly across different temperature treatments whereas tropical mayflies had more obvious

peaks (Fig. 4.2). In almost all populations across both latitudes, a secondary peak was observed

at the 22.5°C and 25°C temperature treatments, and corresponded with stress observed during

experiments (e.g. at 20°C for mid elevation mayflies; Fig. 4.2B, Ecuador). As indicated in the

Methods, we excluded these stressful test temperatures from the analyses of the ascending and descending phases of metabolic rate profiles and dealt with them separately in an analysis of Q_{10} effects.

The comparison of the ascending phases of the profiles revealed significant interactions between temperate and tropical mayflies at all three elevations (Fig. 4.3; Table 4.2). In each case, metabolic rate increased more rapidly in tropical mayflies compared to their temperate counterparts. At low elevation, it is important to note that the significant interaction is driven by the sudden increase in metabolic rate at 10°C (Fig. 4.3 C). Removal of this temperature treatment yielded a non-significant interaction.

Next, we found significant interactions between temperate and tropical descending metabolic rates for high elevation (Fig. 4.4 A; Table 4.3) and for low elevation (Fig. 4.4 C). At mid elevation, although the lines appear to cross, there is no significant interaction (Fig. 4.4 B; Table 3). However, a Student's T-test reveals that oxygen consumption values are different at 17.5°C (t=3.946, p=0.001), but are not different at 20°C (t = -1.006, p = 0.33) suggesting a steeper trend for tropical mayflies compared to temperate ones. Mid elevation tropical mayflies also showed steeper decreases in metabolic rate (Fig. 4.4 B, C) although at high elevation, temperate mayflies appeared to be more sensitive (Fig. 4.4 A).

Finally, an analysis of peaks between metabolic rate profiles revealed that tropical mayflies have higher peak routine metabolic rates that tropical mayflies at any given elevation (Fig. 4.5).

Q_{10} effects

The increase in metabolic rate from 7.5°C to 15°C (non-stressful temperatures) was higher at all elevations and both latitudes compared to the increase between 15°C and 25°C

(stressful) temperatures (Table 4.4). When comparing between latitudes, tropical mayflies showed greater sensitivity to temperature (higher Q_{10} values) at both non-stressful and stressful temperatures than their temperate counterparts (Table 4.4). Notably, at mid and low elevations, tropical mayflies were extremely sensitive to temperature even at non-stressful temperatures (Q_{10} = 8.40 and 5.17, respectively).

DISCUSSION

Overall effects of temperature on routine metabolic rate in mayflies

Our goal was to compare routine metabolic rate profiles in closely related temperate and tropical mayflies to determine differences in sensitivity to temperature and vulnerability to climate warming. Across all mayflies tested, metabolic rate increased with temperature, peaked at what appeared to be stressful temperatures, and then decreased. After the decline in metabolic rate, we noted the presence of secondary peaks at the 22.5°C and 25°C test temperatures for most populations. We interpreted these peaks as a severe stress response. In fact, studies in brown bullhead catfish note large increases in ventilatory effort, which correlate with stress, following an initial decline at high temperatures (Heath 1973). With the exception of temperate low elevation mayflies, the CT_{MAX} values measured in these populations (Shah et al. 2017a) were only a few degrees higher than 25°C (Fig. 4.2). These combined results strongly suggest that all mayflies are likely to experience some stress around 25°C.

We found several lines of evidence showing differences in sensitivity to temperature for tropical and temperate mayflies. First, the ascent and descent of metabolic rates with temperature was generally steeper in tropical mayflies at all three elevations. The steepness suggests a more rapid change in metabolic rate and therefore greater thermal sensitivity (Rowe & Crandall 2017).

Steeper ascending and descending metabolic rate slopes were accompanied by high Q_{10} values and higher peaks, all of which suggest that tropical mayflies function best at a narrower range of temperatures and will struggle to cope with increasing stream temperatures compared to temperate mayflies. Studies of terrestrial ectotherms suggest similar patterns of sensitivity in terrestrial insects (Deutsch et al. 2008), lizards (Huey et al. 2009) and reef fish (Rummer et al. 2014). Second, the strong effect of temperature on tropical mayflies was most apparent in the Q_{10} results, where values were nearly always higher in comparison to temperate mayflies. The particularly high Q_{10} effects for mid and low elevation tropical mayflies at non-stressful temperatures were similar in value to those of North Atlantic stenothermal fishes when experiencing temperatures away from an optimum (Johnston et al. 1991). Finally, relatively high levels of mortality followed experimentation with tropical mayflies at the higher test temperatures, even though none were documented in temperate mayflies. In sum, these results provide evidence suggesting all mayflies are on average better adapted to cool temperatures but that tropical mayflies are far more vulnerable to global warming compared to their temperate relatives as predicted (Janzen 1967; Ghalambor 2006).

Effects of temperature on high elevation mayflies

Although temperate mayflies appear to have shown overall reduced sensitivity to temperature especially during the ascending phase of metabolic rate profiles, high elevation species are a notable exception in the descending phase response. Here, temperate mayflies show greater sensitivity and appear to descend more rapidly into metabolic depression than tropical species. Although CT_{MAX} (29.3°C; Fig. 4.2 A) falls outside the range of temperatures tested, these mayflies showed a secondary increasing trend in metabolic rate at the 22.5°C and 25°C, highlighting that the onset of stress occurs long before reaching CT_{MAX}. Thus at higher

temperatures, high elevation temperate species may be particularly sensitive, similar to tropical mayflies whose CT_{MAX} is about a degree higher than 25°C. Previous work measuring thermal breadth (differences between CT_{MAX} and CT_{MIN}) has also concluded that high elevation mayflies may be at least equally vulnerable to warming as their tropical relatives (Shah et al. 2017a). In fact, Q_{10} values at non-stressful temperatures for both types of mayflies were similar, i.e. around 3, although a difference in sensitivity was observed at stressful temperatures, where tropical mayflies showed greater sensitivity ($Q_{10} = 1.46$) than temperate mayflies ($Q_{10} = 1.07$). The overall similarity in response seen at high elevation between latitudes likely occurs because high elevation in temperate mountains can be far less thermally variable compared to low elevation and maximum temperatures do not rise especially high even at the peak of the summer (Shah et al. 2017a; Shah et al. 2017b). At tropical high elevation a greater amount of thermal variation exists relative to low elevation owing to a lack of canopy cover, creating a similar thermal environment as that experienced in temperate mountain high elevations. Thus, high elevation temperate species are more thermally sensitive than other temperate populations, whereas high elevation tropical species are less thermally sensitive compared to lower elevation tropical populations.

Effect of temperature on mid and low elevation mayflies

Mid and low elevation temperate mayflies showed similar metabolic rate profiles and Q_{10} responses suggested that thermal sensitivity was low. In the Rocky Mountains of Colorado, mid and low elevations can be characterized by high thermal variability, where cold, near-freezing winter temperatures are eventually replaced by high summer temperatures (Clow, 2010). The entirety of this variation is likely to be experienced by a single mayfly nymph that typically hatches at the coldest spring temperatures and emerges during some of the warmest summer

temperatures (Brittain 1990). Reduced sensitivity to temperature, seen as shallower increases and decreases in metabolic rates (Figs. 4.3 B, C, and 4.4 B, C) may be an adaptation to these broad thermal fluctuations, as it can ensure the maintenance of a high aerobic scope (Nilsson et al. 2009) across a wide range of temperatures. Aerobic scope is another way of describing an organism's energy reserves and is the difference between the lowest (basal/resting/routine) metabolic rate when at rest and the highest (maximum) metabolic rate during the highest levels of activity that an organism can achieve while still using O_2 as the final electron acceptor in the production of ATP (Pörtner & Knust 2007). Ectotherms that possess a wide aerobic scope can maintain high performance for various life-sustaining activities. Thus, the higher peak routine metabolic rates in tropical mayflies (i.e. when they are essentially at rest) may provide some evidence that these mayflies are living closer to their maximum active metabolic ceilings and have narrower aerobic scopes. As temperatures rise, tropical species may have less available energy to devote to feeding and growth and may cease to function normally compared to temperate species with wider aerobic scopes. In other studies, aerobic scope has been shown to decrease in tropical species, e.g. reef fish (Nilsson et al. 2009; Munday et al. 2009) and polar fish (Pörtner & Knust 2007) when exposed to high temperatures because basal metabolic rates rise, thereby reducing the distance to maximum metabolic rate. In temperate mid and low elevation streams, the low and relatively invariant routine metabolic rate coupled with a low CT_{MIN} and high CT_{MAX}, may allow mayflies to maintain a wide aerobic scope and dedicate energy to foraging and growth, and lead to survival even at higher temperatures.

At tropical mid and low elevations, relatively stable temperatures result in minimal change in water temperature over the year (Shah et al. 2017a). These streams are usually forested and are not subject to large increases in temperature due to insolation (A. Shah *pers. obs.*),

therefore mayflies could be described as more stenothermal in these streams and sensitivity to temperature is evident in their metabolic rate profiles. Average mid elevation stream temperatures are around 10-11°C (Table 4.1), at which metabolic rates are found to be quite low. A first peak in metabolic rate is seen at 17.5°C, suggesting the onset of stress. At low elevation, a peak is seen at 10°C, but this was not correlated with any observations of stress. Moreover, average stream temperatures at this elevation tend to be around 15°C, thus the increase in metabolic rate at 10°C may be an artefact of our experimental procedure or unusually high levels of microbial activity in some of the chambers. A secondary peak in metabolic rate occurs at 17.5°C, where we might expect to see the onset of stress. Behavioral observations revealed that all insects died after being removed from the 25°C experiment as well as at 5°C, and suggest not only sensitivity to high temperatures but also to low temperatures. Further, CT_{MIN} values were relatively high (Fig. 4.2 B, C) and CT_{MAX} values quite close to 25°C suggesting narrower overall thermal tolerance in these species. Combined, our results suggest that mid and low elevation mayflies may have some of the narrowest thermal breadths as a result of the stable temperatures of streams at these elevations. Thus, tropical mayflies from mid and low elevation streams may be some of the most adversely affected by rising temperatures.

Comparison of metabolic rates and critical thermal maxima as indicators of vulnerability

Some of the results of the present study are surprising because we expect, CT_{MAX}, the commonly used metric for thermal sensitivity (Lutterschmidt & Hutchison 1997; Amarasekare & Savage 2012; Diamond et al. 2012; Piyaphongkul et al. 2012; Ribeiro et al. 2012) to coincide with the descending limb of the metabolic rate profiles (Fig. 4.1). Yet, previously measured CT_{MAX} in many temperate mayfly populations occurs at much higher temperatures than predicted

(Fig.4.1 A, B, C). For example, we found CT$_{MAX}$ to be ~29.3°C in high elevation temperate mayflies (Shah et al. 2017a) but here they began to show signs of extreme stress at even lower temperatures (~17.5°C) as indicated by a metabolic peak at this temperature. This apparent contradiction in results could be due to methodology, as rapid ramping rates or high starting temperatures can overestimate CT$_{MAX}$ values (Rezende et al. 2010; Terblanche et al. 2007). The discrepancy could also arise because CT$_{MAX}$ and metabolic rates are governed by fundamentally different processes (Hochachka 2002) and may therefore respond differentially to temperature. Indeed, the results of the present study may be far more ecologically relevant because insects were held at test temperature for longer periods of time than in standard CT$_{MAX}$ experiments. But despite this apparent discrepancy, the overall interpretation of our results – that tropical mayflies are more vulnerable than their temperate counterparts – remains unchanged.

Impacts of elevated stream temperature on mayflies

For mayflies, there can be several major consequences of warming stream temperatures. First, as indicated before, temperature can have an effect on aerobic scope (Clark et al. 2013; Farrell et al. 2008; Pörtner & Farrell 2008). In tropical mayflies, we measured relatively high standard metabolic rates compared to temperate mayflies. Thus, tropical mayflies may not have a lot of "room" to increase their metabolic rate before approaching their maximum (Nilsson et al. 2009) thereby reducing the range of temperatures over which they can maintain adequate levels of activity. Given the generally low metabolic rates of temperate mayflies, it is possible that they have a wide aerobic scope (Healy & Schulte 2012). A lower aerobic scope may imply less energy for feeding and proper development, and we may therefore predict decreases in tropical mayfly population sizes in warming streams.

An alternative consequence of warming is accelerated growth and early emergence at warmer temperatures as shown in high elevation mayflies in the Rocky Mountains (Harper & Peckarsky 2006). Though we know little of the fitness consequences of rapid growth in mayflies, we may look to other ectotherm research for potential consequences. For example, in Atlantic silverside fish (*Medina medina*), increased growth rate due to warm temperature in cold-adapted populations results in a trade-off with swimming performance (Billerbeck et al. 2001). Similarly, rapid growth could come at the expense of larval swimming or adult flight performance in mayflies. Due to the importance of swimming and flight in dispersal and finding mates (Harker 2009), increased growth rates due to climate warming could reduce fitness especially in tropical mayflies.

Finally, in a study of lab-reared mayflies, individuals raised at extremely high temperatures (~30°C) failed to emerge altogether (Chou et al. 2018). In fact, metabolomic studies on these heat-stressed mayflies showed severe depletion of lipids and acylcarnitines (important compounds involved in metabolism in most living organisms), suggesting that despite access to food, metabolism can fail to produce the required energy at high temperatures (Chou et al. 2018; Chou et al. 2017). The consequences of warming in streams may therefore be dire indeed. High thermal sensitivity may not merely reduce levels of activity by reducing aerobic scope, but can lead to a decline in population sizes through its various negative effects on individual survival and fitness.

Thermal sensitivity can of course be mediated via a suit of mechanisms. For example, behavioral thermoregulation has been shown to be an effective strategy to buffer terrestrial ectotherms from stressful environmental temperatures (Kearney & Porter 2009). However, streams are more confined habitats compared to terrestrial ones. This likely reduces any

advantage of behavioral thermoregulation because individuals seeking respite may have to move great distances before they experience more suitable temperatures. Moreover, when seeking refuge from warm temperatures, moving upstream or higher in elevation to reach cooler areas is preferable. For small mayfly larvae, moving long distances against the downward force of the water may be a challenge, even though some level of short-distance upstream movement has been documented (Bishop & Hynes 1969). Another mechanism to mitigate thermal stress is the ability to acclimate (Hofmann & Todgham 2010; Ghalambor et al. 2007), a physiological strategy employed to decrease sensitivity to temperature. Previous work has shown that temperate mayflies have an ability to acclimate to warmer temperatures, but tropical mayflies, especially those at low elevation lack such a capacity (Shah et al. 2017b). Tropical stoneflies, on the other hand, do display a capacity to acclimate (Shah et al. 2017b), as do other types of tropical aquatic ectotherms (Seebacher et al 2015; Donelson et al. 2012). Thus, the capacity to acclimate varies among different tropical aquatic ectotherms, and may be utilized by some to mitigate the effects of rising water temperatures. Lastly, the presence of standing genetic variation and the evolvability of thermal tolerance traits can increase the chances of adaptation to higher temperatures by natural selection (Skelly et al. 2007; Visser 2008). But to our knowledge, no studies have been explicitly conducted to address the adaptability of mayflies to warmer temperatures.

Insects, which make up a significant portion of the earth's biodiversity, are likely to be the most vulnerable to global climate change (Deutsch et al. 2008). Recent work shows that changes in patterns of insect physiology, phenology, behavior, and geographic rage are already occurring as global temperatures rise (Menédez 2007). However, much of this work focusses on temperate, terrestrial insect systems (Chown et al 2015). Though freshwater ecosystems make up

only 0.8% of the earth's surface, they hold nearly 6% of all described species and are characterized by a high degree of endemism (Dudgeon et al. 2005). Many aquatic macroinvertebrates are essential to the healthy functioning of streams and rivers (Covich et al. 1999) yet, we know very little about how freshwater aquatic insects will respond to increasing temperature, and even less about how tropical stream insects will cope (Chown et al. 2015). Our work indicates that tropical mayflies are more sensitive to warm temperatures, but that temperate high elevation mayflies may be equally vulnerable. While the evolutionary potential for these traits, or the ability of mayflies to shift their ranges to track more suitable temperatures remains to be understood, it is clear that both temperate and tropical mayflies will be imperiled by rising temperatures.

Table 4.1. Site, stream temperature and acclimation temperature information for temperate (Rocky Mountain) and tropical (Andes) streams. We pooled data for streams that occurred at similar elevations to increase our sample size.

Location	Site ID	Elevation classification	Site elevation (m)	Avg. stream temperature during collection (°C)	Acclimation temperature (°C)
Temperate	COP2212	Low	2212	12.5	14
	COP2590	Mid	2590	10.8	10
	COP2798	Mid	2798	7.6	10
	COP3166	High	3166	6.2	6
Tropical	ECP1845	Low	1845	13.5	14
	ECP2003	Low	2003	14.0	14
	ECP2694	Mid	2798	10.0	10
	ECP3683	High	3683	8.6	7
	ECP3898	High	3898	6.6	6

Table 4.2. Output of model testing the interaction between ascending phases of temperate and tropical mayflies across 6 test temperatures (from 5°C to 17.5°C).

HIGH ELEVATION	Df	Sum of Sq.	Mean Sq.	F- value	p-value
Temperature	5	0.006	0.001	38.94	**<0.000**
Latitude	1	0.001	0.001	40.70	**<0.000**
Dry weight	1	0.0001	0.0001	4.37	**0.039**
Latitude x Temperature	5	0.001	0.0001	3.27	**0.001**
Residuals	97	0.003	0.000		
MID ELEVATION					
Temperature	5	0.001	0.000	11.34	**<0.000**
Latitude	1	0.000	0.000	9.52	**0.002**
Dry weight	1	0.002	0.002	72.65	**<0.000**
Latitude x Temperature	4	0.000	0.000	4.17	**0.004**
Residuals	98	0.002	0.000		
LOW ELEVATION					
Temperature	5	0.001	0.000	11.35	**<0.000**
Latitude	1	0.003	0.003	157.69	**<0.000**
Dry weight	1	0.002	0.001	70.25	**<0.000**
Latitude x Temperature	4	0.001	0.000	6.77	**<0.000**
Residuals	152	0.003	0.000		

Table 4.3. Output of model testing the interaction between ascending phases of temperate and tropical mayflies across 2 test temperatures (17.5°C to 20°C).

HIGH ELEVATION	Df	Sum of Sq.	Mean Sq.	F- value	p-value
Temperature	1	0.000	0.000	8.32	**<0.000**
Latitude	1	0.002	0.002	77.13	**<0.000**
Dry weight	1	0.001	0.001	17.78	**0.003**
Latitude x Temperature	1	0.000	0.000	4.04	**<0.000**
Residuals	38	0.001	0.000		
MID ELEVATION					
Temperature	1	0.001	0.001	26.33	**<0.000**
Latitude	1	0.000	0.000	12.79	**0.001**
Dry weight	1	0.001	0.001	17.48	**0.000**
Latitude x Temperature	1	0.000	0.000	2.48	0.124
Residuals	35	0.001	0.000		
LOW ELEVATION					
Temperature	1	0.000	0.000	12.11	**0.001**
Latitude	1	0.001	0.001	37.26	**<0.000**
Dry weight	1	0.000	0.000	7.94	**0.007**
Latitude x Temperature	1	0.000	0.000	6.87	**0.012**
Residuals	35	0.001	0.000		

Table 4.4. A comparison of Q_{10} values calculated for temperate and tropical mayfly metabolic rates between non-stressful temperatures (5°C to 15°C) and stressful temperatures (15°C to 25°C).

	NON-STRESS Q_{10}	*STRESS Q_{10}*
HIGH		
Colorado	3.63	1.07
Ecuador	3.15	1.46
MID		
Colorado	3.15	0.73
Ecuador	5.17	1.59
LOW		
Colorado	2.36	1.34
Ecuador	8.40	1.67
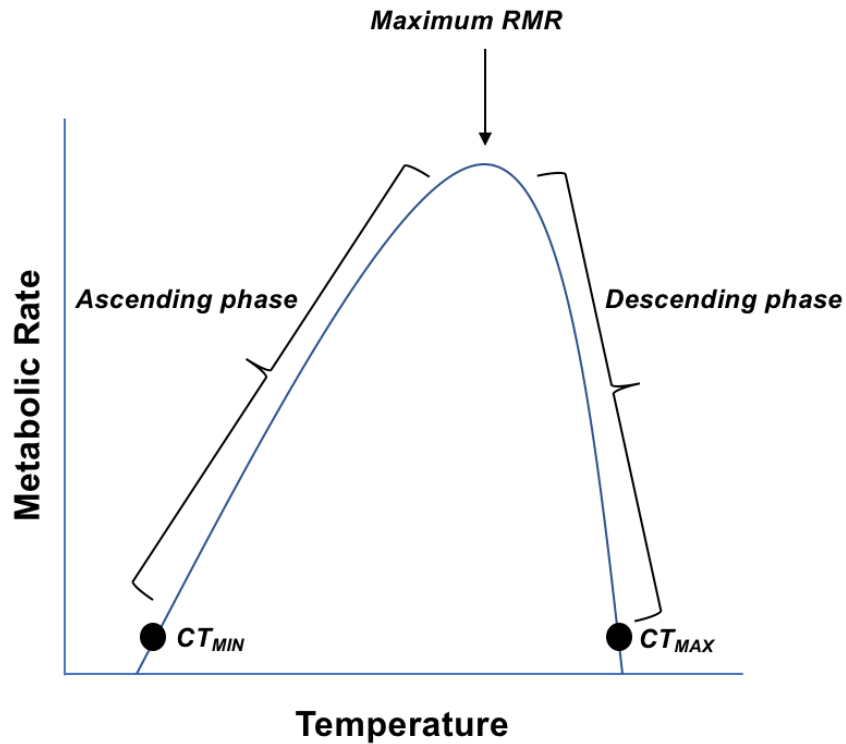
Figure 4.1. Conceptual diagram showing expectations for change in metabolic rate with temperature in an ectotherm. The metabolic rate profile includes an ascending phase, which occurs just beyond the critical thermal minimum (CT_{MIN}). Once temperatures become stressfully high, metabolic rate will reach a maximum and peak. Finally, with still increasing temperature, an ectotherm will descend into metabolic depression and finally approach a critical thermal maximum (CT_{MAX}). Beyond this temperature, the ectotherm is unlikely to survive for long.
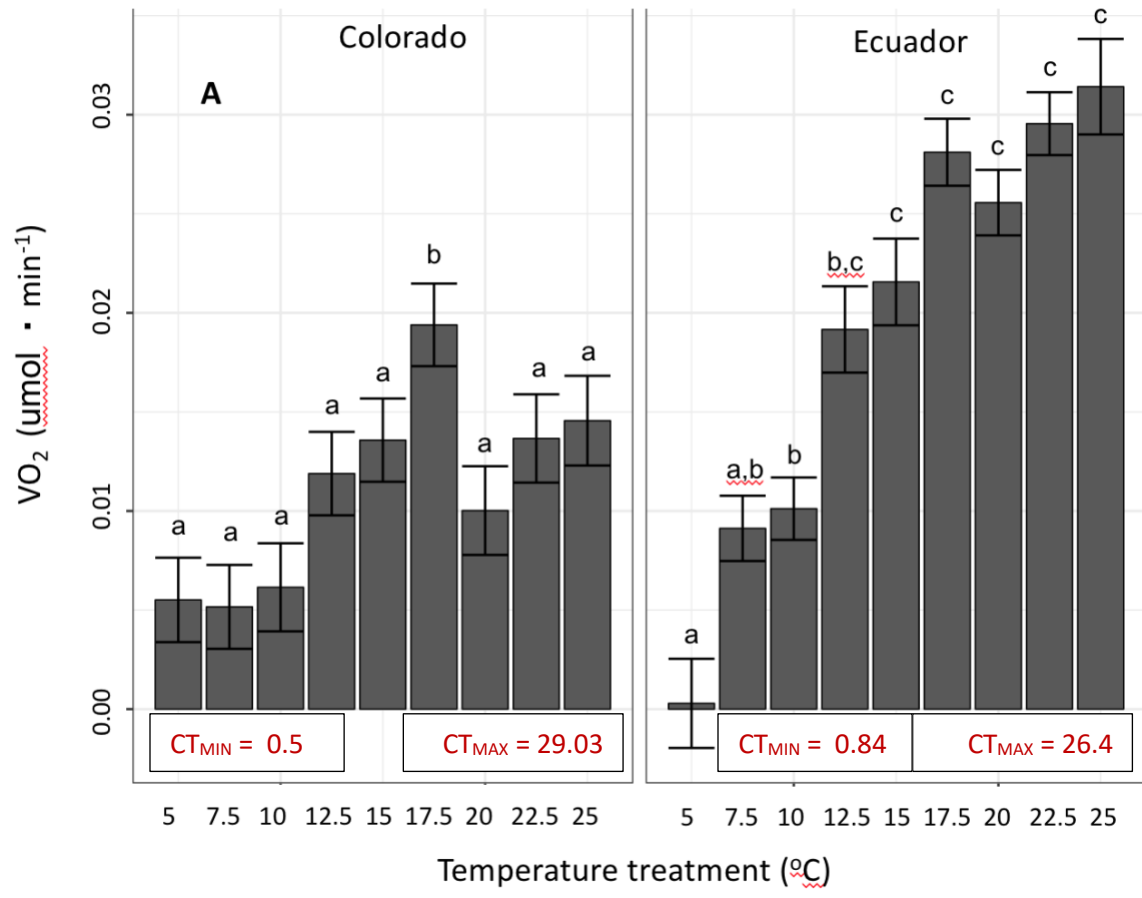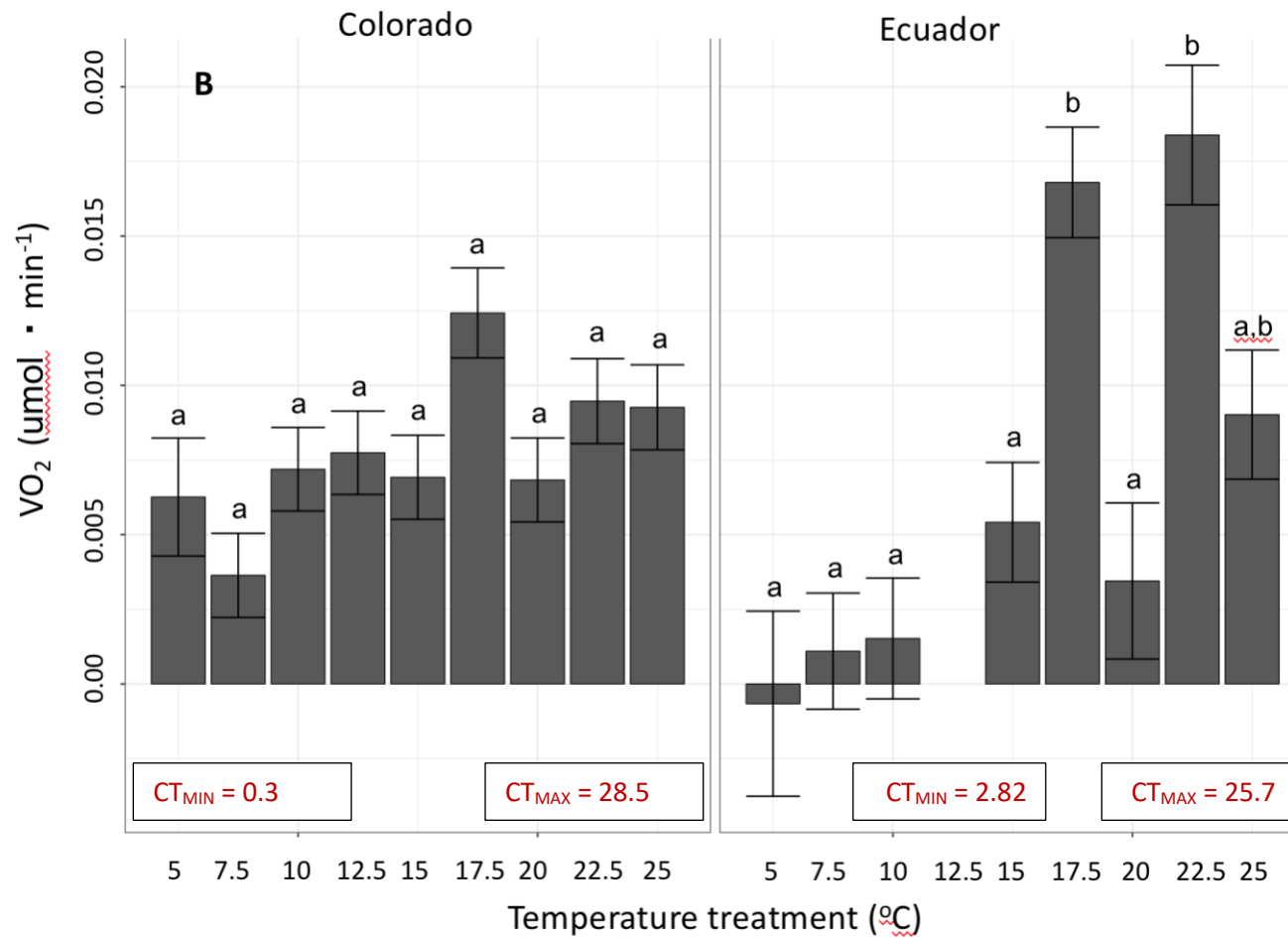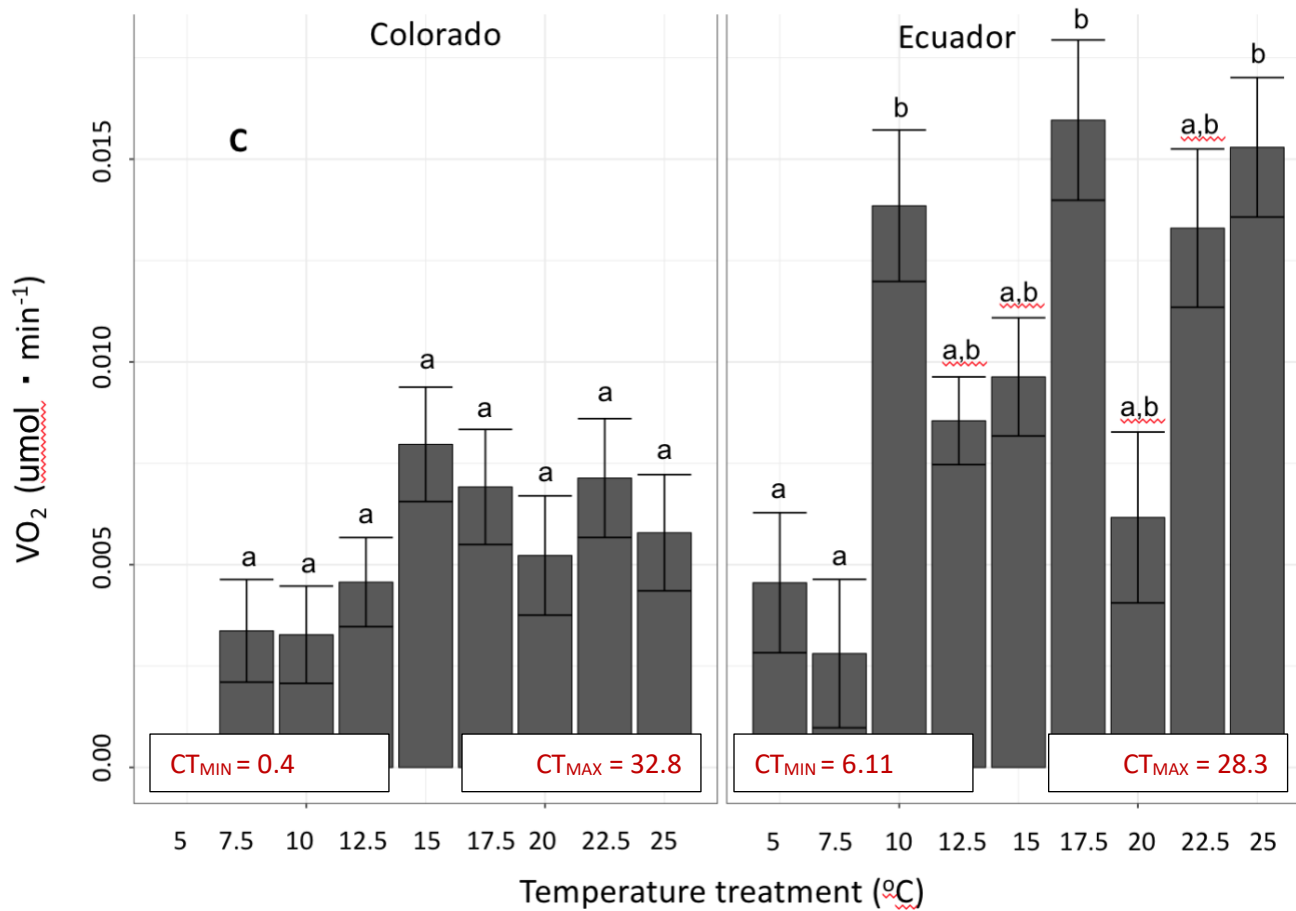
Figure 4.2. Least square means of oxygen consumption rates across temperature in mayflies from temperate and tropical streams. Vertical bars represent standard errors. Comparisons are made between high elevation (A), mid-elevation (B) and low elevation (C). Critical thermal minimum and maximum temperatures measured in a previous study are shown in boxes below bar plots. In tropical mayflies, test temperatures 22.5°C and 25°C were especially stressful although temperature mayflies were also found to exhibit some stress behavior as well.
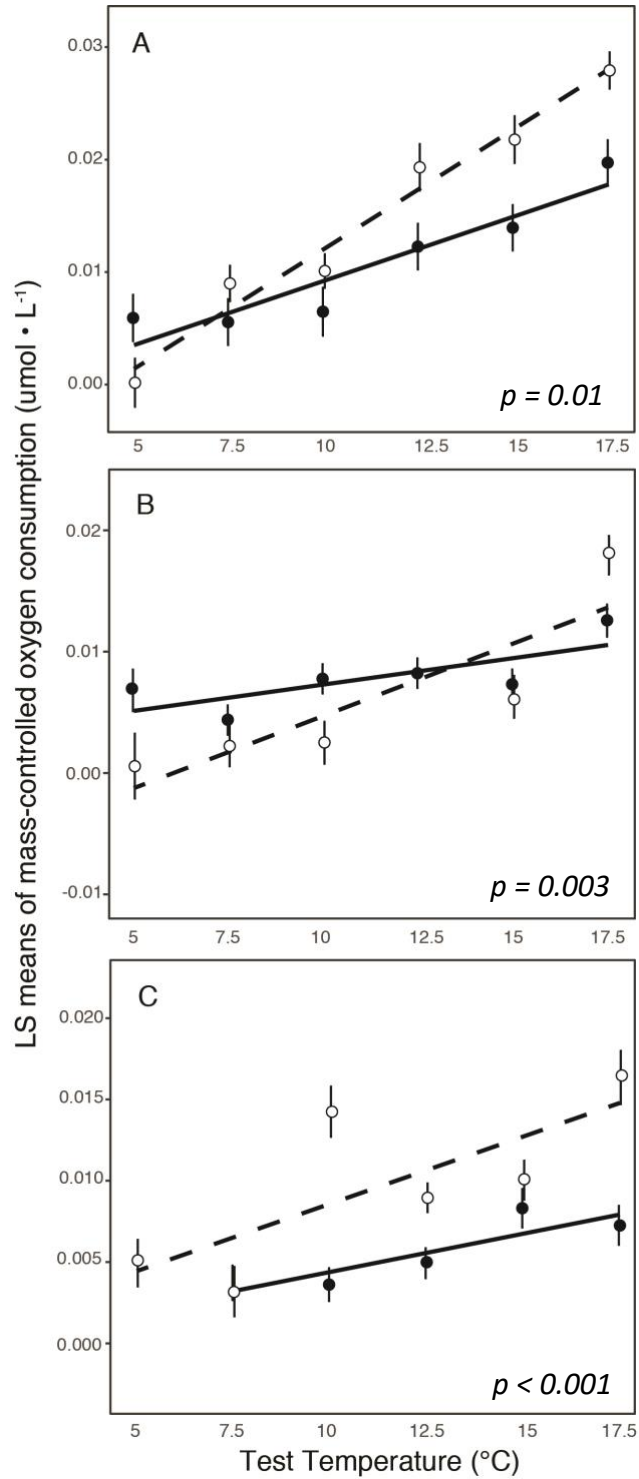
Figure 4.3. A comparison of the ascending phase of metabolic profiles of temperate and tropical mayflies from high (A), mid (B) and low (C) elevations. Values are the least square means of metabolic rate after accounting for body mass in the model.
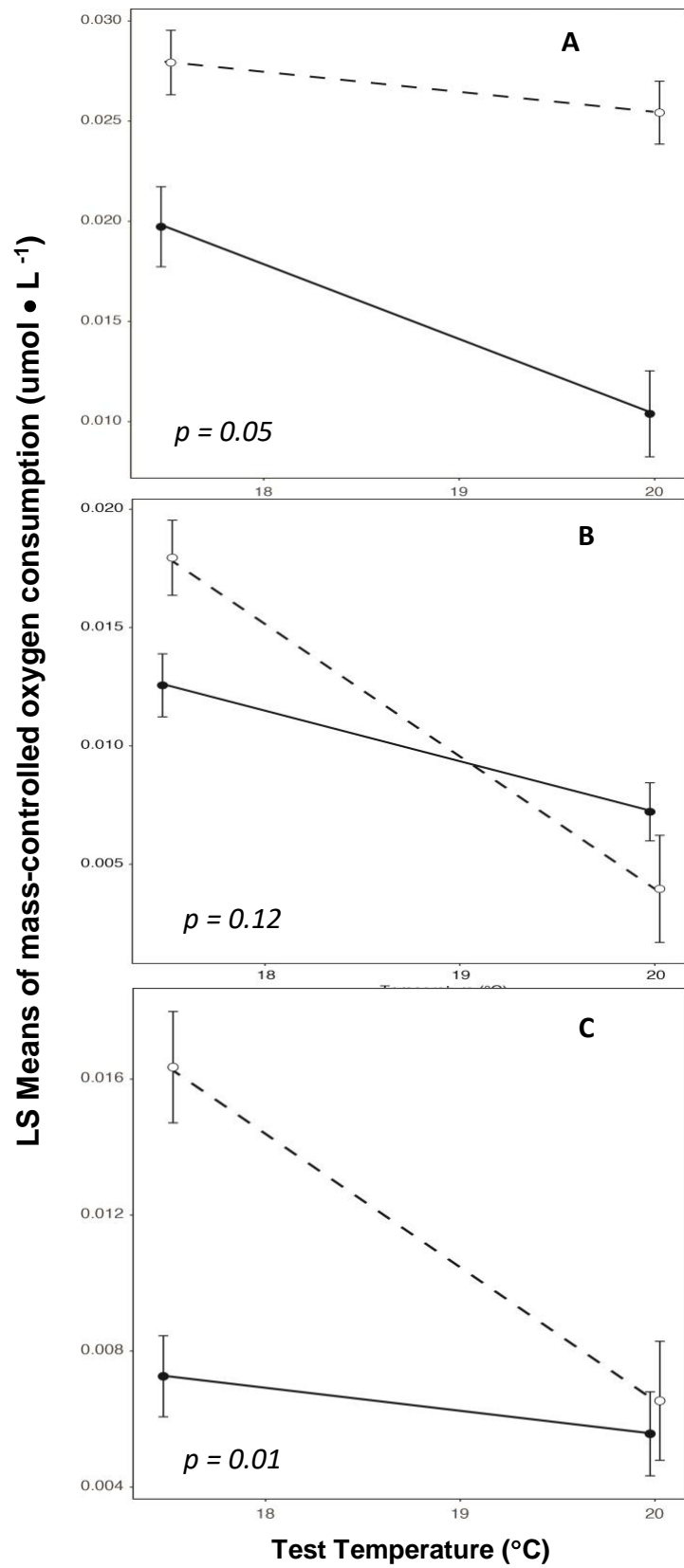
Figure 4.4. A comparison of the descending phase of metabolic profiles of temperate and tropical mayflies from high (A), mid (B) and low (C) elevations. Values are the least square means of metabolic rate after accounting for body mass in the model.
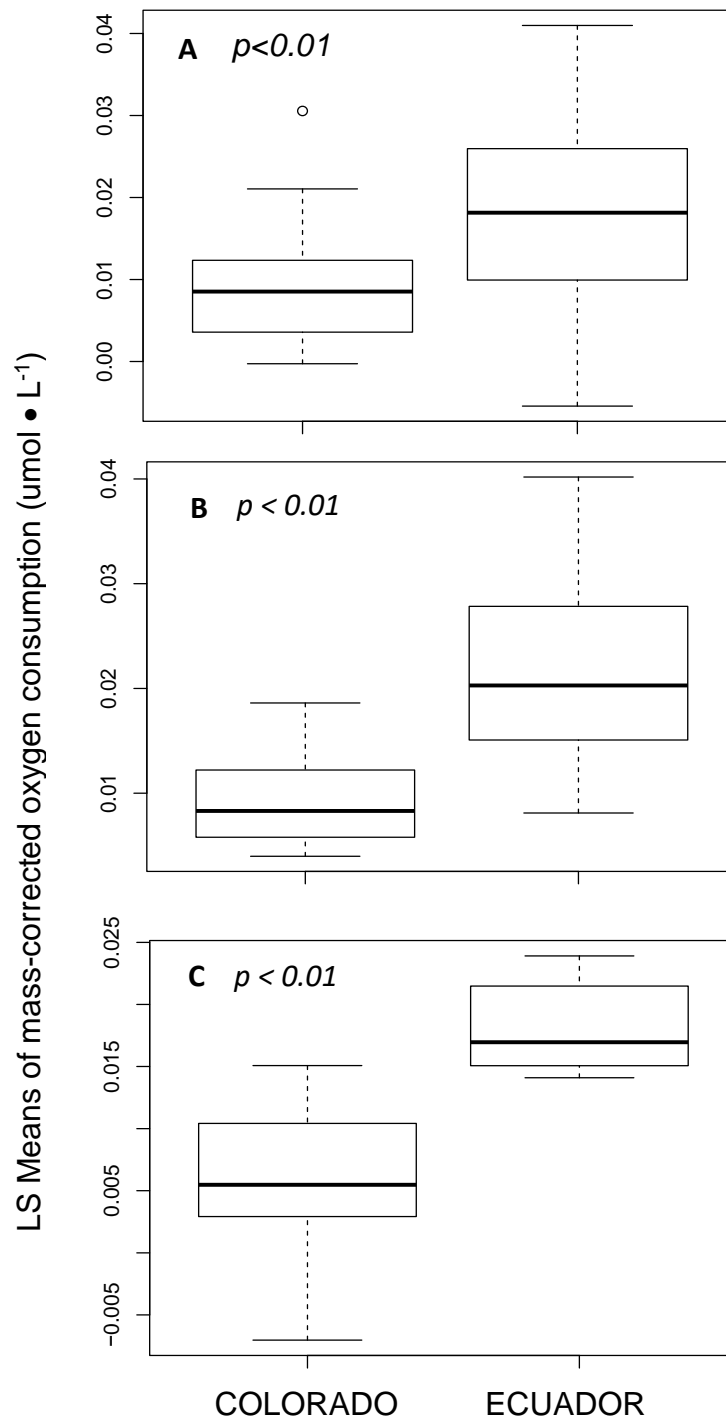
Figure 4.5. Peak routine metabolic rates (i.e. the highest metabolic rate) are compared between temperate and tropical mayflies from high (A), mid (B), and low (C) elevations. In all cases,

tropical mayflies appear to have higher resting metabolic rates suggesting that even during low activity, they experience generally high metabolic rates.

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5. OF MAYFLIES AND MOUNTAINS: STREAM TEMPERATURE IMPACTS POPULATIONS STRUCTURE IN TROPICAL BUT NOT TEMPERATE MOUNTAIN MAYFLIES (BAETIDAE)

SUMMARY

In his seminal paper written in 1967, Janzen posited that tropical mountains are more biodiverse than temperate mountains because of differences in thermal regimes. Due to the wide seasonal temperature fluctuations of temperate mountains, organisms should evolve broad thermal tolerance, and therefore disperse easily across elevation. On tropical mountains, stable annual temperatures with little overlap across elevations should favor narrower thermal tolerance and restrict movement to higher (colder) or lower (warmer) elevations. In previous work, we found support for Janzen's prediction regarding latitudinal variation in thermal tolerance. Here, we test the second prediction by investigating whether greater differences in thermal tolerance across elevation in tropical than in temperate mayflies results in more restricted gene flow. We found greater population structure in tropical populations that was best explained by differences in maximum stream temperature across elevation. In contrast, temperate populations had higher gene flow among populations and genetic structure was not explained by temperature, physiological tolerance, or geographic distance. We conclude that greater gene flow among temperate populations is likely due to broad thermal tolerance. Because tropical populations are more thermally sensitive and have lower genetic diversity due to lower gene flow, they may be more vulnerable to global warming.

INTRODUCTION

Tropical mountains are far more biodiverse than temperate mountains (Meyers et al. 2000). For decades, researchers have sought to explain the underlying ecological and evolutionary reasons for this difference in diversity (Mayr 1963; Janzen 1967; Hoffmann & Blows 1994; Case & Taper 2000; Holt & Keitt 2005). One prevailing hypothesis – the Climate Variability Hypothesis (CVH) – posits that temperature, through its effect on organismal thermal tolerance, is the main driver of these latitudinal differences in biodiversity (Dobzhansky 1950; Pianka 1966; Janzen 1967; Stevens 1989; Fine 2015). Janzen (1967) predicted that the wider seasonal temperature variation experienced in temperate mountains should result in greater temperature overlap across elevations, such that organisms experience a similar range of temperatures regardless of their elevation. Temperate species should therefore evolve broad thermal tolerances, and be more physiologically similar across elevation gradients. In contrast, year-round temperatures on tropical mountains are relatively stable, which results in little overlap in temperature across elevations (i.e. temperatures are always warmer in the lowland tropics and colder at higher elevations). As a consequence, tropical organisms at different elevations experience both a narrow and different range of temperatures which is predicted to lead to the evolution of narrower thermal tolerances.

Such physiological differences should result in differential dispersal ability between temperate and tropical species (Janzen 1967). Specifically, temperature should be a more effective barrier to dispersal in tropical species compared to temperate species because of the increased likelihood of encountering suboptimal temperatures when moving to either higher or lower elevation. Predicted consequences of stratification of temperature, narrower thermal tolerances, and reduced dispersal are smaller elevation range sizes, greater species turnover, and

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higher biodiversity in tropical mountains (Janzen 1967, Ghalambor et al. 2006). Despite the general appeal of this hypothesis, few studies have tested it and we have little knowledge about how physiological traits and dispersal vary in phylogenetically-related, functionally similar species experiencing different thermal regimes.

Because reduced dispersal ability decreases gene flow between populations, it is a key process in population differentiation, speciation, and the generation of species diversity (Martin & McKay 2004; Ghalambor et al. 2006; Fine 2015). Constraints to dispersal across a landscape can occur due to geographic distance between populations (Wright 1943; Rousset 1997), physical barriers, or landscape "resistance" features, which restrict dispersal and gene flow (Clobert et al 2001, Bowler & Benton 2005). However, dispersal can also be restricted because populations are locally adapted to different environments and have minimal gene flow between them (Wang & Bradburd 2014), resulting in high genetic structure. Such "isolation-by-environment" (IBE) has been documented in a number of species (reviewed in Shafer & Wolf 2013), suggesting that it is a common occurrence in nature. Dispersal especially depends on biophysical factors like temperature (Richter 2009) as seen in a variety of organisms such as marine fish (O'Connor et al. 2007), invertebrates (Benestan et al. 2016; Tyler & Young 1998; Velasco & Milan 1998), mammals (Alter et al. 2015; Castillo et al. 2014), and birds (Sokolov 2000). Thus, an important mechanism by which IBE can occur is if organisms exhibit local adaptation in physiological tolerance and moving to a different thermal environment is physiologically stressful. For example, island-dwelling *Anolis* lizards locally adapted to thermally distinct natal islands experience strong selection against dispersal between islands because of maladapted thermal tolerance traits (Logan et al. 2016). Comparing the relationship between environmental temperature, thermal tolerance, and gene flow across elevations in

different climatic regions can provide insights into the mechanisms that underlie population genetic structure and ultimately test if climate variability contributes to latitudinal differences in biodiversity.

Mountain stream insects are an excellent study system in which to test hypotheses related to thermal environment, population genetic structure and range distributions (Gill et al. 2016; Polato et al. 2016; Shah et al. 2017a). Phylogenetically related species occur in freshwater streams along elevation gradients in temperate and tropical mountains, facilitating comparisons. Members of the family Baetidae are commonly found in streams around the world and fulfill similar ecological roles as the primary consumers of algae and periphyton (Hynes 1970). Being exposed to fast-flowing water, larval baetids often passively disperse or "drift" to lower sections of streams. Upon reaching winged adulthood, they can fly upstream or disperse to different drainages (Bilton et al. 2001). Indeed, dispersal is an important feature in mayfly ecology allowing populations to maintain connectivity (Schmidt et al. 1995). Despite commonalities among baetid species, temperate and tropical populations differ markedly in thermal tolerance (Shah et al. 2017a). As ectotherms, mayflies are highly responsive to water temperature, which is well known to play an important role in their life-history because it governs individual body temperature, physiological processes, and affects dissolved oxygen concentrations in streams (Ward & Stanford 1984; Verberk et al. 2011). Mayflies that occupy streams along elevation gradients therefore provide an opportunity to test the effect of thermal tolerance and environmental temperature variation on dispersal, a topic that remains unresolved (Finn et al. 2006).

To date, studies have shown that temperature regimes vary between temperate and tropical streams. For example, Andean streams in Ecuador are characterized by low seasonal

variation compared to Rocky Mountain streams, and average and maximum stream temperatures generally decrease with elevation at both latitudes (Table 5.1) (Shah et al. 2017a). Along with a decrease in temperature, measured critical thermal maximum (CT_{MAX}) values, which are the maximum temperatures tolerated before the onset of locomotor dysfunction, also decrease with elevation. Thus, the lowest elevation populations have the highest CT_{MAX} (i.e., are more warm tolerant), and the highest elevation populations exhibit low CT_{MAX} values (i.e., are less warm tolerant). CT_{MAX} values differ across elevation more in Andean than Rocky Mountain mayflies, suggesting that tropical populations segregated along an elevation gradient are more physiologically different from one another than are temperate populations (Shah et al. 2017a). This pattern results in a strong positive correlation between stream temperature and CT_{MAX} at both latitudes.

If greater altitudinal stratification of temperature restricts dispersal among mayfly populations in the tropics as predicted by the Climate Variability Hypothesis, this gives rise to several testable predictions. First, population genetic structure corrected for geographic distance should be greater in tropical versus temperate populations across the elevation gradient (Slatkin 1987; Bohonak 1999; Baguette et al. 2013). Second, we expect that stream temperature, thermal physiology, or both will best explain genetic population structure in the tropics (Janzen 1967), while genetic structure among temperate populations should be low and best be explained by geographic isolation. Third, differences in temperature along the elevation gradient should generate divergent selective pressures resulting in more localized adaptation among tropical populations compared to temperate populations. We assume that because the two temperate and tropical genera are closely-related and ecologically similar, the genetic architecture underlying thermal tolerance is also similar (i.e. a similar number of loci determine thermal tolerance). If

divergent selection is greater and/or gene flow is more restricted among populations at different elevations in the tropics, then we should detect a stronger signal of divergent selection among mayfly populations at different elevations in the tropics compared to the temperate zone (i.e., detection of more outlier loci with high divergence in allele frequencies compared to neutral loci). Although several studies have compared population structure in temperate versus tropical species (Martin & McKay 2004; Eo et al. 2008; Eber & Brandl 1994; Aulard et al. 2002) and the effect of climate variability on thermal tolerance in related temperate and tropical species (Feder & Lynch 1982; van Berkum 1988; Addo-Bediako 2000; Ghalambor et al. 2006; Deutsch et al. 2008; Shah et al. 2017a), no study of which we are aware has systematically measured thermal tolerance and genotyped the same individuals to directly test the role of thermal tolerance in driving population divergence in species inhabiting temperate and tropical regions.

Here, we combined previously measured CT_{MAX} values (Shah et al. 2017a) as an index of thermal sensitivity with genome-wide single nucleotide polymorphism (SNP) data in temperate and tropical mayflies (Family: Baetidae) to test Janzen's (1967) extension of the Climate Variability Hypothesis or "Why Mountain Passes are Higher in the Tropics". In our previous work, we determined the strength of correlation between stream temperature and CT_{MAX} from our temperate and tropical sites. In the present study, we assessed neutral population genetic structure to investigate how connectivity between sites differs in temperate and tropical climates. Then, we tested the extent to which geographic distance, stream temperature, or physiology correlate with genetic structure among populations. Finally, we tested for evidence of divergent selection by investigating how many outlier loci were present in temperate versus tropical populations, and identifying any association of putative outlier loci with functional regions of the genome.

METHODS

Site Information

We sampled larval mayflies (Family: Baetidae) from small, wadeable tributaries of the main-stem rivers of the Cache la Poudre River (Rocky Mountains, Colorado; Fig. 5.1 A) and the Papallacta River (Andes, Ecuador; Fig. 5.1 B). We focused on two genera of mayflies (*Baetis* in the Rockies and *Andesiops* in the Andes) that are not only phylogenetically and morphologically similar, but also fulfill similar functional roles as grazers of algae and periphyton in streams. *Baetis* spp. were collected from 5 streams in the Rocky Mountains, between 2212m – 3166m, as they became increasingly rare in streams above this elevation in the Cache la Poudre River drainage. *Andesiops* spp. were collected from 6 streams in the Andes, between 2003m – 3898m (Fig. 5.1, Table 5.1). In our previous study, we measured physiological traits in the mayflies (Shah et al 2017a), then dried and preserved a subset for genomic analyses detailed below.

DNA extraction and genomic library preparation

We used double digest restriction-site associated DNA (ddRAD) sequencing (Peterson et al. 2012) to genotype single-nucleotide polymorphisms (SNPs) for each population (i.e. stream site). We sequenced a total of 158 baetids (Genus: *Baetis*) from 5 populations in the Rockies and a total of 65 baetid mayflies (Genus: *Andesiops*) from 6 populations in the Andes.

We isolated total genomic DNA from each dried insect using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. We then generated ddRAD DNA sequence libraries following methods suggested in Peterson *et al.* (2012). Individuals were barcoded and multiplexed in shared project pools of 288 individuals per library, following the protocols of Polato et al (2017) and Poff et al. (2018). In this modified protocol based on the

original ddRAD method (Peterson et al. 2012), digestion and adapter ligation steps are combined, and size selection is performed using magnetic beads (Ampure XP, Beckman-Coulter) to clean and select fragments from 300 to 1000 bp. We sequenced 100 bp single-end reads on an Illumina HI-SEQ 2500.

Sequence processing, assembly, and genotyping were performed using *Stacks v 1.42* (Catchen et al. 2013), for each latitude separately (Andes and Rockies). After demultiplexing, low quality reads were filtered out using default values in *process_radtags*. For each assembly, the *denovo_map* script was used with the following parameters (as in Poff et al. 2018) and based on initial parameter permutations as recommended by Paris *et al.* (2017): a minimum of 3 reads was required to create a stack (*-m*), 2 mismatches were allowed between loci when processing an individual (*-M*), 4 mismatches were allowed between loci when building the catalog (*-n*), and highly repetitive RAD-tags were removed or broken up in *ustacks*. We generated the initial SNP output using minimal filters in the program *populations* (Catchen et al. 2013), exporting a single random SNP per locus, and SNPs present in at least 20% (-r 0.2) of any given population (-p 1). We then removed loci that were genotyped in < 50% of individuals, individuals with > 50% missing data, and SNPs with minor allele frequencies < 0.01 using Plink! v. 1.07 (Purcell et al. 2007). After filtering we had a final SNP matrix for *Baetis* from the Rockies that retained 4 sites, 117 individuals, and 545 SNPs, and had a genotyping rate of 83%. The final SNP matrix for *Andesiops* from the Andes that retained 5 sites, 48 individuals, and 695 SNPs and had a genotyping rate of 87%.

Population structure analyses

We first analyzed our genomic data using a principal component analysis (PCA) to examine population structure in the Rockies and Andes. The PCA is unbiased because it does not

consider pre-defined populations (grouped by stream site in our data) to determine population structure. Thus, it is possible to visualize genetic structure among populations based solely on genetic differentiation. We performed PCA on the putative neutral loci dataset with outlier loci removed (see "Detecting outlier loci" below) in the package *adegenet* in R (Jombart and Ahmed 2011) . Then, using the PCA loadings, we conducted a second analysis of population structure known as a discriminant analysis of principal components (DAPC) in the same program. This analysis maximizes the variation found among pre-defined populations, i.e. loadings include alleles which show the greatest between-population differences and the smallest within-population differences. The DAPC is a powerful analysis because it also provides membership probabilities of individuals, making it possible to infer gene flow and admixture among populations. DAPC was also performed with putative neutral loci. Plots of population structure were output to show levels of admixture among the populations in the Rockies and Andes. We then calculated observed and expected heterozygosity (H_o and H_E). Lastly, we used HP-Rare 0.1(Kalinowski 2005) to estimate allelic richness (A_R) values for each population (Table 5.2).

Neutral landscape genomic analyses

To avoid the influence of loci under selection, we only used putative neutral loci as our index of connectivity for these analyses. We tested which of our three possible explanatory variables – Euclidean distance between sites, differences in temperature between sites, and CT_{MAX} differences between populations – best predicted F_{ST}. We employed Mantel tests (Mantel 1967), which are used to test the significance of correlations between two matrices, (i.e. F_{ST}; Legendre & Legendre 1998). An insufficient numbers of sites precluded the use of statistical analyses such as Maximum-Likelihood Population Effects (MLPE). We tested the effects of distance, temperature, and physiology on pairwise genetic differentiation (F_{ST}) among

populations at each latitude. Using the package *vegan* in R (version 3.4.0), we ran three simple

Mantel tests between F_{ST} and the three predictor variables: Euclidian distance (to test for effects

of geographic distance), temperature differences (to test for IBE), and differences in CT_{MAX} (to

test for the effect of physiology on population structure). Euclidian distances were calculated

using the package *geosphere* (R Core Team 2017), temperature differences were calculated as

the difference between (maximum or average) stream temperatures between sites, and

physiological distance was calculated as differences in population averages of CT_{MAX}.

Detecting outlier loci

We performed genome scans with the *pcadapt* package in R (Luu et al. 2017) to test for

potential outlier loci among populations separately for both latitudes. We used the which uses the

Mahalanobis statistic, a robust method for inferring loci putatively under divergent selection

(Luu et al. 2017). We used the qvalue approach implemented in the R package *qvalue* (Storey

and Tibshirani 2003) to test for potential outliers, using alpha scores of 0.1 based on the

transformed Mahalanobis statistic p-values (Luu et al. 2017). To compute the z-scores, we

visually determined K based on the screeplot (Jackson 1993). We ignored loci with minor allele

frequencies < 0.05 to reduce false discovery rates. Sequences of outlier loci were retrieved and

used in the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) to investigate

associations with any functional regions of the genome.

RESULTS

Population structure and inferred gene flow

Principle component analysis (PCA) plots suggested greater overlap and lower

population structure in Rocky Mountain mayfly populations (Fig. 5.2 A) than in Andean ones

(Fig. 2B). In the Andes, at high elevation, there appears to be some population substructure, where some individuals from ECP3683 do not cluster with others from that population (Fig. 5.2 B). Similarly, discriminant analysis of principal components (DAPC) plots revealed low genetic structure in Rocky Mountain populations (mean $F_{ST} = 0.073$; Table 5.3), seen as greater overlap among individuals from different populations (Fig. 5.3 A) and more shared cluster membership within individuals among populations (Fig. 5.4 A). Mayflies from high elevation (3166m and 2798m) showed a strong signal of admixture with the mid-elevation individuals (2590m). These results suggest that there is considerable gene flow across the elevation gradient, seen most clearly at the mid-elevation site (2590m) where high and low elevation individuals appear to meet and possibly hybridize (Figs. 5.3 A, 5.4 A).

In the Andes, populations remain relatively distinct, exhibiting high genetic structure (mean $F_{ST} = 0.264$; Table 5.3), with the exception of the highest elevation sites (3898m and 3683m) where there appears to be considerable admixture between the populations (Figs. 5.3 B and 5.4 B) even though some substructure exists. The greatest genetic differentiation occurs between high and low elevation populations, with the mid-elevation population (2694m) clustering closer to the low elevation populations (Fig 5.3 B).

Overall within-population variation was low in the Andes and higher in the Rockies (Table 5.2). This pattern was evident in the measures of observed heterozygosity (H_O) and allelic richness (A_R), both of which were higher for Rocky Mountain mayflies (Table 5.2). Expected heterozygosity (H_E) was somewhat higher than average in two tropical populations (2694m and 3683m).

Effects of environmental variables on F_{ST}

We found that maximum stream temperature, CT_{MAX}, and geographic distance are highly correlated for the tropical and temperate groups (Table 5.4). Despite these strong correlations among the three parameters, we found that maximum stream temperature is the most significant explanatory variable for population structure in Andean mayfly populations (Table 5.5; Fig. 5.5). Although a borderline significant correlation was noted between geographic distance and F_{ST}, no significant correlation was found for physiology and F_{ST}. Because physiology and stream temperature are so tightly correlated, separating their effects is not statistically possible with our study design. In temperate populations we found no statistically significant explanatory variable in Mantel tests (Table 5.5; Fig. 5.5).

Presence of putative outlier loci

We found a greater proportion of putative outlier loci in the Andean dataset (149 out of 742 loci = 20.1%) than the Rocky Mountain dataset (25 out of 545 loci = 4.6%). A BLAST search for association of these loci with any functional regions of the genome yielded no interpretable associations.

DISCUSSION

Understanding the mechanisms that shape differences in dispersal ability and gene flow has fundamental implications for patterns of population genetic differentiation, speciation, and species diversity. While several processes can lead to patterns of genetic differentiation across landscapes, such as historic demography and variation in effective population size (Wright 1969), reduced dispersal and gene flow are thought to be key in generating and maintaining spatial genetic structure (Bohonak 1999) and adaptive divergence (Garant et al. 2007). Numerous

studies address how landscapes influence gene flow, but still the causes of variation in gene flow remain poorly understood (Manel et al. 2003; Storfer et al. 2007; Storfer et al. 2010). For example, in some mayfly populations, gene flow shapes population structure (Hughes 2007; Finn et al. 2011), but the mechanism by which gene flow varies is unclear. Here, we tested if reduced thermal tolerance and increased changes in temperature across elevation gradients resulted in greater population structure in the tropics (Janzen 1967, Ghalambor 2007). We found support for this hypothesis, as temperate mayfly populations were less genetically differentiated from each other, and dispersal appears to be less constrained by temperature across the elevation gradient. In contrast, populations of tropical species were more genetically distinct from one another across the elevation gradient, suggesting that it is physiologically challenging to disperse to higher or lower elevation sites where temperatures are very different (Janzen 1967, Ghalambor et al. 2007).

Dispersal, temperature, and population structure

Mayflies can potentially disperse from natal sites as larvae through downstream drift or as winged adults via flight. During the larval stage, drift is more frequently the mode by which mayflies disperse within drainages (Bergey & Ward 1988) and occurs within the stream network (Meffe & Vrijenhoek 1998). Sometimes moving up to 2 km as larvae (Hershey et al. 1993), this is arguably the most important stage for aquatic insects as they develop and grow in streams for several months before emerging as winged adults. Dispersal by flight is shown to occur within and between drainages as well (Hershey et al. 1993; Schmidt et al. 1995), but adults live shorter lives (48-72 h) and likely fewer disperse at this stage compared to the larval stage (Waters 1972). Flying adults are also thermally sensitive as flight activity ceases below 7°C (Shipp et al. 1988; Briers et al. 2003) in temperate zones, possibly restricting adult dispersal from lower elevations

to very high elevations. Temperature can therefore affect both larvae and adults, and jointly

contribute to genetic structure in populations across elevation.

At both latitudes in our study, we inferred relatively low dispersal between the lower

elevation populations (~2,500m) and the high elevation populations (~3300m; see Table 5.3) .

Given the apparent absence of any physical barriers between these elevations, the genetic

differences we found may be a result of a combination of geographic distance and differences in

habitat type and environmental temperatures. For example, the landscape above 3000m in the

Andes changes dramatically, as mountain forests give way to the open grasslands of the high

elevation *páramo* region. Insects inhabiting streams in the *páramo* could be subject to much

greater variation in temperature, as there is little or no canopy cover to buffer streams from

extreme diel variation in temperature (Shah et al. 2017a). These populations can therefore be

isolated at high elevation as our results suggest. In fact, our results corroborate those from a

study examining population structure among *páramo* baetid populations in the Andes that found

no genetic structure among high elevation populations within drainages, but only between

drainages (Finn et al. 2016). Similarly, high elevation alpine streams in the Rockies can be

relatively isolated and exposed resulting in a thermally harsher habitat and a different suite of

selective pressures for resident mayflies (Finn et al. 2006; DeChaine & Martin 2004; Polato et al.

2016).

Population genetic structure in the Andes appeared to be best explained by the

differences in maximum stream temperature between sites in Andean but not Rocky Mountain

populations. The seasonally stable temperatures at each elevation in the Andes results in little

overlap in temperature across elevations, making each elevation thermally distinct from the

elevation above or below it. Evidence for how stream temperature can determine thermal

tolerance was seen in our previous work where different taxonomic orders exhibited similar physiological trait values despite being highly phylogenetically distinct (Shah et al. 2017a). Other studies that have assessed thermal limits in terrestrial insects (Addo-Bediako et al. 2000) or aquatic ectotherms (Sunday et al. 2012) have also found that different species living in the same environment exhibit similar levels of thermal tolerance. In the Colorado Rockies, high summer stream temperatures and near-freezing winter temperatures result in each elevation experiencing on average, similar temperatures (Shah et al. 2017a). From the time a Rocky Mountain baetid mayfly hatches from an egg in the early spring, to the moment it emerges from the stream as a winged adult in the late summer, the insect experiences some of the coldest (spring) and hottest (summer) stream temperatures measured in a given year (Brittain 1990). This thermal "experience" should result in broad thermal tolerance, and permit mayflies to move easily between warm low and cold high elevation streams for the several months that they are in larval form. In fact, we find evidence of this movement in the Rockies because there exists a zone of overlap between the high and low elevation species around 2500m. Here, both *B. bicaudatus* from high elevation and *B. tricaudatus* from low elevation can be found (Polato et al. 2016; A. Shah *pers. obs.*). Although we inferred high levels of dispersal among Rocky Mountains mayfly populations, we did not find complete panmixia perhaps simply due to the large distances between sites.

Teasing apart the relative importance of stream temperature, CT_{MAX}, and distance among streams was difficult given the naturally strong correlations among these predictor variables, and also the low statistical power due to the small number of sites in our study. However, stream temperature was found to be a significant explanatory variable for F_{ST} in Andean mayflies. However, Thermal tolerance is a strong candidate mechanism by which stream temperature

affects population genetic structure (Janzen 1967) and other studies have shown the importance of stream temperature on thermal tolerance. For example, our previous work has shown that stream temperature range predicts thermal breadth in both temperate and tropical aquatic insects (Shah et al. 2107a). Further, tropical mayflies exhibited reduced thermal plasticity, with low elevation species showing the greatest sensitivity to increasing temperatures (Shah et al. 2017b). However, at high elevation in the tropics, some level of thermal plasticity was detected (Shah et al. 2017b). This may be the reason we find that although different in CT_{MAX}, the two tropical high elevation populations are genetically very similar. We suspect that there may be some gene flow between these populations given their ability to withstand a wider range of temperatures and the relative proximity of those two sites. A final line of evidence showing that temperature affects physiology is that low elevation larval mayflies exhibited the highest swimming performance only at temperatures that closely matched their natural stream temperatures (Bacmeister et al. *unpublished*). Thus, stream temperature plays an important role in restricting movement along the stream network by driving differences in thermal tolerance traits. This is illustrated in the tropical mayfly populations of the present study where differences in pairwise CT_{MAX} values were greater in the Andes than in the Rockies, indicating that tropical populations are more physiologically different from one another compared to temperate populations.

Outlier loci, temperature, and thermal tolerance

If differences in stream temperature regime are more extreme across elevation in the Andes, then stronger divergent selection and reduced gene flow should enable higher levels of adaptive divergence and result in the detection of a higher number of putative targets of natural selection in Andean populations than in Rocky mountain populations (Funk et al. 2005; Funk et al. 2016). A much higher proportion of loci were high F_{ST} outliers in the Andes compared to the

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Rockies, which may suggest stronger divergent selection pressure among these populations. Similarly, greater local adaptation has been found for a variety of tropical taxa compared to temperate taxa (Chen & Schemske 2015; McKenzie et al. 2013). While in many cases gene flow is reduced due to landscape effects, for aquatic species, populations are connected via stream networks with fewer major barriers. Adults are able to fly between adjacent streams and larvae can often passively move downstream with flow (Bilton et al. 2001). Thus, within a drainage, thermal regime at different elevations has the potential to be a strong selective force leading to population differentiation. We were unable to find clear associations of the putative outlier loci with functional regions of the genome, possibly because of our small sample size and the absence of reference genomes for any closely related species. Nevertheless, our data clearly support many of the predictions of the CVH, thus providing a platform to base future studies on outlier loci and differences in heat tolerance between temperate and tropical species.

Impact of climate change on mayfly populations

Dispersal to more suitable habitat may be key to the success of many organisms in a warming world (Castillo et al. 2014). Range shifts have been widely documented where species move to higher elevation or latitude in response to warming (Parmesan 1996; Pounds et al. 1999; Hill et al. 2002; Parmesan & Yohe 2003). However, in species that appear to be physiologically confined to some environments, range shifts may not necessarily be possible, and a surprisingly low number of tropical, low elevation species have been found to expand their ranges (Parmesan et al. 1999; Thomas & Lennon 1999; Hill et al. 2002). Simulations of climate change scenarios conducted by Valladares et al. (2014) forecast extinction for populations that have limited-to-no dispersal, are genetically distinct from neighboring populations, or are locally adapted. Although our results suggest that tropical high elevation mayfly populations are greatly differentiated from

their low elevation counterparts, previous work has shown that they have wider thermal breadths (Shah et al. 2017a) and a slightly higher capacity to acclimate than their low elevation relatives (Shah et al. 2017b). But given the isolation of high elevation streams (Finn et al. 2011; Polato et al. 2016), and the lack of appropriate habitat at still higher elevations, these populations may be limited by the lack of "better" high elevation habitat to which they can disperse. On the other hand, low elevation tropical populations may be limited simply due to their narrow physiological capabilities, thus being unable to move to higher elevations on account of the unfavorable temperatures they would experience.

While the forecast for temperate mayflies may be more optimistic because of better dispersal ability and lower thermal sensitivity, high elevation species may still encounter difficulty in locating good quality high elevation habitat where they can expand their ranges. More studies jointly investigating the thermal physiology and genetic structure of populations are needed to better understand how populations will respond to climate change. As vital primary consumers in mountain stream ecosystems, mayflies are integral to stream health. The loss of mayfly populations and species may therefore have the potential to drastically change community composition and dynamics as global temperatures rise.

Table 5.1. Sample origin, site identification code, coordinates, elevation, average temperature, maximum temperature and average CT$_{MAX}$ of mayflies collected for this study. Average and maximum temperatures were calculated using data collected from loggers that were placed in streams for 8 months to 1 year.

Location	Site ID	Latitude	Longitude	Site elevation (m)	Average temperature (°C)	Maximum temperature (°C)	CT$_{MAX}$ (°C)
ROCKIES	COP2212	40.7034642	-105.5846997	2212	4.84	21.4	30.55
	COP2590	40.92771743	-105.6743979	2590	4.64	18.1	30.70
	COP2798	40.8133392	-105.7088208	2798	2.83	11.5	28.95
	COP3166	40.62347946	-105.7080039	3166	2.04	11.9	29.03
ANDES	ECP1845	-0.4503404	-77.8907438	1845	13.53	16.9	28.22
	ECP2003	-0.44924	-77.943	2003	13.95	15.81	27.57
	ECP2694	-0.3763895	-78.0747191	2798	10.02	13.17	25.79
	ECP3683	-0.2869309	-78.1153583	3683	8.57	11.96	26.60
	ECP3898	-0.3465564	-78.1997769	3898	6.57	10.8	24.66

Table 5.2. Summary statistics for number of individuals, observed (H_o) and expected (H_E) heterozygosity, inbreeding coefficients (F_{IS}) and allelic richness (A_R) in each mayfly population from the Colorado Rocky Mountains ("CO") and the Ecuador Andes ("EC").

Site Code	N	H_O	H_E	A_R
COP2212	54	0.080	0.089	1.42
COP2590	56	0.070	0.109	1.50
COP2798	48	0.072	0.092	1.49
COP3166	76	0.068	0.084	1.48
ECP1845	12	0.030	0.064	1.23
ECP2003	16	0.036	0.033	1.22
ECP2694	20	0.021	0.099	1.28
ECP3683	26	0.031	0.119	1.34
ECP3898	23	0.032	0.051	1.20

Table 5.3. Pairwise F_{ST} values of neutral loci for Andean and Rocky Mountain mayflies.

ROCKIES	COP2212	COP2590	COP2798	
COP2212				
COP2590	0.044			
COP2798	0.123	0.071		
COP3166	0.116	0.065	0.018	
ANDES	ECP1845	ECP2003	ECP2694	ECP3683
ECP1845				
ECP2003	0.099			
ECP2694	0.279	0.367		
ECP3683	0.263	0.324	0.194	
ECP3898	0.337	0.414	0.233	0.094

Table 5.4. Correlations among elevation, CT_{MAX}, and maximum stream temperature in the Rocky Mountains (A) and Andes (B). The three variables in our study were all highly correlated with one another at both latitudes.

Rockies	Max. temperature	CT_{MAX}	Elevation
Max. temperature			
Physiology	0.99		
Elevation	-0.86	-0.80	

Andes	Max. temperature	CT_{MAX}	Elevation
Max. temperature			
Physiology	0.95		
Elevation	-0.94	-0.82	

Table 5.5. Summary statistics for simple Mantel tests to examine the effects of landscape and physiological variables on mayfly populations structure in the Andes.

	Rocky Mountains		Andes	
	r	*P-value*	*r*	*P-value*
Maximum temperature	-0.191	0.75	**0.869**	**0.008**
CT$_{MAX}$	0.675	0.250	0.546	0.092
Distance	-0.480	0.833	**0.766**	**0.051**
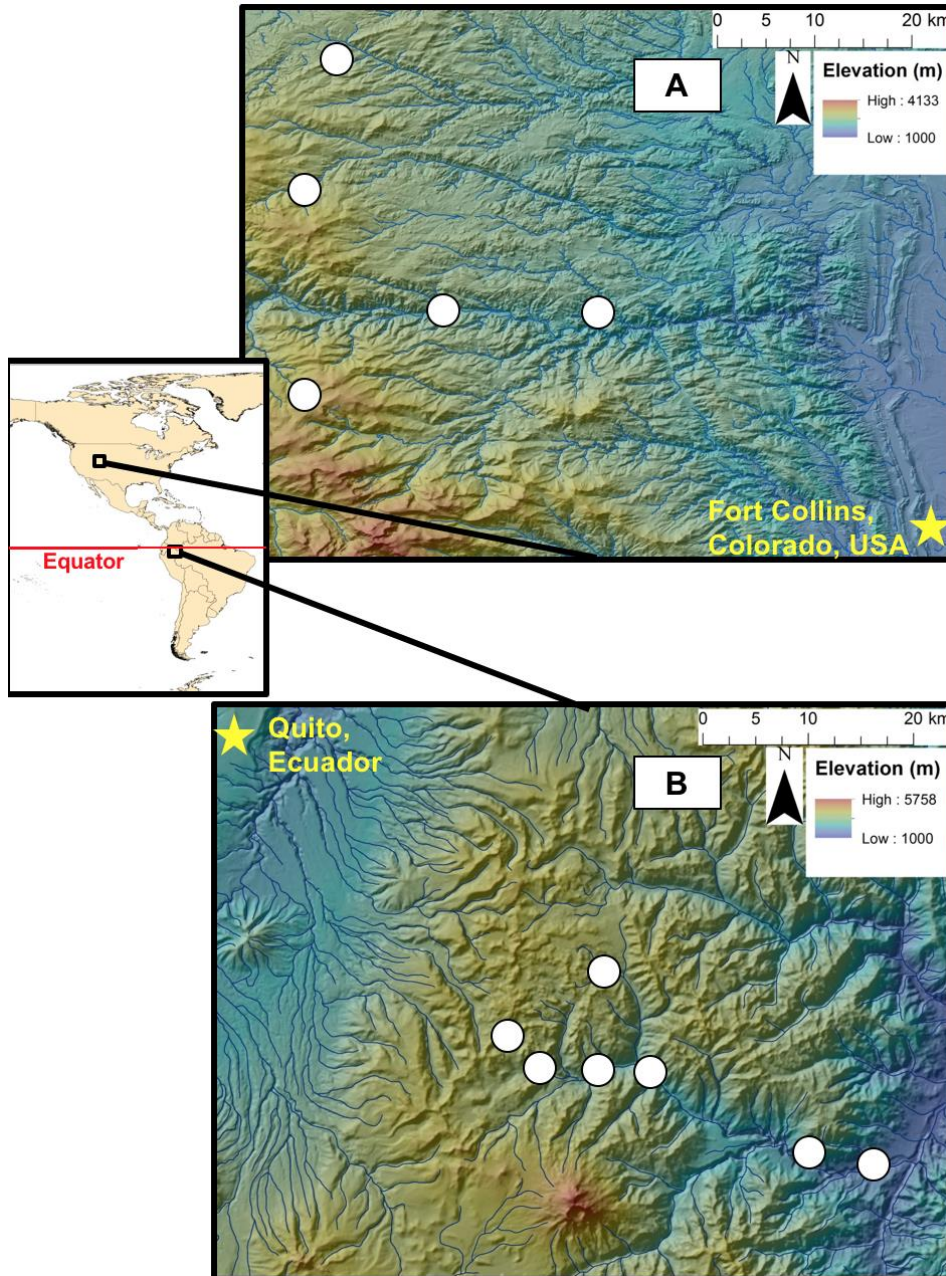
Figure 5.1. Stream sites (white circles) from which mayflies were collected in the Cache la Poudre river drainage in the Rocky Mountains, Colorado (A) and the Papallacta river drainage in the Andes, Ecuador (B). Sites in Colorado spanned between ~1900m and 3200m and in Ecuador between ~ 800m and 3800m. Sites were ~200m – 400m apart in elevation at both latitudes.
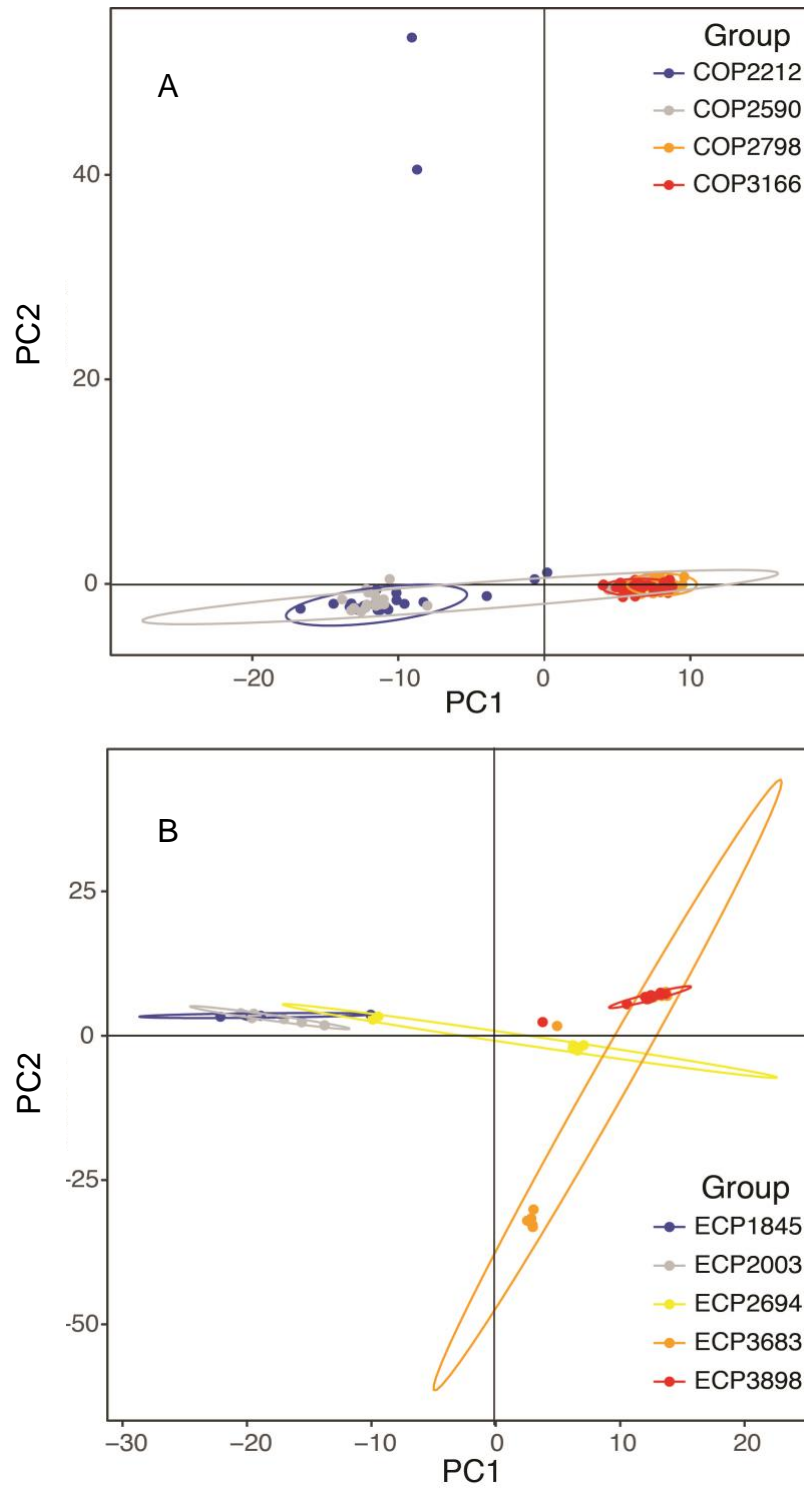
Figure 5.2. PCA plots for mayfly population in the Rockies (A) and Andes (B) based on analysis of putative neutral loci. Numbers in the site identification labels indicate the elevation of the site. For example, COP2798 is a Rocky Mountain stream site at 2798m a.s.l.
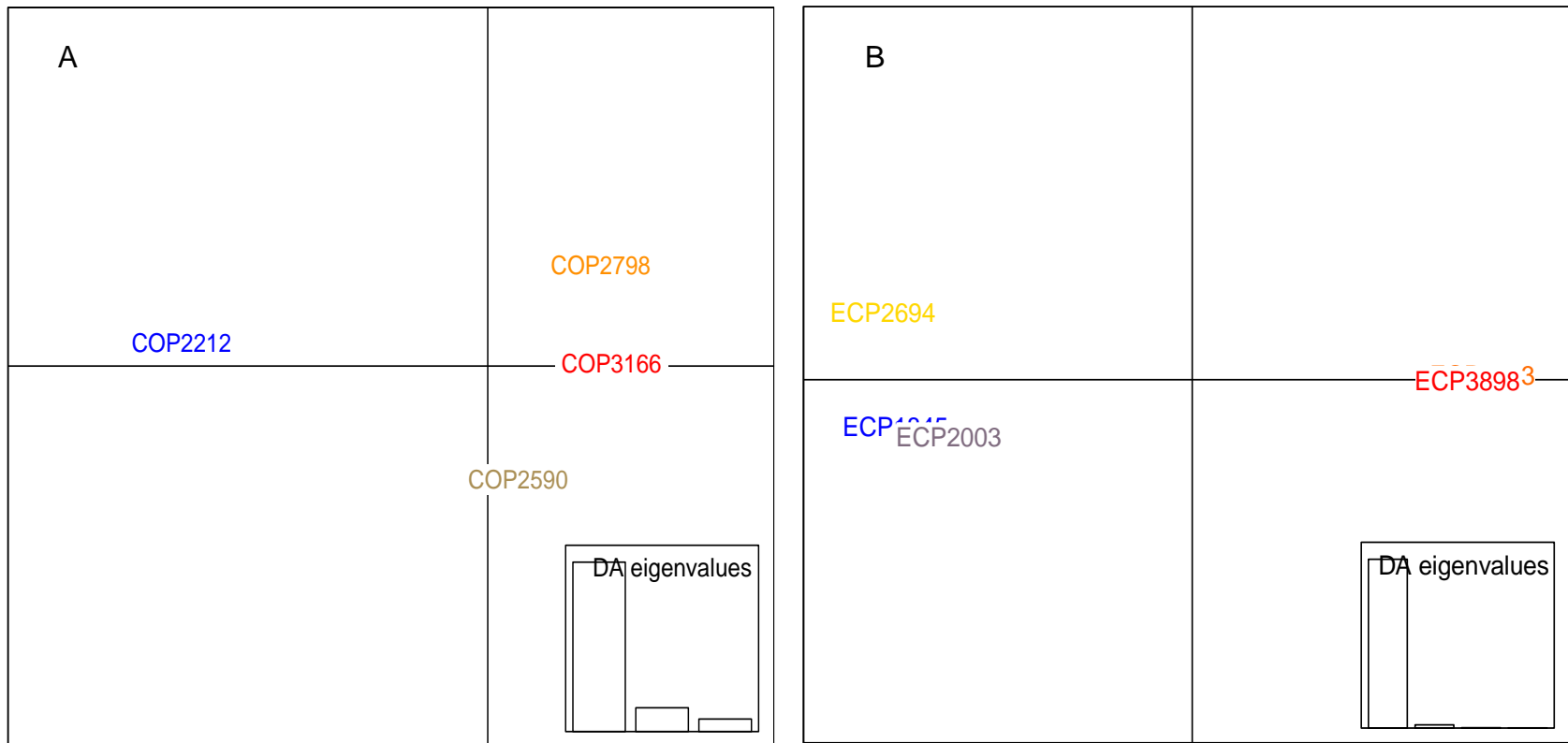
Figure 5.3. DAPC plots for mayfly populations in the Rockies (A) and Andes (B) based on analysis of putative neutral loci.
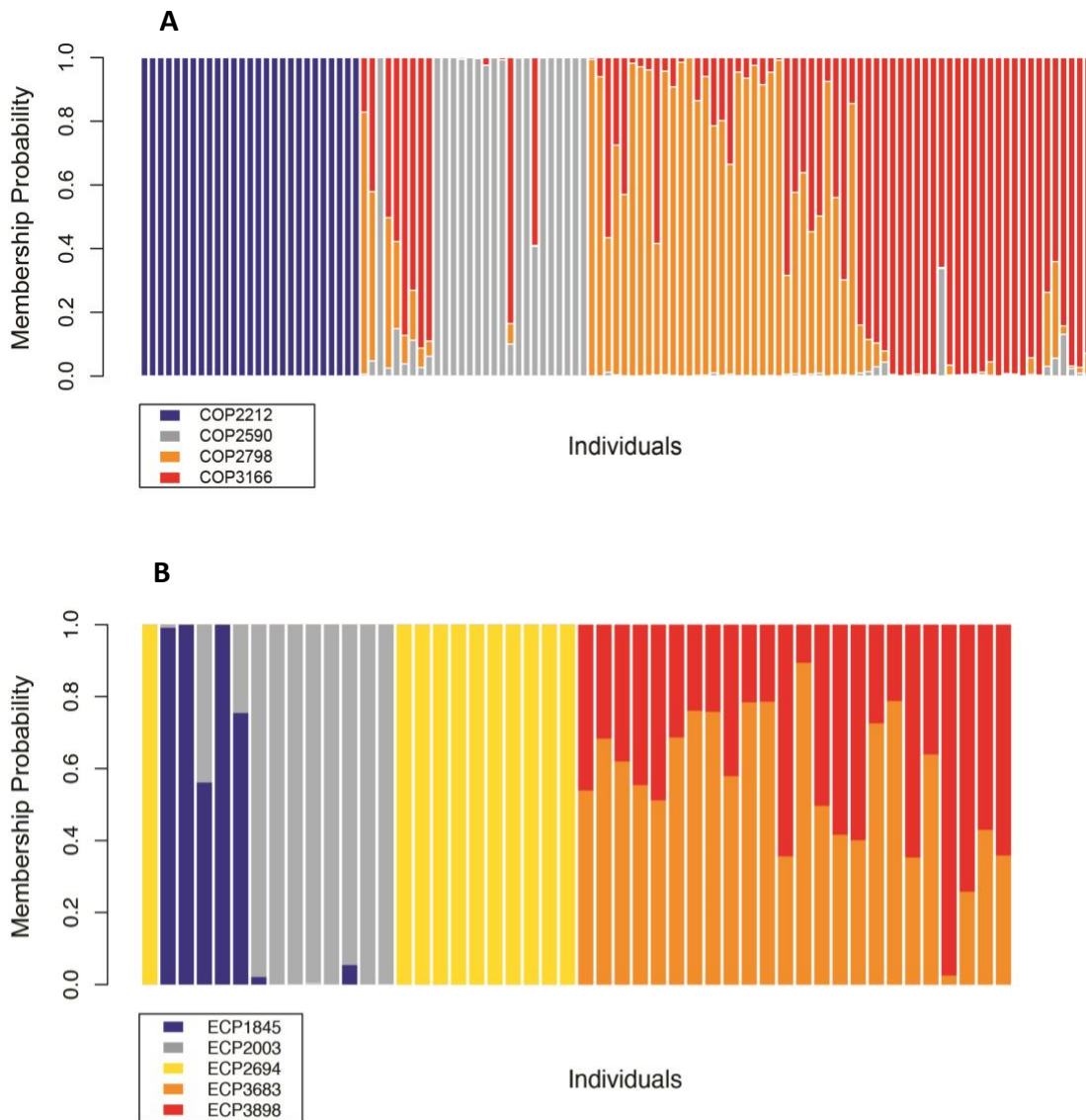
Figure 5.4. STRUCTURE-like plots of DAPC analysis for a global picture of the composition of clusters in the Rocky Mountains (A) and Andes (B). Each individual is represented by a vertical colored bar. The same color in different individuals indicates that they belong to the same cluster. Population codes are given beneath each plot.
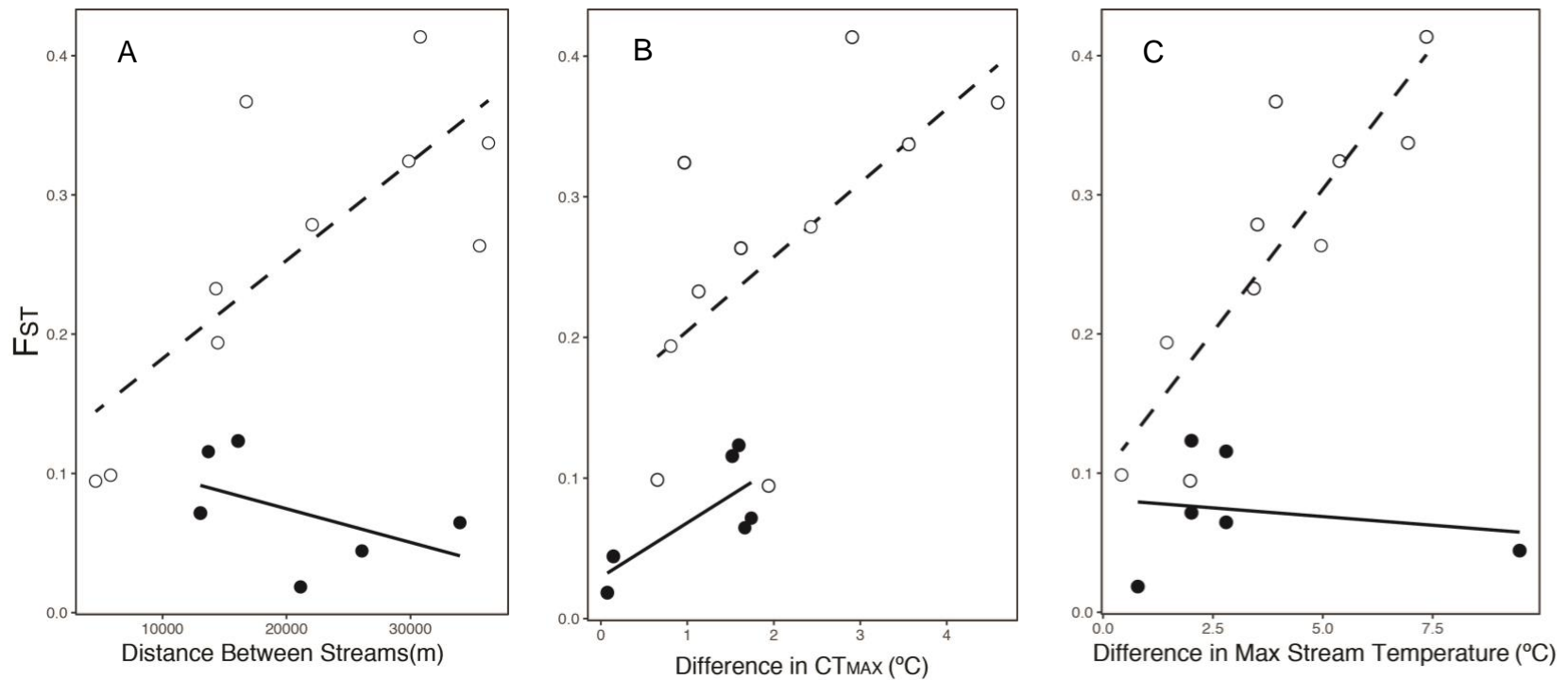
Figure 5.5. Scatterplots of F_{ST} calculated using neutral SNPs versus pairwise differences in distance (A), physiology (B), and temperature (C) in the Rocky Mountains (closed circles and solid lines) and Andes (open circles and dashed lines). In the Andes, we found a significant relationship between F_{ST} and maximum temperature (r = 0.869; p = 0.009) and a borderline significant relationship between F_{ST} and distance between streams (r = 0.766; p = 0.05).

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METHODS

Maximum Critical Thermal Limit CT$_{MAX}$

Experiments were carried out in a 45 L insulated water bath. We ramped the temperature of the water bath using a temperature controller (16C-2, Dwyer Instruments Inc., Michigan City, Indiana) at the rate of ~0.3°C min^{-1}. This moderate rate of increase prevents shocking individuals with too rapid a temperature change and prevents possible acclimation to the experimental temperature with too slow a change (Dallas & Rivers-Moore 2012). We monitored percent oxygen saturation to ensure it was no less than 70% to mimic the high levels of oxygen found in our study streams.

In each experiment, we tested up to 12 individuals (mean = 8.34, min = 4) per MTU per stream site. We placed individual insects in semi-circular mesh containers, 6 cm in diameter, and observed them for sub-lethal behavioral changes as we ramped temperature. We followed the criteria used in previous critical thermal limit studies which found that behaviors such as the loss of righting response, spasms, and an inability to cling to substrate provide repeatable estimates of critical thermal limits in a variety of aquatic insect species (See Appendix Table S1.2; Calosi, Bilton & Spicer 2008; Dallas & Rivers-Moore 2012). In our experiments, sub-lethal behavior varied among species but remained highly repeatable within populations. When individuals approached CT$_{MAX}$ we noted the temperature and transferred individuals to cool, aerated water for recovery. Because critical limits are not lethal in the short term, we excluded data from

insects that did not recover, and used data from only those insects that resumed normal swimming or activity (n=847 individuals, 62 species).

Minimum Critical Thermal Limit CT$_{MIN}$

To measure CT$_{MIN}$, we ramped temperature down using a thermoelectric cooler (TEC) Peltier plate (225 W) attached to an aluminum heat sink and powered by a 12V enclosed single output DC power supply. We prolonged the onset of ice-formation in the bath by vigorously circulating the water with a pump (302 L per hour). We ramped water temperature down at the rate of ~0.2 °C min^{-1}. Each CT$_{MIN}$ trial consisted of testing a maximum of 9 individuals per MTU (mean = 8.5, min = 5) that were placed in acrylic containers with holes and monitored for loss of righting response to determine CT$_{MIN}$. As with CT$_{MAX}$, only those individuals that fully recovered from the thermal stress when returned to normal temperatures were used in analyses. After recovery from experiments, all insects were stored in 96% ethanol, dried for 24h at 56 °C, and weighed to obtain individual body mass estimates.

DNA Barcoding

We DNA barcoded (Hebert et al., 2003) 323 specimens from CT$_{MAX}$ experiments by sequencing the standard animal DNA barcode, Cytochrome c Oxidase subunit I, using protocols from the Canadian Center for DNA barcoding (Hajibabaei *et al.* 2005; Ivanova, DeWaard & Hebert 2006b; Ivanova *et al.* 2006a). All DNA barcodes are publicly available on the Barcode of Life Database (S Ratnasingham & Hebert, 2007) in the dataset "Critical Thermal Limits of Mountain Stream Insects" (DS-CTLMSI; DOI: dx.doi.org/10.5883/DS-CTLMSI). Refined single linkage clustering (Sujeevan Ratnasingham & Hebert, 2013) of these records resulted in the identification of 36 putative species (21 Colorado; 15 Ecuador).

Phylogenetic Methods

PGLS requires a phylogeny with branch lengths to determine the expected covariance in trait values among taxa. Because no comprehensive phylogeny for aquatic insects exists, we estimated our own using available DNA barcode data and constraints from the aquatic insects systematics literature (see Appendix Fig. S1.3). For the alignment, we randomly chose one sequence from among available sequences for each putative species as determined by DNA barcoding at each site (population) or previous work (Gill et al. *unpublished*). This taxon-sampling scheme was used to account for potential among population differences in physiological trait values. We aligned these sequences using MAFFT (Katoh, 2002) using strategy G-INS-i with offset value 0.1 and all other options set as default. We used jModelTest2 (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003) and Akiake's Information Criterion to determine that the $GTR + \Gamma$ model of nucleotide substitution was most appropriate. We ran four simultaneous analyses with four chains for 50,000,000 generations in MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) through the CIPRES science gateway (Miller, Pfeiffer, & Schwartz, 2010). We checked convergence by ensuring that the average standard deviation of split frequencies was < 0.01, that effective sample sizes for parameters were >200, and by plotting the -ln likelihood scores against generation time in Tracer v 1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). We discarded the first 25% of trees as burn-in before constructing the 50% majority rule consensus tree.

Phylogenetic Generalized Least Squares Regression

We used PGLS (Grafen, 1989) fit with the Ornstein–Uhlenbeck model (OU; (Butler & King, 2004; Hansen, 1997; Appendix Table S1.4) of trait evolution to control for phylogenetic signal in CT_{MAX}, CT_{MIN}, and thermal breadth using the gls function in the R package (R Core Team

2013) nlme (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2015). Because our tree was not ultrametric, we modeled variance heterogeneity using the option "weights" in gls (Paradis, Claude, & Strimmer, 2004). The model parameter α was determined using maximum likelihood optimization. PGLS models predicting CT_{MAX}, CT_{MIN}, and thermal breadth included latitude as the main effect, elevation and dry weight as covariates, and an interaction between latitude and elevation.

The use of DNA barcoding for species delimitation allowed us to determine if multiple species existed within MTUs for each site. Because CT_{MAX} and CT_{MIN} experiments took place at different times and DNA barcoding data were only available for CT_{MAX} specimens, we sometimes did not have measures of critical thermal limits for all taxa identified using DNA barcoding. Consequently, we ran PGLS for CT_{MAX}, CT_{MIN}, and thermal breadth separately for sets of taxa for which data were available. To assign CT_{MIN} data to taxa delimited by barcoding of CT_{MAX} specimens, we used two decision rules: 1) if a single species was identified within a site using DNA barcoding and the MTU associated with available CT_{MIN} data was concordant with the barcode identification, then available CT_{MIN} data was associated with that taxon, and 2) if multiple species were found within a site using DNA barcoding and the MTU associated with available CT_{MIN} data was concordant with the barcode identification, then CT_{MIN} data was randomly split among each putative species for each site.

Phylogenetic and PGLS Results

All putative species represented by multiple records (populations) were well supported as monophyletic (posterior probabilities >0.99) by the phylogenetic analysis. Together, our DNA barcodes and constraints from the aquatic insects systematics literature allowed us to generate a

well-resolved phylogeny with branch lengths determined as the relative divergence among taxa

(Appendix Fig. S1.3). Results of PGLS are reported in Appendix Table S1.3.

Table S1.1 Temperate and tropical aquatic insect orders and families selected for study. Starred families occurred in tropical streams as well as temperate streams and at multiple elevations.

	Stream elevations where present (m)	Order	Family	Functional Feeding Guild
Temperate (Colorado, U.S.A.)	1992 - 3166	Ephemeroptera	Baetidae*	Collector-gatherers, scrapers
	1992, 2590, 2798	Ephemeroptera	Ephemerellidae	Collector-gatherers, facultative predators
	1992, 2798	Ephemeroptera	Heptageniidae	Scrapers
	1992, 2798, 3166	Plecoptera	Chloroperlidae	Predators
	1992, 2212, 2590	Plecoptera	Perlidae*	Predators
	1992, 2798, 3166	Plecoptera	Perlodidae	Predators
	1992, 2212, 2590	Trichoptera	Hydropsychidae*	Collector-filterers
	1992, 2212, 2798, 3166	Trichoptera	Rhyacophilidae	Predators
Tropical (Papallacta, Ecuador)	1845-3898	Ephemeroptera	Baetidae*	Collector-gatherers, scrapers
	1845	Ephemeroptera	Leptophlebiidae	Collector-gatherers, filterers
	1845, 2003, 2694, 2957	Plecoptera	Perlidae*	Predators
	1845, 2003, 2694, 2957	Trichoptera	Hydropsychidae*	Collector-filterers
	2694	Trichoptera	Hydrobiosidae	Predators

Table S1.2 Behavioral responses of aquatic insect taxa to warming in CT_{MAX} experiments.

	Taxon	Response
EPHEMEROPTERA	Baetis, Myobaetis, and Andesiops spp.	Activity declines with warming and insects begin "drifting" at the surface. Intermittent leg spasms and abdominal swaying occur when approximately 5-6°C away from CT_{MAX} temperature. Loss of righting response and inability to cling to substrate occur at CT_{MAX} temperature.
	Baetodes spp.	Drifting and leg spasms occur soon after water temperature begins to rise. Loss of righting response occurs at CT_{MAX}.
	Drunella spp.	Gill beats are rapid with initial warming. Close to CT_{MAX}, gill beats slow considerably. Will often swim to surface, and then drift down. Loss of righting response occurs at CT_{MAX}.
	Epeorus and Rhithrogena spp.	Underside of body turns deep red in most individuals with onset of heat stress. Clinging and righting ability are lost at CT_{MAX}.
PLECOPTERA	Sweltsa and Suwalia spp.	Usually very active at the start of experiment. Movement slows considerably as temperature rises. Eventually, movement stops entirely and loss of righting occurs at CT_{MAX}.
	Hesperoperla, Megarcys, Kogotus, Anacroneuria spp.	Active at the start of the experiment and will often attempt to escape. "Pushups" commence as water warms and activity speeds up even more, but is decidedly less coordinated at higher temperatures. Appear "disoriented" and mandibles are often held open just before CTmax. Megarcys and Kogotus often arch backward before losing the ability to right themselves at CT_{MAX}. For all others, loss of righting response occurs at CT_{MAX}.
TRICHOPTERA	Arctopsyche and Leptonema spp.	Insects often start building retreats with saliva. Show "aggression" if an object is brought close and will "snap" with mandibles. This response mellows as temperature increases. Body undulations become common with warming and some insects will emerge from their retreats. Individuals curl up into tight balls close to CTmax, and will often lose the ability to cling with anal prolegs. Loss of righting is the ultimate response to CT_{MAX}.
	Rhyacophila and Hydrobiosid spp.	At warmer temperatures insects will curl into a tight ball and lose the ability to cling with the anal prolegs. Loss of righting response occurs at CT_{MAX}.

Table S1.3 Summary of results from phylogenetic generalized least squares regression (PGLS) for CTMax, CTMin, and thermal breadth fit with an Ornstein-Uhlenbeck (OU) model of trait evolution (maximum likelihood used to determine α). PGLS was used to control for shared evolutionary history while comparing physiological metrics across latitude. The explanatory variable "latitude" was dummy coded "0" for Colorado and "1" for Ecuador. We also included elevation, dry weight, and the interaction between latitude and elevation in the model. Values are presented as parameter estimates ± standard error (SE).

	Intercept (± SE)	p	Latitude (± SE)	p	Elevation (± SE)	p	Dry Weight (± SE)	p	Latitude X Elevation (± SE)	p	α
CT$_{MAX}$	35.816 (± 2.372)	0.000	-7.520 (± 2.739)	0.007	-0.003 (± 0.001)	0.011	36.779 (± 24.367)	0.135	0.002 (± 0.001)	0.150	301.703
CT$_{MIN}$	0.467 (± 1.254)	0.711	11.778 (± 1.386)	0.000	0.000 (± 0.000)	0.939	-18.023 (± 10.772)	0.101	-0.003 (± 0.001)	0.000	180.778
Thermal Breadth	38.645 (± 4.565)	0.000	-26.885 (± 5.028)	0.000	-0.004 (± 0.002)	0.037	104.759 (± 38.574)	0.009	0.008 (± 0.002)	0.000	315.315

Table S1.4 Model selection output for PGLS analysis. The Ornstein–Uhlenbeck (OU) model has greater support (lower AIC values, significantly higher log likelihood values) compared to Brownian Motion models for CT_{MAX}, CT_{MIN} and thermal breadth.

	Model	AIC	logLik	Likelihood Ratio	p-value
CT_{MAX}	BM	565.7473	-276.8736		
	OU	399.1047	-192.5524	168.6425	<0.0001
CT_{MIN}	BM	218.9899	-103.495		
	OU	128.7872	-57.3936	92.20273	<0.0001
Thermal Breadth	BM	356.0358	-172.0179		
	OU	259.6978	-122.8489	98.33807	<0.0001
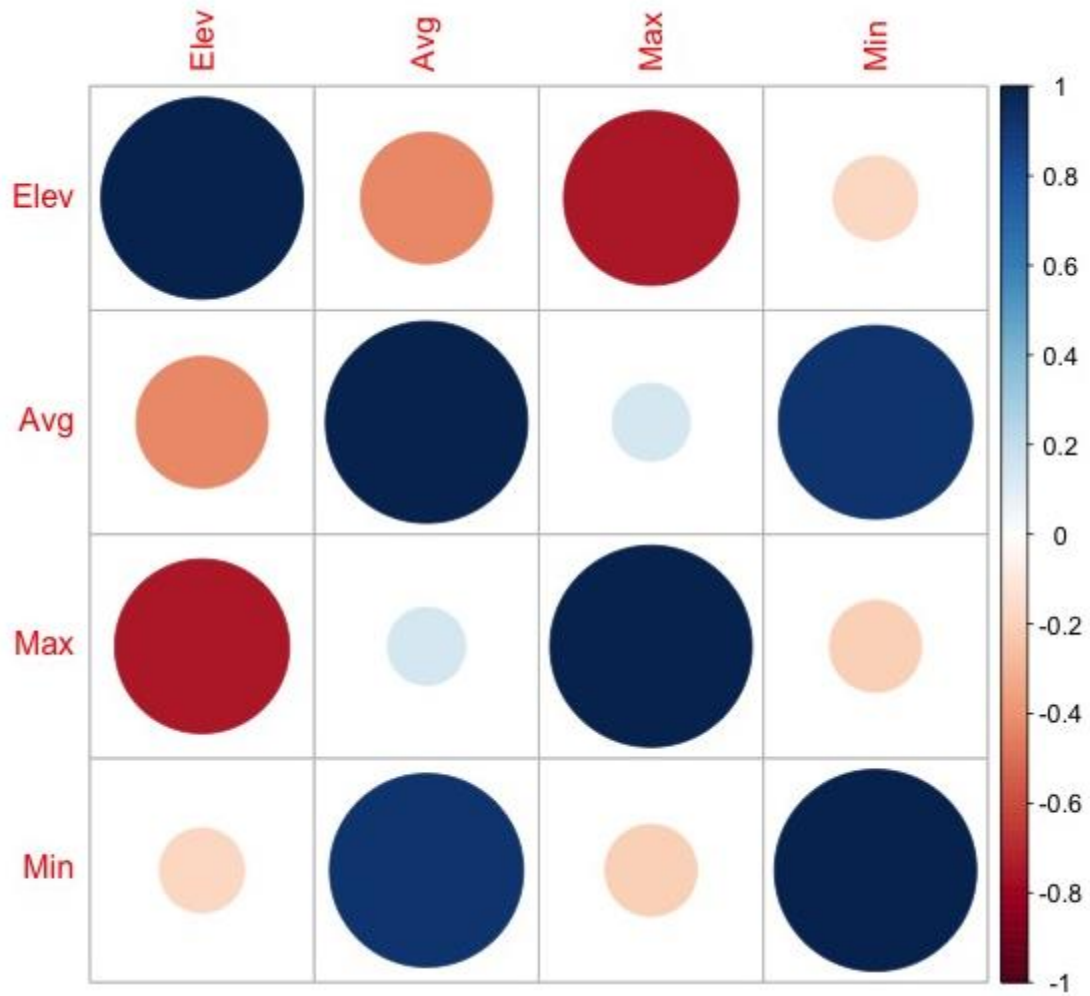
Figure S1.1 Correlation matrix for elevation, average temperature, maximum temperature, and temperature range. We used the high correlation between elevation and stream temperature parameters shown here as justification for using elevation in our PGLS models.
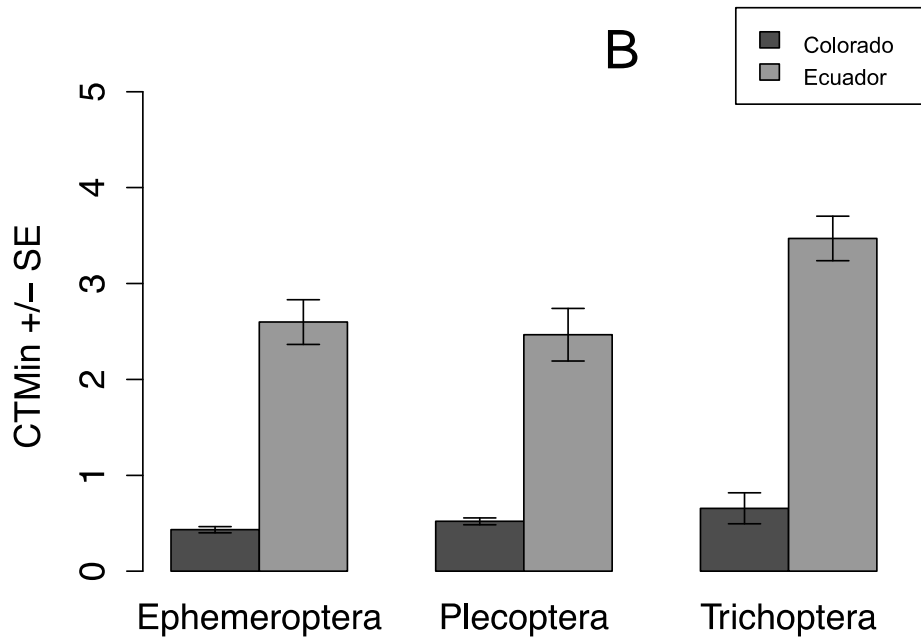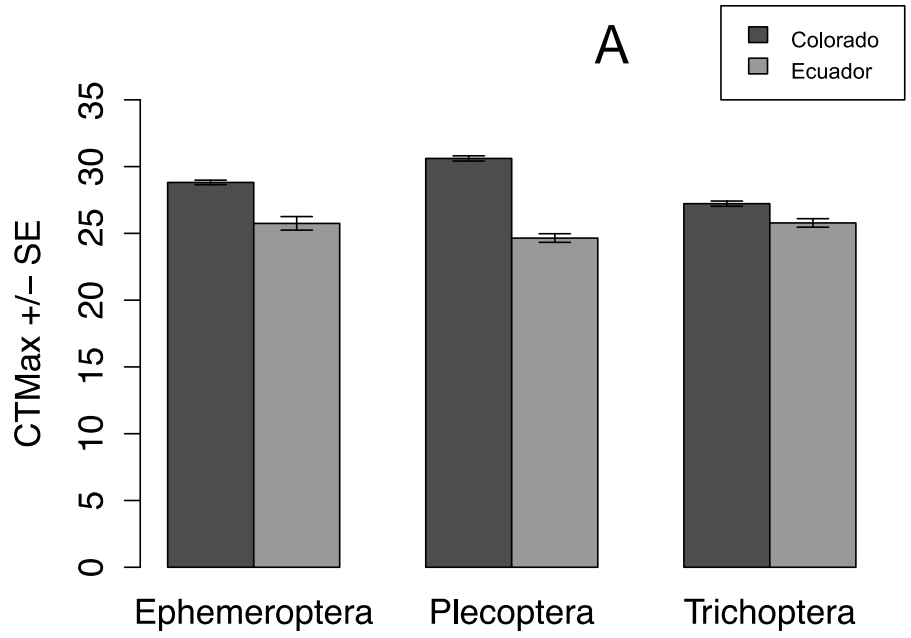
Figure S1.2 Bar plots of the raw data for CTmax (A) and CTmin (B) for insects from Colorado (dark grey) and Ecuador (light grey), split by order. In general, CTmax values were higher and CTmin values were lower in Colorado insects from all orders compared to those from Ecuador.

Constraint	Node Reference	Citation
Genus *Baetodes*	1	Needham & Murphy 1924
Baetodes + Prebaetodes	2	Lugo-Ortiz & McCafferty 1996
Baetodes + Prebaetodes + Mayobaetis	3	Lugo-Ortiz & McCafferty 1996
Genus *Andesiops*	4	Lugo-Ortiz & McCafferty 1999
Family Baetidae	5	Ogden et al. 2009
Genus *Baetis*	6	Leach 1815
Genus *Drunella*	7	Ogden et al. 2009
Order Ephemeroptera	8	Ogden et al. 2009
Drunella + Leptohyphes	9	Ogden et al. 2009
Drunella + Leptohyphes + Thraulodes	10	Ogden et al. 2009
Genus *Anacroneuria*	11	Terry & Whiting 2003
Anacroneuria + Kogotus + Megarcys	12	Terry & Whiting 2003
Genus *Kogotus*	13	Terry & Whiting 2003
Family Perlodidae	14	Terry & Whiting 2003
Order Plecoptera	15	Terry & Whiting 2003
Genus *Sweltsa*	16	Terry & Whiting 2003
Family Chloroperlidae	17	Terry & Whiting 2003
Genus *Suwallia*	18	Terry & Whiting 2003
Plecoptera + Trichoptera	19	Misof et al. 2014
Genus *Leptonema*	20	Holzenthal et al. 2006
Family Hydropsychidae	21	Holzenthal et al. 2006
Order Trichoptera	22	Holzenthal et al. 2006

Figure S1.3 Bayesian 50 percent majority rule consensus tree constructed using DNA barcodes. Smaller numbers adjacent to internal nodes are posterior probabilities. Larger numbers correspond to constraints imposed on our tree topology based on the literature: 1 Lugo-Ortiz & McCafferty 1999; 2 Leach 1815; 3 Waltz & McCafferty 1985; 4 Needham & Murphy 1924; 5–6 Lugo-Ortiz & McCafferty 1996; 7–13 Ogden et al. 2009; 14–24 Terry & Whiting 2003; 25–31 Holzenthal et al. 2006; 32 Misof et al. 2004.

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APPENDIX I LITERATURE CITED

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Method for calculating metabolic rate for each insect.

We extracted average MR values from the Unisense program (see Appendix Fig. S2.1 for schematic) raw data output using R-code. We wrote code to draw a regression line through all of the data and then used the slope of that line as the MR for that particular individual (see example data Appendix Fig. S2.2). We then calculated "control corrected MR" by subtracting average control (or background) MR rates from individual insect MR values. To correct for body size, we divided the metabolic rate by dry weight of each insect. Note that in statistical analyses, we used dry weight as a covariate in the model and control-corrected MR as a response variable. However, for graphing purposes, we used control and mass corrected MR values.

Table S2.1 Sample sizes and mortality shown for each test temperature at each elevation for tropical mayflies.

Latitude	Elevation	Test_Temp	N_total	N_stress	N_death	Notes
Ecuador	High	5	7	0	0	
Ecuador	High	7.5	14	0	0	
Ecuador	High	10	14	0	0	
Ecuador	High	12.5	7	0	0	
Ecuador	High	15	7	0	0	
Ecuador	High	17.5	14	**~5**	**2**	Dead after experiment
Ecuador	High	20	14	0	0	
Ecuador	High	22.5	14	0	**14**	Dead after experiment
Ecuador	High	25	6	0	**6**	Dead after experiment
Ecuador	Mid	5	3	0	0	
Ecuador	Mid	7.5	8	0	0	
Ecuador	Mid	10	7	0	0	
Ecuador	Mid	12.5	n/a	0	0	
Ecuador	Mid	15	7	0	0	
Ecuador	Mid	17.5	9	0	0	
Ecuador	Mid	20	4	**4**	**2**	Dead after experiment
Ecuador	Mid	22.5	5	0	5	Dead after experiment
Ecuador	Mid	25	6	**6**	**1**	Dead after experiment
Ecuador	Low	5	9	**9**	**9**	Dead after experiment

Ecuador	Low	7.5	8	0	0	
Ecuador	Low	10	8	0	0	
Ecuador	Low	12.5	30	0	0	
Ecuador	Low	15	15	0	0	
Ecuador	Low	17.5	7	0	0	
Ecuador	Low	20	6	0	0	
Ecuador	Low	22.5	7	0	0	
Ecuador	Low	25	12	0	**3**	Dead during acclimation

Table S2.2 Sample sizes and mortality shown for each test temperature at each elevation for temperate mayflies.

Latitude	Elevation	Test_Temp	N_total	N_stress	N_death	Notes
Colorado	High	5	8	0	0	
Colorado	High	7.5	8	0	0	
Colorado	High	10	7	0	0	
Colorado	High	12.5	8	0	0	
Colorado	High	15	8	0	0	
Colorado	High	17.5	8	0	0	
Colorado	High	20	7	0	0	
Colorado	High	22.5	7	**2**	0	
Colorado	High	25	7	4	0	
Colorado	Mid	5	7	0	0	
Colorado	Mid	7.5	14	0	0	
Colorado	Mid	10	14	0	0	
Colorado	Mid	12.5	14	0	0	
Colorado	Mid	15	14	0	0	
Colorado	Mid	17.5	13	0	0	
Colorado	Mid	20	14	0	0	
Colorado	Mid	22.5	14	0	0	
Colorado	Mid	25	14	0	0	
Colorado	Low	5	n/a	0	0	

Colorado	Low	7.5	17	0	0	
Colorado	Low	10	20	0	0	
Colorado	Low	12.5	22	0	0	
Colorado	Low	15	14	0	0	
Colorado	Low	17.5	14	0	0	
Colorado	Low	20	13	0	0	
Colorado	Low	22.5	13	0	0	
Colorado	Low	25	14	0	0	

O2 reduction current converted to a signal

O2 Microsensor

45-Quart cooler (water bath)

Signal amplified by Oxy-meter and converted by computer program to O2 concentration levels.

MR-Rack

Rack with chambers

Oxy-meter

Computer

Water level

800 rpm

Stirrer control

Glass stopper

Capillary to fit O2 probe

4mL respirometry chamber

Test insect

Mesh structure for insect to rest

Plastic ring with mesh top to prevent contact between insect and rotating magnetic stirrer
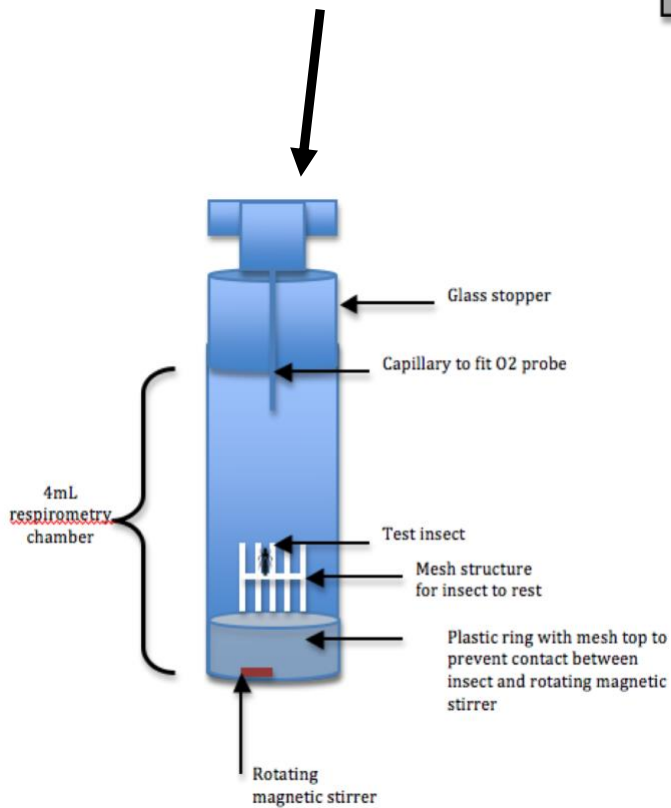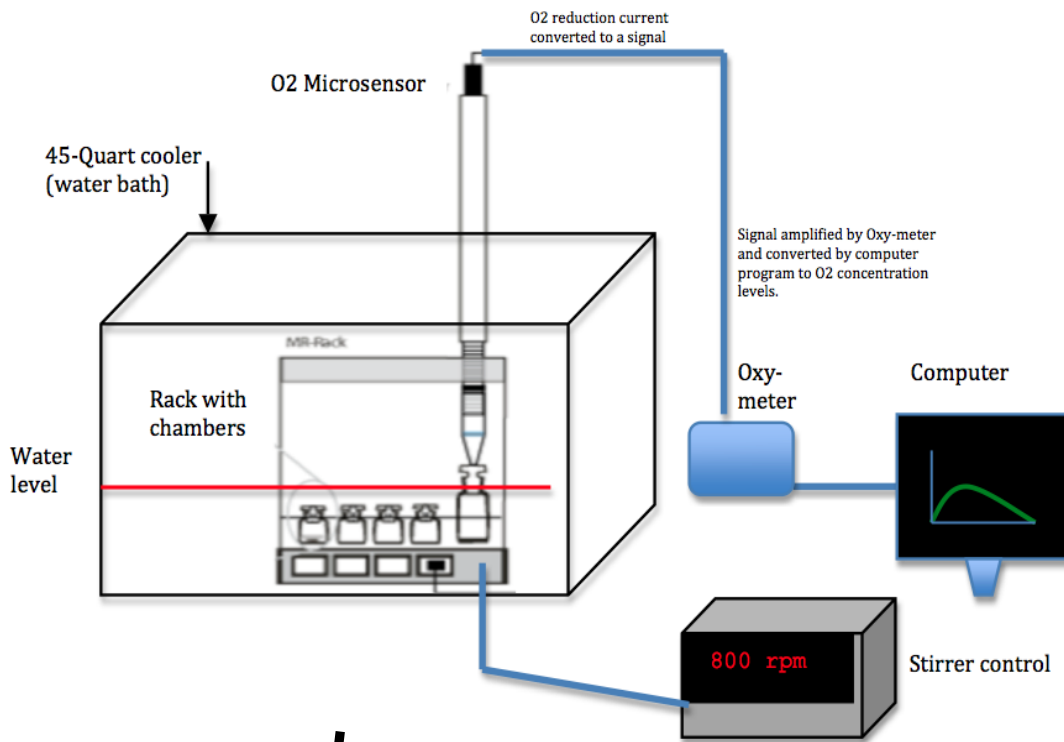
Rotating magnetic stirrer

Figure S2.1 Schematic representation of closed-respirometery system used to measure standard metabolic rate of temperate and tropical mayflies. The set-up of a single chamber is also shown.
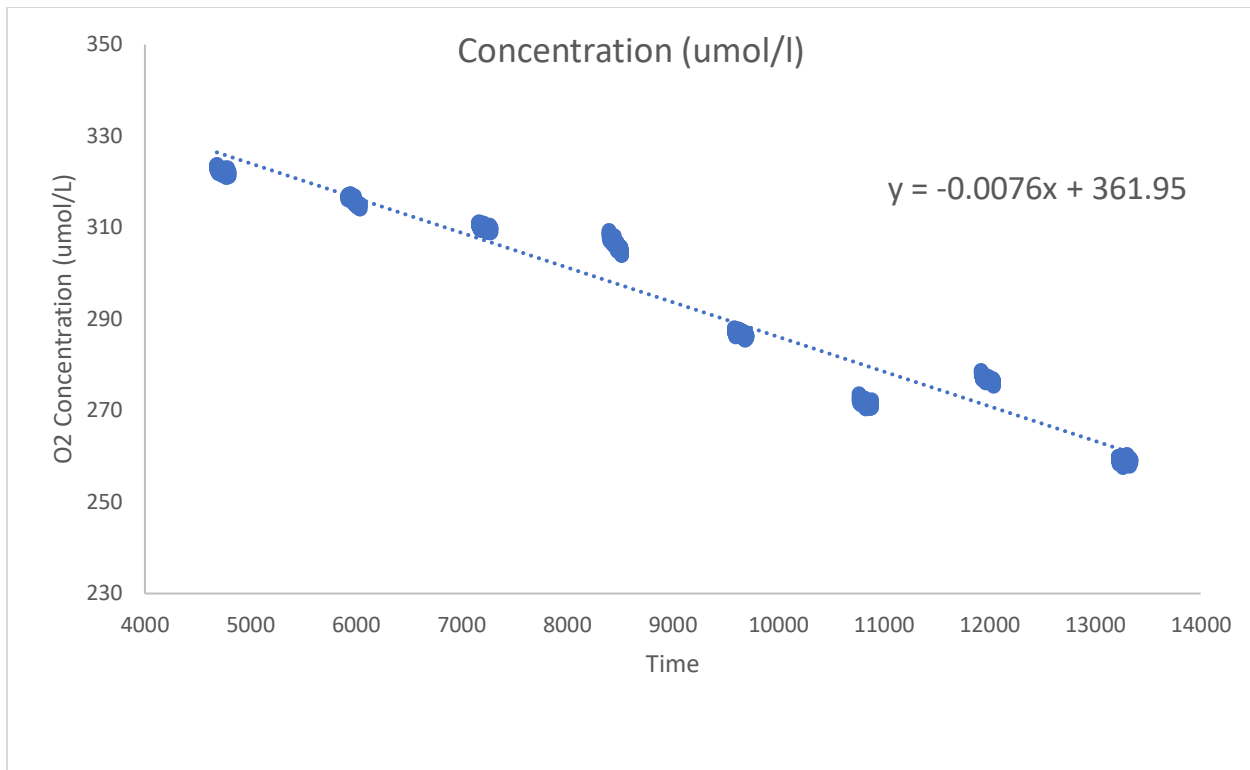
Figure. S2.2 Graph of raw output from Unisense software program for a single chamber (insect). Oxygen concentration is shown as a function of time. Each cluster of points represents a 2-min period over which oxygen concentration was measured every 0.5 sec. In this case, concentration was measured a total of 8 times. We drew a regression line through all of the data to arrive at a single, average respiration/metabolic rate value for this insect, i.e. 0.0076 umol/L.